

# Chapter 13

## Three-Dimensional Traction Force Microscopy of Engineered Epithelial Tissues

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### Abstract

Several biological processes, including cell migration, tissue morphogenesis, and cancer metastasis, are fundamentally physical in nature; each implicitly involves deformations driven by mechanical forces. Traction force microscopy (TFM) was initially developed to quantify the forces exerted by individual isolated cells in two-dimensional (2D) culture. Here, we extend this technique to estimate the traction forces generated by engineered three-dimensional (3D) epithelial tissues embedded within a surrounding extracellular matrix (ECM). This technique provides insight into the physical mechanisms that underlie tissue morphogenesis in 3D.

**Key words** Micropatterning, Biomechanics, Engineered tissue

### Abbreviations

2D	Two-dimensional
3D	Three-dimensional
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DVC	Digital volume correlation
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
HBSS	Hanks' balanced salt solution
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
TFM	Traction force microscopy

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## 1 Introduction

### 1.1 History and Importance of Traction Force Microscopy (TFM)

Many fundamental biological processes are driven by an interplay between mechanical and biochemical signals, including cell proliferation [1], differentiation [2–4], and epithelial-mesenchymal transition (EMT) [5, 6]. Quantifying the mechanical forces that cells exert, however, and assessing their role in the vast array of molecular signaling networks that regulate cell behavior is a tremendous challenge. This is partially owing to the fact that mechanical loads act across hierarchical length scales. (For example, should we be measuring forces at the level of the cytoskeleton, the level of the organism, or somewhere in between?) In addition, cells are usually ill suited to traditional mechanical tests, as they are small and often mechanically inaccessible, both when embedded in a dense meshwork of ECM and when interconnected with neighboring cells in an intact epithelium.

Still, despite these challenges, significant progress has been made in the study of mechanical forces in biology with the advent of traction force microscopy (TFM). This technique involves cells (or tissues) cultured on or embedded within a flexible substratum that can deform in response to cell-generated tractions. If the mechanical properties of the substratum are well characterized, then the traction forces that generated the observed deformations can be calculated (*see Note 1*).

When TFM was first invented, deformations were restricted to two dimensions (2D), since cells were typically cultured on thin films [7–10]. TFM was later expanded to three-dimensional (3D) systems: first, by accounting for 3D deformations of the underlying substratum during 2D cell culture [11], and then later by studying individual cells embedded in 3D gels [12]. Single cells have been estimated to exert forces on the order of 10 nN on their surrounding substrata [11].

### 1.2 Recent Contributions

We were among the first to fully extend 3D TFM into a multicellular context. Engineered mammary epithelial tissues can be embedded within 3D collagen gels [13] containing fluorescently labeled polystyrene microspheres, which are used to track the deformations of the gel during culture and thereby estimate the traction stresses exerted by the developing tissues [14]. Here we present a detailed protocol for these experiments, which can be readily extended to other types of cells, gels, and tissues.

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## 2 Materials

### 2.1 PDMS Stamps

1. Polydimethylsiloxane (PDMS) (Sylgard 184, Ellsworth Adhesives).
2. PDMS curing agent (Sylgard 184, Ellsworth Adhesives).

3. Lithographically patterned silicon master.
4. Plastic weigh boat.
5. 100 mm Petri dishes (Fisher Scientific).
6. 70 % (v:v) Ethanol.
7. Razor blade.

## **2.2 Micropatterning Materials**

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

1. 10× Hanks' balanced salt solution (HBSS).
2. Cell culture media: 1:1 Dulbecco's modified Eagle's medium: Ham's F12 Nutrient Mixture (DMEM/F12 (1:1), Hyclone) supplemented with 2 % fetal bovine serum (FBS), 5 µg/mL insulin, and 50 µg/mL gentamicin.
3. Phosphate-buffered saline (PBS).
4. 0.1 N NaOH.
5. Bovine type I collagen (non-pepsinized; Koken, Tokyo, Japan).
6. 1 µm diameter fluorescent polystyrene beads (Invitrogen).
7. 1 % (m:v) bovine serum albumin (BSA) in PBS. Store at 4 °C.
8. Curved stainless steel tweezers (#7 Dumont).
9. 35 mm tissue culture dishes (Fisher Scientific).
10. 15 mL conical tube (Fisher Scientific).
11. 1.5 mL Eppendorf Safe-Lock Tube (Eppendorf).
12. Circular #1 glass coverslips, 15 mm in diameter (Bellco Glass Inc.).
13. Vybrant DiI (or DiO) cell-labeling solution (Invitrogen).
14. 0.05 % 1× Trypsin–EDTA (Invitrogen).
15. 70 % (v:v) Ethanol.
16. Ice.

