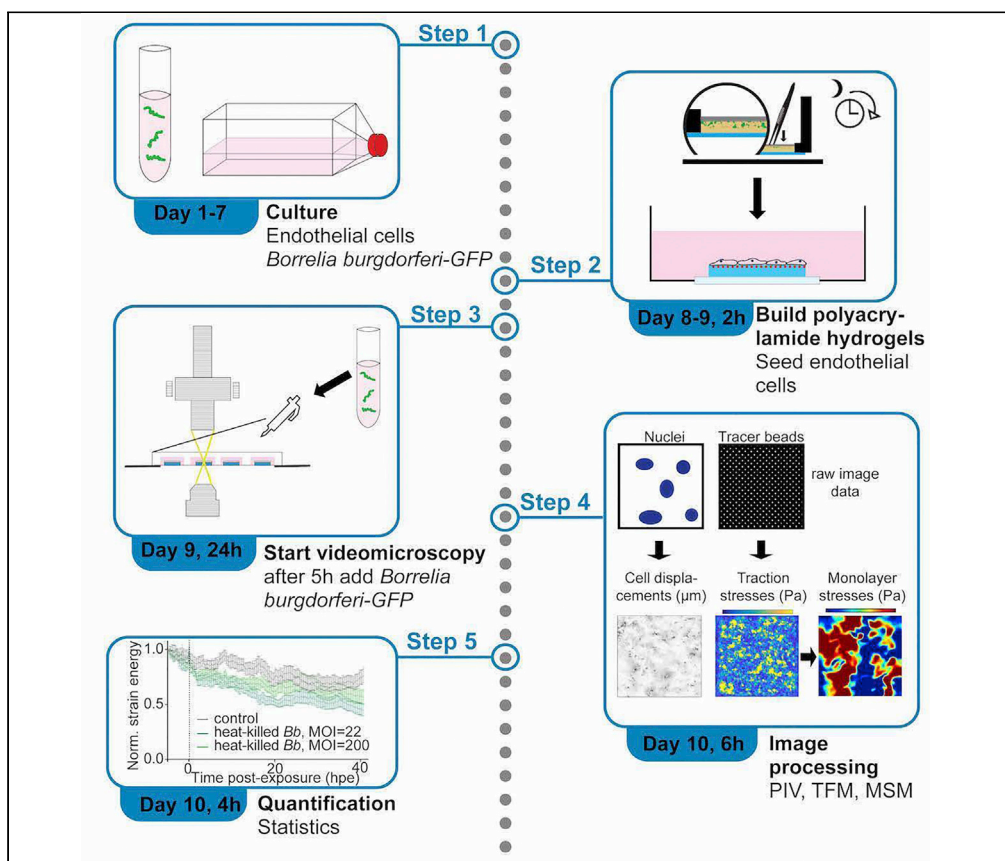# STAR Protocols

Protocol

Spatiotemporal characterization of endothelial cell motility and physical forces during exposure to *Borrelia burgdorferi*

Marie Muenkel, Raul Aparicio-Yuste, Michal Caspi Tal, Peter Kraiczy, Effie E. Bastounis

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Highlights

Exposure of endothelial cells (ECs) to live or heat-killed *B. burgdorferi* (Bb)

Measure changes in the kinematics and dynamics of *Bb*-exposed ECs

Quantify motility of *Bb*-exposed ECs through PIV on the image of EC nuclei

TFM and MSM allow measuring *Bb*-exposed EC traction and monolayer stresses

Cell motility and biomechanics are critical in various (patho)physiological processes, including the regulation of vascular barrier integrity, which can be subverted by bacterial pathogens. Here, we present a protocol on how to expose endothelial cells (ECs) to vector-borne *Borrelia burgdorferi* (Bb) and characterize EC kinematics and dynamics during exposure to live or heat-inactivated *Bb* through traction force and monolayer stress microscopy. Modifications to this protocol may be necessary for studying how different cell types interact with *Bb* or other microorganisms.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Spatiotemporal characterization of endothelial cell motility and physical forces during exposure to *Borrelia burgdorferi*

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https://doi.org/10.1016/j.xpro.2022.101832

SUMMARY

Cell motility and biomechanics are critical in various (patho)physiological processes, including the regulation of vascular barrier integrity, which can be subverted by bacterial pathogens. Here, we present a protocol on how to expose endothelial cells (ECs) to vector-borne *Borrelia burgdorferi* (*Bb*) and characterize EC kinematics and dynamics during exposure to live or heat-inactivated *Bb* through traction force and monolayer stress microscopy. Modifications to this protocol may be necessary for studying how different cell types interact with *Bb* or other microorganisms.
For complete details on the use and execution of this protocol, please refer to Yuste et al. (2022).[1]