## **2.3 Confocal Fluorescence Microscopy**

1. CCD camera attached to an inverted spinning disk confocal microscope.
2. 0.05 % (v:v) Triton X-100 in PBS.

## **2.4 Tracking Bead Displacements**

1. Imaris, Version 7.6.3 (Bitplane).

## **2.5 Calculating Average Displacement Fields**

1. MATLAB (The Mathworks).

## **2.6 Reconstructing Tissue Geometry**

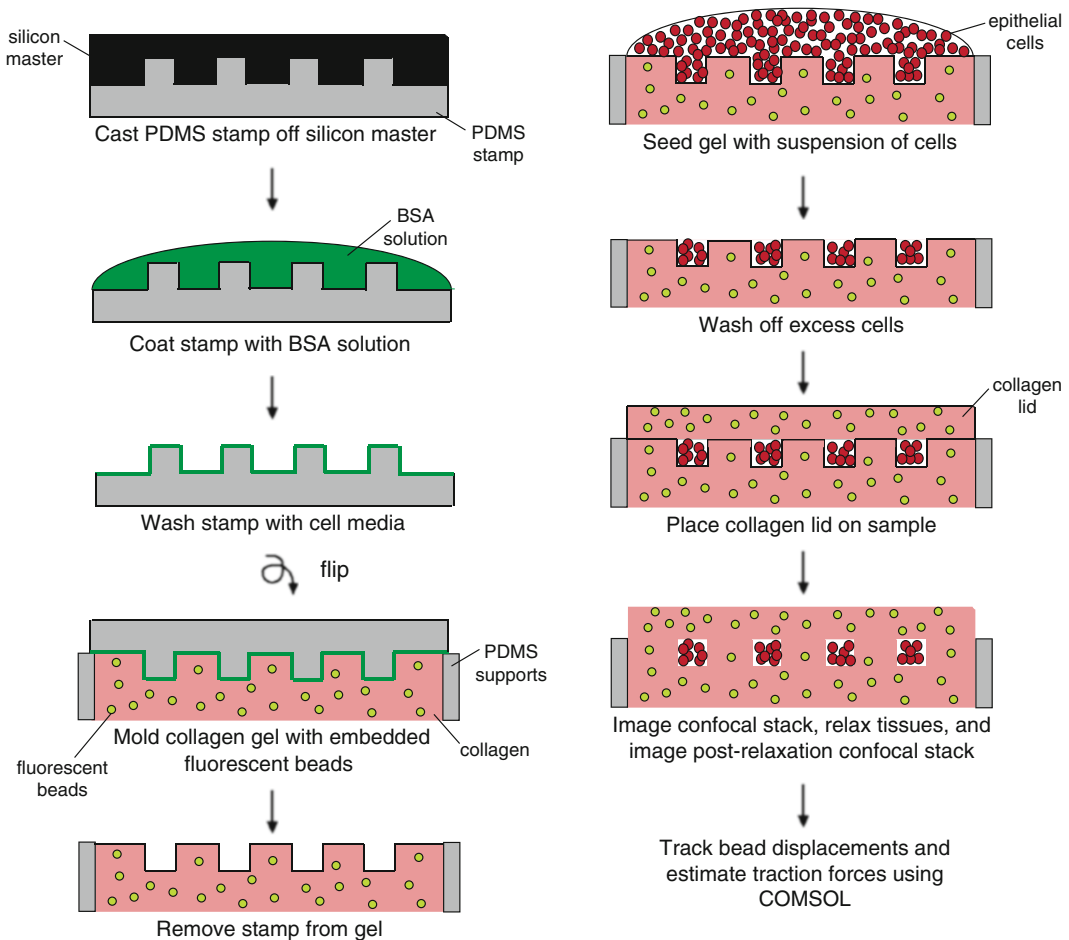
1. Inventor Professional (AutoDesk, Inc.).

## **2.7 Computing Traction Forces**

1. COMSOL Multiphysics, Version 4.2a (COMSOL AB).

### 3 Methods

Here, we describe a 3D engineered tissue model used to quantify the traction forces exerted by tissues on their surrounding ECM (Fig. 1). Collagen matrices containing fluorescent beads with multiple tube-shaped cavities of defined geometry are created using a microlithography-based technique. The cavities are then seeded with epithelial cells labeled with fluorescent vital dye (DiI). The tissues and surrounding fluorescent beads are then imaged in 3D using confocal fluorescence microscopy both before and after treatment with Triton X-100. Bead displacements are tracked in 3D, and the resultant traction stresses are calculated using computational modeling.



**Fig. 1** Schematic of procedure for preparing samples

### **3.1 Preparation of PDMS Stamps for 3D Micropatterning**

1. Mix the PDMS prepolymer and curing agent at a 10:1 (w:w) ratio. Aim for a total weight of approximately 60 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 large weigh boat. Cure the PDMS in an oven at 60 °C for at least 2 h.
2. Once the PDMS is cured, remove the rims from the weigh boat and carefully peel the PDMS from the silicon wafer, removing any PDMS on the bottom of the master. Using a clean razor blade, remove any excess PDMS from around the imprinted features, leaving the remaining PDMS in a circular shape.
3. Using a clean razor blade, cut the polymerized PDMS into stamps (~5 mm cubes), making one stamp for each sample. Place the stamps feature side up in a 100 mm Petri dish.
4. Create supports for the PDMS stamps by spreading 2–3 g of PDMS on a 100 mm Petri dish using a spin coater (*see Note 2*). Cure the PDMS as described above. Using a razor blade, cut out two (~5 mm square) supports per PDMS stamp and place the supports in the Petri dish containing the PDMS stamps.
5. In a biosafety cabinet (cell culture hood), sterilize stamps and supports by washing briefly with 70 % ethanol and aspirating the excess liquid. Allow residual ethanol to evaporate completely (~2 min).

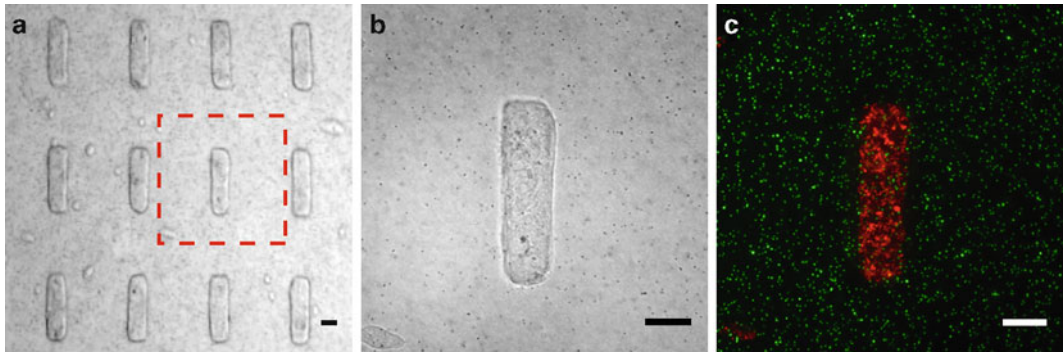
### **3.2 Micropatterning of 3D Epithelial Tissues**

1. In a biosafety cabinet, coat four PDMS stamps with approximately 200  $\mu\text{L}$  of 1 % BSA in PBS (*see Note 3*). Leave the BSA-coated stamps at 4 °C for a minimum of 4 h to eliminate air bubbles.
2. Aspirate BSA from the four PDMS stamps.
3. Add cell culture media to the stamp surfaces, aspirate, and repeat (200  $\mu\text{L}$  per four stamps per wash should be sufficient).
4. Using curved stainless steel tweezers, place the PDMS supports in the 35 mm tissue culture dishes (two supports per stamp, separated by a distance slightly less than the length of the stamp).
5. Wash four circular glass cover slips (15 mm in diameter, #1) with 70 % ethanol and aspirate the excess liquid. Place these in a 100 mm Petri dish.
6. In a chilled 1.5 mL Eppendorf tube, prepare a neutralized solution of collagen. Add 50  $\mu\text{L}$  10 $\times$  HBSS, 30  $\mu\text{L}$  0.1 N NaOH, 30  $\mu\text{L}$  cell culture media, and 400  $\mu\text{L}$  of stock collagen for a final concentration of 4 mg/mL (*see Note 4*). Mix slowly by pipetting up and down; try not to introduce bubbles. If bubbles are introduced, centrifuge the mixture. If fluorescent beads are to be used for TFM, add them to the collagen mixture at this time at a high concentration ( $\sim 4 \times 10^8$  beads/mL).
7. Distribute 200  $\mu\text{L}$  of the collagen mixture evenly on the surfaces of the PDMS stamps (approximately 50  $\mu\text{L}$  per stamp).

8. Using the curved tweezers, flip the stamps upside down onto the supports in the tissue culture dishes and place the dishes into the incubator at 37 °C for approximately 30 min. Add 50 µL of the collagen mixture onto each of the circular cover slips (these will be the collagen caps that are placed on top of the patterned tissues) and place these into the incubator at 37 °C for approximately 30 min.
9. Aspirate the media from a 100 mm tissue culture dish that is 40 % confluent with epithelial cells. Add 10 µL Vybrant DiI (or DiO) (Invitrogen) in 2 mL of fresh culture media to the tissue culture dish and incubate at 37 °C for 15 min.
10. After the incubation, aspirate the media containing DiI or DiO, wash the cells once with PBS, and trypsinize the cells using 2 mL of trypsin.
11. Add 8 mL of cell culture media to the trypsinized cells, place in a 15 mL conical tube, and centrifuge at 800 rpm for 5 min.
12. After the centrifugation step, aspirate the supernatant and resuspend the cells in 250–400 µL of cell culture media for a final cell concentration of  $\sim 10^6$ – $10^7$  cells/mL (*see Note 5*).
13. Remove the tissue culture dishes containing the PDMS stamps and collagen from the incubator and lift the PDMS stamps off of the collagen using the curved tweezers (*see Note 6*); the stamps can now be discarded.
14. Add 30 µL of cell suspension onto each collagen gel. While observing under a microscope, shake the dishes so that the cells settle into the patterned collagen (*see Note 7*).
15. After  $\sim 5$  min (or whenever the cells have settled into the wells of the pattern), wash the stamps with 430 µL of cell culture media by tilting the tissue culture dish on its side and allowing the media to pour over the stamp (*see Note 8*). Aspirate the wash from the tissue culture dish and repeat.
16. Place the collagen containing the cells in the incubator at 37 °C for 15 min. Then, place the glass cover slips with collagen caps on top of the cell-containing gels so that the cells are completely embedded in collagen (Fig. 2). Place the dishes in the incubator at 37 °C again for 15 min.
17. Add cell culture media slowly ( $\sim 2.5$  mL) on top of the glass cover slip until the cell-containing gels are covered in media, and place the dishes in the incubator at 37 °C.