BEFORE YOU BEGIN

This protocol describes specific steps to measure the changes in human skin microvascular endothelial cells (HMEC-1) kinematics and dynamics before and after prolonged exposure to *Borrelia burgdorferi* (*Bb*) (Figure 1). A monolayer of HMEC-1 cells is exposed to live or heat-inactivated *Bb* and the changes in HMEC-1 kinematics and dynamics are measured via microscopy followed by image processing. Depending on the desired endothelial cell (EC) type to be used, the most appropriate culture conditions and seeding density to achieve an integral EC monolayer will have to be determined empirically and the protocol will need to be adjusted accordingly.

Preparation of *Bb* culture media

⏱ Timing: 1 h

For our studies we have been using infectious *Bb* strains Bb1286[5] and GCB726,[6] both of which were generated from the B31 5A4 NP1 *Bb* strain. Thus, both strains are cultured in the same growth medium. The difference between Bb1286 and GCB726 is in the GFP construct that was introduced to make the bacteria fluorescent.
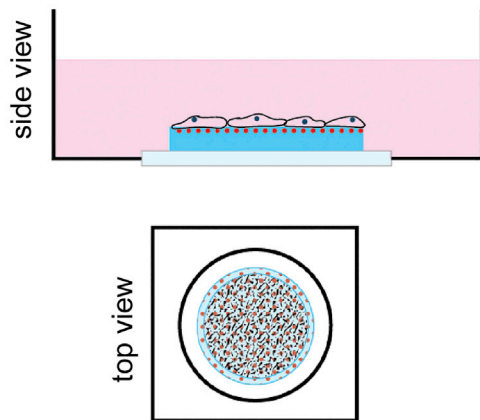
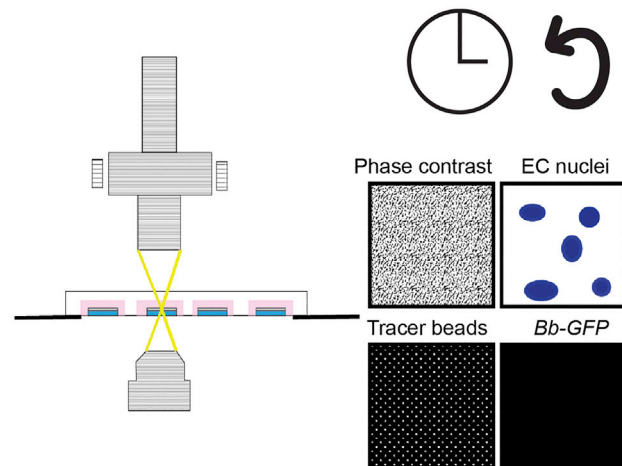**A** Prepare cultures of *Borrelia burgdorferi* (*Bb-GFP*) and ECs
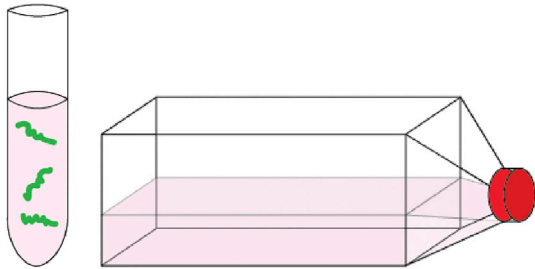
B Manufacture polyacrylamide hydrogels with tracer beads

side view

top view
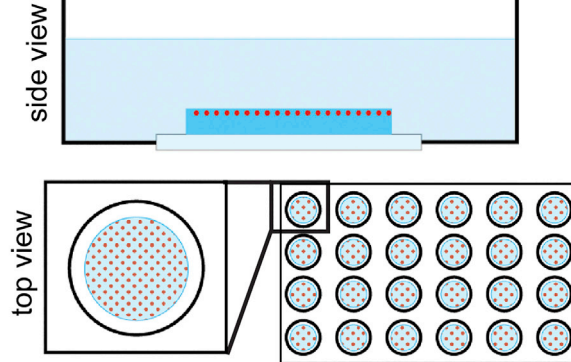
C Seed ECs on collagen I coated-hydrogels