### **3.3 Confocal Time-Lapse Imaging of Fluorescent Bead Displacements and Tissue Morphogenesis**

1. Monitor the positions of the fluorescent beads by collecting confocal stacks of the tissues: 120 images (spaced 1 µm apart).
2. Relax the tissues by adding 0.05 % Triton X-100 in PBS overnight.
3. Capture a second stack of post-relaxation images (120 images, 1 µm apart) of fluorescent beads the next day (*see Note 9*).



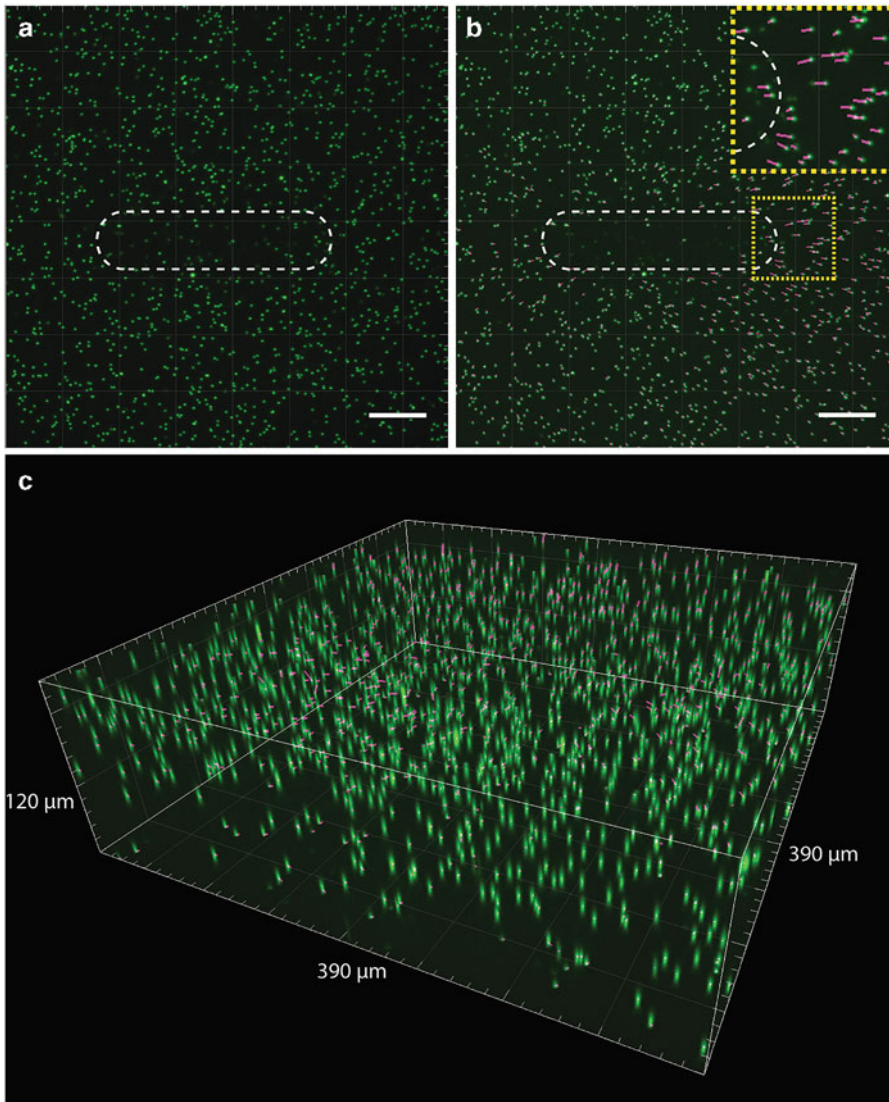
**Fig. 2** (a) Bright-field image of micropatterned epithelial tissues. (b) Higher magnification view of micropatterned tissue presented in (a). (c) Confocal fluorescence image (maximum z intensity) of tissue shown in (b); 1  $\mu\text{m}$  diameter fluorescent microspheres (green), Dil-labeled cells (red). Scale bars = 50  $\mu\text{m}$

4. To account for experimental noise in the motion of the fluorescent beads, monitor the positions of the beads in cell-free collagen gels (see **Note 10**).

**3.4 Tracking Bead Displacements**

1. For each tissue, import 3D image stacks into Imaris (see **Note 11**). There should be two time points—before and after treatment with Triton X-100.
2. Correct for rigid body drift between image stacks.
  - (a) Select Edit  $\rightarrow$  Properties, and input voxel dimensions.
  - (b) In Surpass view, use Spots filtering to select a region of beads far away from the tissue.
  - (c) Track these spots using the Autoregressive Motion routine.
  - (d) Highlight all tracks in the “Edit Track” window and select “Correct Drift.” This should be largely rigid body motion (i.e., only translations and rotations).
  - (e) In Surpass view, the newly drift-corrected image stack should appear.
3. Quantify 3D bead displacements in the entire imaging volume using the Spots filter and the Autoregressive Motion tracking routine (Fig. 3) (see **Note 12**).
4. Export the tracked displacements as a spreadsheet. The following quantities will be used for further analysis:

Track Position Start X	Track Position Start Y	Track Position Start Z	Track Displacement X	Track Displacement Y	Track Displacement Z
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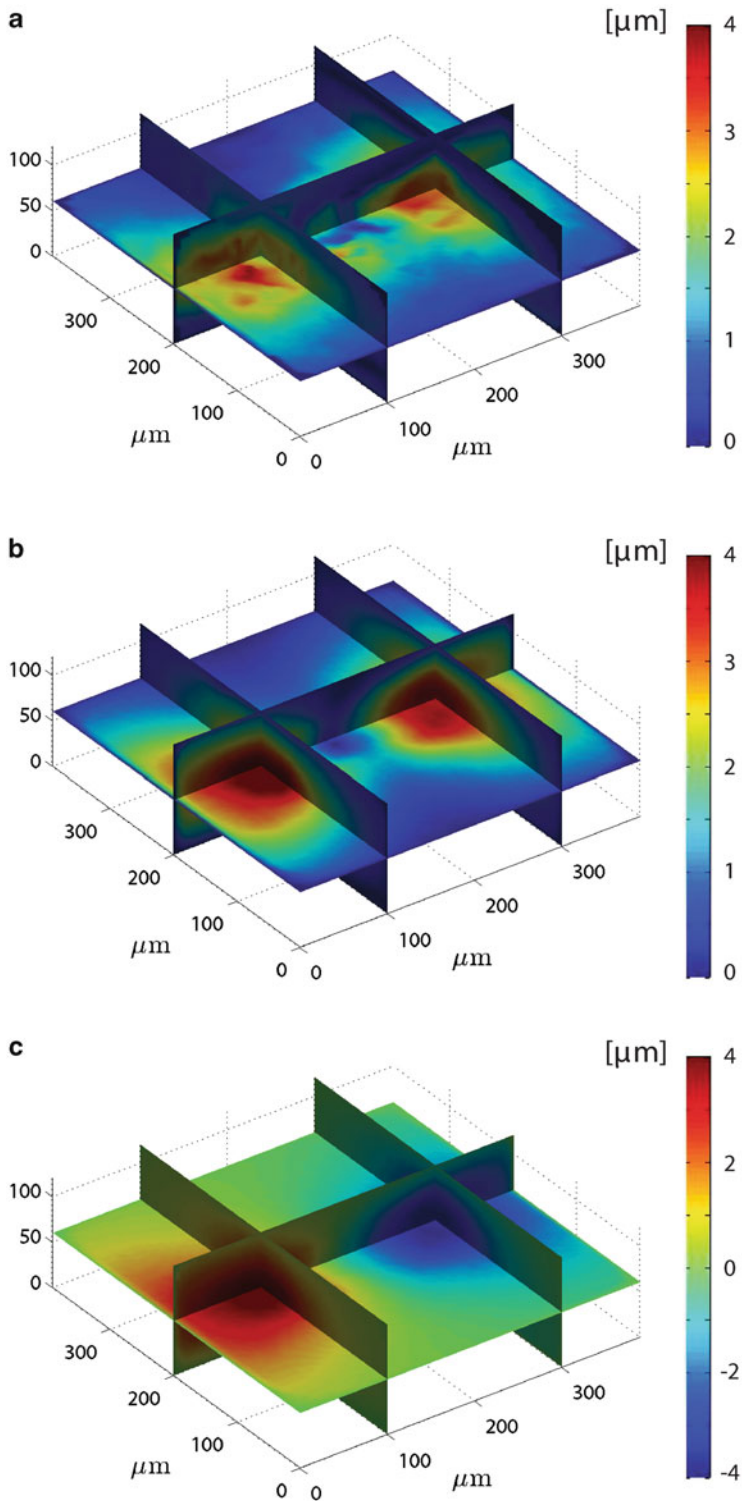