side view

top view

D After 24 h start time-lapse image acquisition

Phase contrast EC nuclei

Tracer beads *Bb-GFP*

E 5 h later add live or heat-inactivated *Bb-GFP*

Phase contrast EC nuclei

Tracer beads *Bb-GFP*
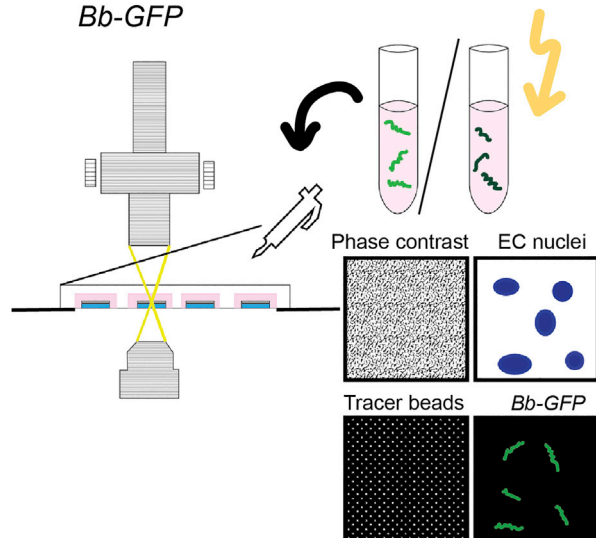
F Analysis of EC kinematics and dynamics based on time-lapse image data

EC nuclei Tracer beads

raw time-lapse image data

PIV to calculate EC displacements

TFM to calculate traction stresses
0 50 100 [Pa]

MSM to calculate monolayer stresses
0 200 400 [Pa]

Figure 1. Schematic of assay for probing EC kinematics and dynamics prior and after exposure to *Bb*

The main steps for seeding and exposing HMEC-1 cells to *Bb* and monitoring via microscopy and image processing changes in HMEC-1 kinematics and dynamics are depicted.
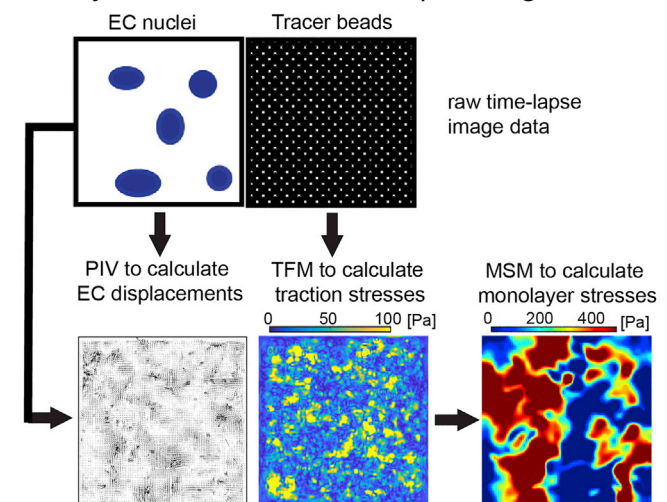
(A) Culture of HMEC-1 and *Bb-GFP*.

(B) Manufacturing of polyacrylamide hydrogels with embedded fluorescent tracer beads on multi-well plates.

(C) Seeding of HMEC-1 on hydrogels for 24 h.

(D) Initiation of time-lapse multi-channel and multi-positional image acquisition.

(E) Addition of live or heat-inactivated *Bb* at ∼ 5 h post-initiation of videomicroscopy.

(F) Image processing to determine alterations in EC motility, traction and monolayer stresses.

1. Preparation of stock antibiotic solutions.

   Note: Type 1 ultrapure water was used for the preparation of reagents.

   a. Dissolve 0.5 g gentamicin sulfate into 10 mL of sterile water to prepare a 50 mg/mL stock solution.
   b. Dissolve 0.5 g of kanamycin into 10 mL of sterile water to prepare a 50 mg/mL stock solution.
   c. Use a 0.2-μm syringe filter to sterilize the antibiotic solutions and store stock solutions at −20°C for 1 year.

   Note: Lower grades of water may be suitable depending on your cultures.

2. Preparation of *Bb* culture media.
   a. Supplement BSK-H medium containing sodium bicarbonate with 5.4% rabbit serum.
   b. Aliquot the solution into 50 mL falcon tubes and store at −20°C for future usage.
   c. When performing experiments thaw supplemented media and add gentamicin and kanamycin from stock solutions to a final concentration of 50 μg/mL and 100 μg/mL, respectively.
   d. Store at 4°C and use for the next 2–3 months.