**Fig. 3** (a) Maximum z intensity image of confocal stack showing fluorescent microspheres before treatment with Triton X-100; the epithelial tissue outline is indicated with a *white dashed line*. (b) Maximum z intensity image of confocal stack showing fluorescent microspheres after treatment with Triton X-100; bead displacements are shown in *purple*, and the tissue outline is indicated with a *white dashed line*. The *inset* shows a higher magnification view of the *boxed region*. (c) 3D reconstruction of bead trajectories shown in (b) with indicated dimensions. *Scale bars* = 50  $\mu\text{m}$

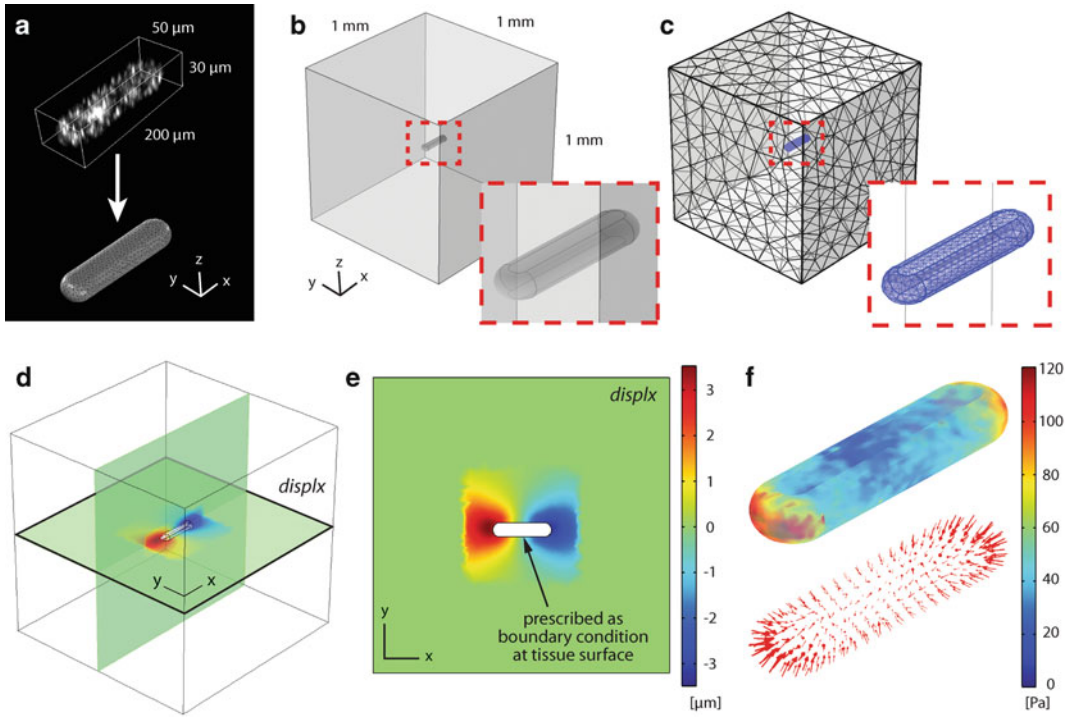
### 3.5 Computing Average Displacement Field (Using Data from Multiple Tissues)

1. Use the *griddata* subroutine in MATLAB to interpolate the exported displacement data across a 3D grid spanning the entire imaging volume. Use Track Position Start X, Y, Z to define a point cloud, and then interpolate each component of the displacement field (e.g., Track Displacement X) separately. These data are often fairly noisy (Fig. 4a).





**Fig. 4** (a) Plot of the interpolated total displacement field for a single tissue. (b) Plot of the interpolated total displacement field averaged over 20 tissues. (c) Plot of the averaged x component of the displacement field



**Fig. 5** (a) Tissue geometry was reconstructed in AutoDesk Inventor from measurements of fluorescently labeled tissues in Imaris. Adapted from [14]. (b) Model geometry used to compute traction forces in COMSOL Multiphysics. (c) Finite element mesh. (d, e) Imported displacement field prescribed as boundary condition along the surface of imported tissue geometry. Here, the x component of the displacement field “displx” is shown. (Compare to Fig. 4c.) (f) Model-computed traction stresses

2. To increase the signal-to-noise ratio, each component of the displacement field can be averaged across multiple tissues (Fig. 4b, c), but only if the displacement data from multiple tissues are properly aligned using the fluorescently labeled tissue geometry. Especially for small displacements, subtle alignment errors can produce significant artifacts in the averaged displacement field.
3. Ensure that the data for each component of the displacement field is organized in a format compatible with the finite element package used to compute the resultant traction forces.

**3.6 Reconstructing and Exporting Tissue Geometry**

1. Using Imaris, measure average morphological parameters (e.g., tissue length, height) for several fluorescently labeled tissues (Fig. 5a).
2. Use the measured parameters to draw a 3D surface in AutoDesk Inventor Professional. Save the reconstructed geometry as an Inventor .ipt file (Fig. 5a).

### 3.7 Calculating Traction Forces

1. Construct a 3D finite element model to compute the traction forces exerted by the engineered tissues. Although other commercial software packages can be used, the following protocol employs COMSOL Multiphysics Version 4.2a (*see Note 13*).
  - (a) Open COMSOL Multiphysics and create a New (File → New...) model file.
  - (b) Select “3D” for Space Dimension, “Solid Mechanics (solid)” for Add Physics, and “Stationary” for “Select Study Type” (*see Note 14*). Click the “Finish” icon.
2. Create model geometry.
  - (a) Click “Geometry 1” and specify the appropriate units for length.
  - (b) Right-click “Geometry 1” in the Model Builder window, and click “Import.” Under the “Geometry import:” pull-down menu, select “3D CAD file,” then click “Browse...” to locate the Inventor .ipt file containing the exported tissue geometry, and click “Import” (*see Note 15*). Ensure that “Solids” and “Surfaces” are checked under “Objects to import” and that “Form solids” is selected under the “Import options” pull-down menu. (If the length units specified in the CAD file are correct, ensure that “From the CAD document” is selected from the “Length unit” pull-down menu.)
  - (c) Right-click “Geometry 1” in the Model Builder window, and click “Block.” Under the “Size and Shape” menu, select values for “Width,” “Depth,” and “Height” that are significantly larger than those of the imported tissue geometry. This block will represent the surrounding collagen gel. (The appropriate size will depend on how far the displacement field propagates into the gel. The displacements should all decay to zero before the outer boundary of the block. Here, we specified a cube with sides of 1 mm.) Click the “Build Selected” icon.
  - (d) Right-click “Geometry 1” in the Model Builder window, and under the “Transforms” menu, click “Move.” Under “Input objects:,” click on the imported tissue geometry and click the “+” icon. Select “impl” (i.e., the imported tissue geometry) and specify values for “x,” “y,” and “z” that move the tissue to a location within the surrounding gel that will coincide with the position of the tissue in the measured displacement field exported from Imaris. (The two must be aligned to ensure that the correct experimental displacements are interpolated along the model tissue surface.)

- (e) Right-click “Geometry 1” in the Model Builder window, and under the “Boolean Operations” menu, click “Difference.” Under “Objects to add,” click on the block and click the “+” icon. Under “Objects to subtract,” click on the imported tissue geometry and click the “+” icon.
  - (f) Right-click “Geometry 1” in the Model Builder window, and click “Build All.” This final geometry (Fig. 5b) represents the geometry of collagen gel surrounding the tissue.
3. Import the (averaged) experimental displacements.
- (a) Right-click “Global Definitions” in the Model Builder window, and under the “Function” menu, select “Interpolation.”
  - (b) Under “Data Source,” select “File” and click “Browse...” to locate the data file containing the x component of the averaged gel displacements. (Ensure that the units of the grid points specified in the imported displacements file match the units in the model.)
  - (c) Select the appropriate data format and assign the function a name. (Here we use “displx”.) Check the box for “Use space coordinates as arguments.” Under the Extrapolation pull-down menu, select “Specific value” and input “0” for “Value outside range.”
  - (d) Repeat and create separate interpolation functions for the y and z components of the averaged gel displacements. (Here we name these functions “disply” and “displz,” respectively.)
  - (e) Right-click “Definitions” under “Model 1” in the Model Builder window, and select “Variables.” Select “Boundary” from the “Geometric entity level” pull-down menu. Ensure that “Manual” has been selected from the “Selection” menu.
  - (f) Select the surfaces bounding the imported tissue geometry and define the following variable:

$$\text{tract sqrt}(\text{solid.Tax}^2 + \text{solid.Tay}^2 + \text{solid.Taz}^2)$$

4. Define mechanical properties and boundary conditions
- (a) Expand “Solid Mechanics (*solid*)” in the Model Builder window, and click “Linear Elastic Material Model 1.”
  - (b) Ensure that the entire model geometry is selected. Under the “Linear Elastic Model” menu, select “Isotropic” and specify the Young’s modulus and Poisson’s ratio as follows (*see Note 16*):