   Note: You should avoid repeated freezing and thawing cycles.

Preparation of HMEC-1 culture media

⊙ Timing: 1–2 h

We used HMEC-1 as model ECs because they are dermal microvascular ECs and therefore most similar to the first type of ECs *Bb* interacts with after skin infection occurs through a tick bite. Additionally, we chose those cells because we have previously characterized in great detail their mechanobiology and response to infection with intracellular bacterial pathogens.[7,8] Finally, since HMEC-1 are immortalized (i.e., cell line), they can be easily cultured or genetically manipulated.

3. Preparation of HMEC-1 cell culture media.
   a. Prepare 2 mL stocks of 250 μg/mL hydrocortisone.
      i. Weigh 25 mg of hydrocortisone.
      ii. Add 100 mL of autoclaved water and mix well.

   Note: If the hydrocortisone powder does not dissolve well in the water, place the solution briefly in a 37°C water bath.

      iii. Use 0.2 μm vacuum bottle top filters and pass through the filter the whole solution.
      iv. Aliquot 2 mL of the solution in 15 mL falcon tubes and store at −20°C for up to a year.
   b. Prepare 5 mL stocks of 200 mM L-glutamine.
      i. Weigh 2.95 g of L-glutamine.
      ii. Add 90 mL autoclaved water and mix well.

    iii. Adjust to a final volume of 100 mL.

    iv. Use 0.2 μm vacuum bottle top filters and pass through the filter the whole solution.

    v. Aliquot 5 mL of the solution in 15 mL falcon tubes and store at −20°C for up to a year.

  c. Prepare 500 μL stocks of 10 μg/mL epidermal growth factor (EGF).

    i. Reconstitute 100 μg of lyophilized EGF in 10 mL autoclaved water.

    ii. Mix well and use a 0.2-μm syringe filter to sterilize.

    iii. Aliquot 500 μL of the solution in 1.5 mL microcentrifuge tubes and store at −20°C for up to a year.

  d. Prepare HMEC-1 media using the previously made stocks.

    i. In 442.5 mL of MCDB-131 medium, add 50 mL fetal bovine serum, a 5 mL stock of 200 mM L-glutamine, a 500 μL stock of 10 μg/mL EGF and a 2 mL stock of 250 μg/mL hydrocortisone.

Note: For live-microscopy media (in case you do not have a CO_2 incubator unit at your microscope) supplement instead Leibovitz's L-15 with 10% fetal bovine serum, 10 ng/mL of EGF, and 1 μg/mL hydrocortisone. L-15 already contains 2 mM L-glutamine, so you do not need to add L-glutamine.

    ii. Aliquot the MCDB-131 medium with supplements (from here on referred as full MCDB-131 medium) in 50 mL falcon tubes and store at −20°C.

    iii. Keep only 1–2 50 mL falcon tubes at 4°C when culturing HMEC-1 cells.

Culturing and passaging of HMEC-1 cells

🕑 Timing: 30 min to 1 h

4. Prewarm HMEC-1 culture media in a 37°C water bath.
5. Whenever cells become confluent, passage them by splitting them 1:3 to 1:6.
  a. For passaging, remove and discard the culture medium and rinse the cells once with PBS.
  b. Add 2 mL of 0.05% trypsin-EDTA in the T-75 flask and place the flask in the incubator for 5–10 min or until the cells are detached from the surface of the flask.

⚠ CRITICAL: To avoid clumping of cells do not shake the flask while waiting for the cells to detach but rather let them sit in the incubator for the appropriate amount of time until they are detached from their plastic substrate. Mechanical agitation will lift the cells, but it is possible that they might detach in clumps. This part is particularly important when seeding cells in coverslips or hydrogels where one wants to ensure homogeneous cell spatial distribution in the monolayer.

  c. When cells have detached, add culture media up to a final volume of 12 mL and mix cells by gently pipetting.
  d. Add appropriate volumes of the cell suspension to new flasks (so that the cell density as compared to the confluent flask is diluted between 1:3 and 1:6), and supplement with appropriate volumes of full culture medium.

Preparation of 10% ammonium persulfate and 0.5 M HEPES pH=7.5

🕑 Timing: 30 min

These stock solutions are needed for the preparation of the polyacrylamide hydrogels to conduct traction force microscopy (TFM).

6. Preparation of 10% ammonium persulfate.

a. Weigh 1 g of ammonium persulfate.
b. Dissolve the powder in 10 mL autoclaved water.
c. Aliquot 1 mL of the solution in 1.5 mL microcentrifuge tubes and store at −20°C.

Note: Working solutions are stored at 4°C for up to a week. This did not impact the resulting stiffness of our hydrogels as evidenced by previous atomic force microscopy (AFM) measurements performed.[7] Nevertheless, other protocols suggest always making the APS solution fresh.

7. Preparation of 0.5 M HEPES pH=7.5.
    a. Weigh 119.15 g of HEPES powder and place in a 2 L glass flask.
    b. Add 800 mL of autoclaved water.
    c. Add a magnetic stir bar and start stirring by placing the flask on a magnetic stir plate until the powder is dissolved.
    d. Adjust the pH to 7.5 by adding the appropriate volume of 10 N NaOH.

Alternatives: Use NaOH pellets to adjust the pH.

    e. Adjust the final volume to 1 L.
    f. Use 0.2 μm vacuum bottle top filters and pass through the filter the whole solution.
    g. Prepare a working solution of HEPES 50 mM by mixing the 1:10 the 0.5 M stock solution with water and filter sterilize too.
    h. Store at 21°C–24°C the stock solution (0.5 M) and at 4°C the 50 mM HEPES solution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Collagen I rat tail	Fisher	Cat# A1048301
Acrylamide 40% solution	Sigma	Cat# A4058
Bisacrylamide solution 2%	Fisher	Cat# BP1404250
Aminopropyltriethoxysilane	Sigma	Cat# 919302
FluoSpheres™ Carboxylate-Modified Microspheres, 0.2 μm, yellow-green fluorescent (580/605), 2% solids	Invitrogen	Cat# F8810
MCDB131, no glutamine	Gibco	Cat# 10372019
Leibovitz's L-15 medium, no phenol red	Thermo Fisher Scientific	Cat# 21083027
Fetal bovine serum	Gemini Bio-Products	Cat# 900108
Hydrocortisone	Sigma	Cat# H0888
hEGF	Sigma	Cat# EG9644
L-Glutamine	Fisher	Cat# SH3003401
Hoechst 33342, trihydrochloride	Invitrogen	Cat# H3570
BSK-H Medium	Sigma	Cat# B3528
Rabbit serum	Sigma	Cat# R4505
Gentamicin sulfate	MP Biomedicals	Cat# 194530
Kanamycin monosulfate	MP Biomedicals	Cat# 150029
Glycerol bidistilled (99.5%)	VWR Chemicals	Cat# 24388.295
0.05% Trypsin/EDTA	Gibco	Cat# 25300096
HEPES, Free acid	J.T. Baker	Cat# 4018-04
Ammonium persulfate (APS)	Fisher	Cat# BP17925
Tetramethylethylenediamine (TEMED)	Sigma	Cat# T9281-1 25 ML
Sulfo-SANPAH	ProteoChem	Cat# c1111-100mg
Sodium hydroxide pellets	Fisher	Cat# S318-500
Dimethyl sulfoxide (DMSO)	J.T. Baker	Cat# 9224-01