$E$	Young's modulus	User defined	750 Pa
$\nu$	Poisson's ratio	User defined	0.2

- (c) Right-click “Solid Mechanics (*solid*)” and select “Prescribed Displacement.” Under “Boundary Selection,” ensure that “Manual” is selected, and select the surfaces bounding the tissue by clicking on them individually and selecting “+” (Fig. 5d, e).
- Check the box next to “Prescribed in x direction” and input the following (*see Note 17*):
 
$$u_0 - \text{displx} * 10^{(-6)}$$
  - Repeat for displacements in the y and z directions, using “disply” and “displz,” respectively.
- (d) Right-click “Solid Mechanics (*solid*)” and select “Fixed Constraint.” Under “Boundary Selection,” ensure that “Manual” is selected, and select each of the outer surfaces of the box representing the collagen gel.
5. Create model mesh.
- Right-click “Mesh 1” in the Model Builder window. Select “Free Triangular” under “More Operations.”
  - Under “Geometric entity level,” ensure that “Boundary” is selected from the pull-down menu and that “Manual” is selected from the “Selection” menu.
  - Select the surfaces bounding the imported tissue geometry.
  - Right-click “Free Triangular 1” and select “Size.” Under the “Predefined” list for “Element Size,” select “Fine.”
  - Right-click “Mesh 1,” and select “Free Tetrahedral.” Ensure that “Remaining” is selected as the “Geometric entity level.”
  - Right-click “Free Tetrahedral 1” and select “Size.” Under the “Predefined” list for “Element Size,” select “Coarser.”
  - Right-click “Mesh 1” and select “Build All” (Fig. 5c).
6. Solve model and plot results.
- Right-click “Study 1” in the Model Builder window and select “Compute.” Once the solution converges, results can be plotted in the “Results” tab. (Ensure that the scale factor for deformations in all plots is set to 1.)
  - To plot traction stresses, right-click “Results” and create a “3D Plot Group.” Right-click the “3D Plot Group” and select “Surface.” Under “Expression,” type “tract” (defined above) and click Plot (Fig. 5f).

- (c) Ensure that model displacements reasonably reproduce those observed experimentally. If they do not, a more accurate description of the mechanical properties of the surrounding gel may be needed. Alternatively, nonlinear effects due to large deformations may likewise need to be included in the analysis.

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## 4 Notes

1. It is important to note that forces are not measured directly here. Instead, they are calculated from the observed deformations of flexible substrata. The accuracy of the computed forces is thus highly dependent on the measured mechanical properties of these flexible materials, which are (typically) only characterized for a specific set of loading conditions. Moreover, simplifying assumptions such as material linearity, isotropy, and homogeneity are often assumed. These assumptions are often made for convenience and only approximate the actual behavior of real materials. The traction forces computed using TFM are thus only estimates.
2. The thickness of the supports will determine how close the epithelial tissues are to the bottom of the tissue culture dish.
3. In a biosafety cabinet, place four dots of BSA in the four corners of each stamp and one in the center, and then spread with a pipet tip so that BSA covers the entire stamp.
4. When using a new bottle of collagen (5 mg/mL), check the pH of the final mixture. The pH should be 8–8.5; adjust the volume of NaOH accordingly.
5. Err on the side of less media; a more concentrated cell suspension will promote faster and more homogeneous settling of the cells into the collagen cavities.
6. Gently lift the PDMS stamps off the collagen as vertically as possible so as not to disturb the pattern.
7. To improve settling of the cells into patterned wells, place the tissue culture dishes next to a bench top centrifuge set to 700 rpm. The vibrations from the centrifuge shake the cells into the collagen cavities.
8. It may be easier to wet the bottom support first so that the wash flows straight down.
9. The elastic recoil happens quickly after adding the detergent (as soon as the detergent diffuses in and lyses the cells). Full relaxation/viscous flow is complete after treating with detergent overnight.
10. This accounts for noise owing to bead movement (which is negligible in these dense gels) as well as for inaccuracies in the

Imaris tracking algorithm. In a single sample, signal-to-noise was typically 5:1, but averaging data from multiple samples attenuated the ratio to ~20:1.

11. Other (free) tracking applications are available. Their implementation, however, requires that the user is proficient with different programming languages (e.g., MATLAB). Examples include Crocker and Grier's 3D particle tracking algorithm [15], and Franck and colleagues' digital volume correlation (DVC) [16].
12. Filters (using quantities such as track length) can be used to screen errant tracks.
13. Although the steps outlined in this protocol are specific to COMSOL Multiphysics Version 4.2a, a similar workflow can be developed in both older and newer versions of the software.
14. In selecting a "Stationary" study type, we thereby neglect inertial effects, which are typically negligible in problems such as cell migration and tissue morphogenesis.
15. Several other CAD formats are compatible with COMSOL. If one would prefer not to use Inventor, consult the COMSOL Multiphysics User's Guide for a list of these alternatives [17].
16. As only a first approximation, we assume linear elastic material properties for the surrounding collagen gel [14].
17. In our analysis, displacements were interpolated in micrometers and therefore converted into meters. The negative sign is owing to the fact that displacements were tracked from before to after treatment with Triton X-100—that is, from the deformed to the undeformed configuration of the gel, which here must be reversed.

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