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
HMEC-1 cells	Reed et al.[9]	Previously obtained from Centers for Disease Control, Biological Products Branch
Experimental models: Organisms/strains		
Borrelia burgdorferi (strain Bb1286)	Dunham-Ems et al.[5]	N/A
Borrelia burgdorferi (strain GCB726)	Moriarty et al.[6]	N/A
Software and algorithms		
ImageJ	Schneider et al.[10]	https://imagej.nih.gov/ij/
MicroManager	N/A	https://www.micro-manager.org/
MATLAB	MathWorks	http://www.mathworks.com/products/matlab/?requestedDomain=www.mathworks.com
GraphPad Prism v6	GraphPad	http://www.graphpad.com/scientific-software/prism/
PIV	This paper	https://github.com/ebastoun/Correlation_length_of_movement_of_epithelial_cells_in_monolayer
TFM	Lamason et al.[2]	N/A
Monolayer stress microscopy, MSM	This paper	https://github.com/ebastoun/Monolayer-Stress-Microscopy
NIS-Elements (RRID: SCR_014329)	Nikon Instruments Inc.	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
Other		
24-Well plates with glass bottom	MatTek	Cat#P24G-1.5-13-F
T-75 Flasks	Falcon	Cat#353118
50 mL Conical tubes	Falcon	Cat#352070
15 mL Conical tubes	Falcon	Cat#352196
Disposable serological pipettes (1 mL, 2 mL, 5 mL, 10 mL)	Falcon	various
Pipette tips 1–200 µL	Denville	Cat#P1122
Pipette tips 101–1,000 µL	Denville	Cat#P1126
Powder-free examination gloves	Microflex	Cat#XC-310
0.2 µm Vacuum bottle top filters	Thermo Fisher Scientific	Cat#566-0020
20 mL Syringes	BD	Cat#302830
0.2 µm Syringe filters	Thermo Fisher Scientific	Cat#723-2520
10 cm Culture plates	Falcon	Cat#351029
Biosafety cabinet	Baker	Cat#SG504
Hemocytometer	Sigma	Cat#Z359629
Bacterial incubator	Thermo Scientific	Cat#IGS180
CO_2 culture Incubator	NuAire	Cat#NU-8700
Vacuum chamber/degasser	Bel-Art	Cat#42025
Cage incubator (for the microscope)	Haison	Custom
Pipettor	Drummond	Cat#4-000-110
Pipet tips (10 µL, 200 µL, 1,000 µL)	Gilson	Cat#F144802, F123601, F123602
pH meter	METTLER TOLEDO	Cat#30019028
1 L Flask	Fisherbrand	Cat#FB5011000
Autoclave	AMSCO	Cat#3021
Stir magnet plate	Bellco	Cat#7760-06000
Magnet stirring bars	Bellco	Cat#1975-00100
Water purification system 2121B	Aqua Solutions	https://www.aquaa.com/

Note: We used an inverted Nikon Eclipse Ti2 a Prime BSI sCMOS camera (Teledyne Photometrics) for all time-lapse recording including the traction force microscopy experiments. The microscope was controlled by the NIS Elements software (Nikon). Any inverted epifluorescence or spinning disk confocal microscope (for fast time-lapse imaging) should work for the purposes of the protocol presented below.

MATERIALS AND EQUIPMENT

HMEC-1 MCDB-131-based culture media		
Reagent	Final concentration	Amount
Hydrocortisone (250 µg/mL)	1 µg/mL	2 mL
L-glutamine (200 mM)	2 mM	5 mL
EFG (10 µg/mL)	10 ng/mL	0.5 mL
FBS	10%	50 mL
MCDB-131 media	N/A	442.5 mL
Total	N/A	500 mL

Once you mix, aliquot in 50 mL falcon tubes and store at −20°C.

STEP-BY-STEP METHOD DETAILS

Culturing of *Bb* and preparation of heat-inactivated *Bb*—Days 1–7

⊙ Timing: 1 h

Bb are grown until they reach mid log phase which is approximately 5×10^7 bacteria/mL in complete BSK-H medium. Depending on the frozen stock this may require 1–2 weeks. For simplicity here we assume they require 1 week. Once they reach mid log phase, bacteria are exposed as live, highly viable bacteria to a monolayer of HMEC-1, or are first heat-inactivated and then added onto the HMEC-1.

1. Add 9 mL of complete BSK-H medium on a 15 mL falcon tube.
2. Further add 1.5 mL of frozen with glycerol *Bb* stock culture.
3. Place the tube at 33°C until the concentration of *Bb* reaches $2–5 \times 10^6$ bacteria/mL (mid log phase).

   Note: Typically, this will take approximately one to two weeks, but precise timing depends on the freezing method and the specific *Bb* strain used. For counting bacteria either use darkfield microscopy or (epi)fluorescence microscopy if *Bb* constitutively expresses GFP. To precisely count their number, use a hemocytometer or another type of counting chamber.

4. Once you reach that number, dilute the *Bb* solution to 1×10^3 bacteria/mL.
5. Grow *Bb* in fresh complete BSK-H at 33°C until a density of approximately 5×10^7 *Bb*/mL is reached, in which case highly viable *Bb* can be exposed to host cells.

   Note: How many *Bb* you need will ultimately depend on your desired multiplicity of infection (MOI). Herein we seed ECs on glass coverslips (or hydrogels built on them) of 24-well plates. To achieve a confluent monolayer, we aim for 4×10^5 cells the day of infection. Thus, to achieve an MOI of 10 *Bb*/cell in each well one needs to add 4×10^6 *Bb*. After counting, spirochetes should be added to ECs as quick as possible to avoid aggregation or clumping of *Bb*.

6. To prepare heat-inactivated *Bb*, incubate the desired amount of bacteria from step 5 at 56°C for 30 min.
7. Subsequently, store heat-inactivated *Bb* at 4°C.

   ⚠ CRITICAL: This step is critical to avoid drastic damage of the *Bb* membrane or morphology that can occur at lower temperatures (e.g., −20°C).

Manufacturing of 3 kPa polyacrylamide hydrogels for EC seeding—Days 7–8
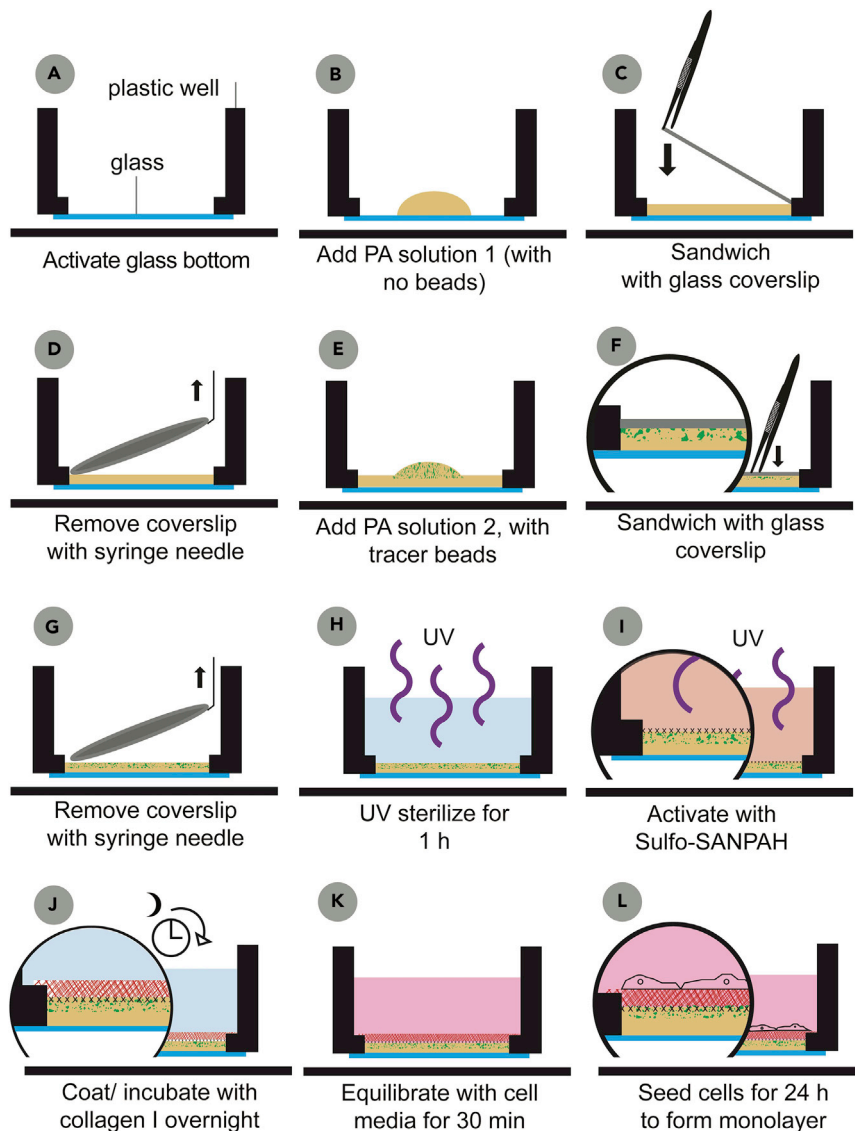
⊙ Timing: 3–4 h

Figure 2. Schematic of the specific steps for building polyacrylamide hydrogels to perform TFM on ECs exposed to Bb

(A) Glass coverslips are chemically treated to allow subsequent polyacrylamide hydrogel attachment.

(B) 3.6 μL of polyacrylamide Solution 1 is placed on each glass coverslip of the multi-well plate.

(C) Solution 1 is sandwiched with a 12-mm circular glass coverslip.

(D) Coverslip is removed with a hooked needle syringe until polymerization occurs.

(E) 2.4 μL of polyacrylamide Solution 2 with tracer beads is added on top of the bottom layer.

(F) Solution 2 is sandwiched with a 12-mm circular glass coverslip.

(G) Water is added, and the coverslip is removed.

(H) UV exposure for 1 h ensures sterilization.

(I) Sulfo-SANPAH solution is added on the hydrogels and irradiation under UV for 10 min is followed.

(J) Hydrogels are washed with HEPES and incubated for 16–18 h with collagen I.

(K) Hydrogel is equilibrated with cell culture medium.

(L) HMEC-1 are seeded and allowed to form a monolayer for 24 h.

The protocol below describes how to activate glass coverslips of 24-well glass bottom plates to then build 3 kPa double-layered polyacrylamide hydrogels with embedded tracer beads to conduct traction force microscopy (TFM) as well as how to sterilize them, coat them with collagen I, and equilibrate them prior to EC seeding (Figure 2).

8. Activation of glass coverslips of 24-well glass bottom plates (Figure 2A).
    a. Add 500 µL of 1 M NaOH on glass coverslips of 24-well glass bottom plates (13 mm-diameter wells) and incubate at 21°C–24°C for 30 min.
    b. Rinse the wells once with ultrapure water.
    c. Add 500 µL of 2% (3-aminopropyl)triethoxysilane in 95% ethanol to each well and incubate at 21°C–24°C for 5 min.
    d. Rinse the wells once with ultrapure water.
    e. Add 500 µL of 0.5% glutaraldehyde in water to each well and incubate at 21°C–24°C for 30 min to 2 h.
    f. Rinse the wells once with ultrapure water.
    g. Dry the coverslips by placing the multi-well plate on a 60°C warm metal plate with the lid of the plate slightly off, or at 21°C–24°C until dry.

    △ CRITICAL: Make sure that the temperature is not higher than 60°C, otherwise the plastic of the multi-well plate will melt.

    ⯀⯀ Pause point: You can store the activated glass coverslips and build the hydrogels at a later point. In that case you do not need to dry them, water will evaporate over time.

9. Fabrication of two-layered 3 kPa polyacrylamide hydrogels and hydrogel sterilization (Figures 2A–2H).
    a. In 1.5 mL microcentrifuge tubes prepare two aqueous solutions (thereon referred as Solution 1) that contain 5% acrylamide and 0.1% bisacrylamide, by adding in a 1.5 mL microcentrifuge tube 152.6 µL water, 23.125 µL of 40% acrylamide, and 9.25 µL of 2% bisacrylamide.

    Note: You can make hydrogels of different stiffnesses by adjusting the concentration of acrylamide and bisacrylamide accordingly.[7,8]

    △ CRITICAL: The solvent can impact the final stiffness of the hydrogel as pH can impact the polymerization reaction. We used water as the solvent as in other studies[8,2,11,12] and previously performed atomic force microscopy measurements to confirm that the expected and resulting stiffness of the hydrogels is similar.[7]

    b. In 1.5 mL microcentrifuge tubes prepare two more aqueous solutions (thereon referred as Solution 2) that contain 5% acrylamide, 0.1% bisacrylamide and 0.04% of 0.2 µm tracer fluorescent beads, by adding in a 1.5 mL microcentrifuge tube 149.6 µL water, 23.125 µL of 40% acrylamide, 9.25 µL of 2% bisacrylamide and 3 µL of 0.2 µm tracer fluorescent beads.

    Optional: Degas all four solutions using vacuum for 15 min to eliminate oxygen that inhibits polymerization.

    c. Pipet 1.2 µL of 10% APS stock and 0.8 µL of TEMED to Solution 1 to initiate polymerization and mix well by pipetting up and down.
    d. Immediately after add 3.6 µL of Solution 1 to the center of each glass coverslip of the 24-well dish (Figure 2B).
    e. Act fast and using your forceps cover/sandwich the polyacrylamide droplets with a 12 mm untreated circular coverslip (Figure 2C).
    f. Let Solution 1 sit for 20 min or until it fully polymerizes.

    △ CRITICAL: If the solution in the tube polymerizes before you manage to cover all wells of the 24-well plate, then you can activate Solution 1 in the second ''backup'' tube you previously prepared and repeat the above steps so that all wells of the 24-well plate are processed. I.e., you can add polyacrylamide Solution 1 from the first tube to the number of

wells you can manage (e.g., 6 wells), and the "backup" tube can be used for the remaining wells.

g. Gently tap a syringe needle to a surface to create a small hook at its tip to facilitate the removal of the coverslips.
h. Lift all the coverslips using the syringe needle and your forceps (Figure 2D).

△ CRITICAL: Do not add water to facilitate the removal of the coverslip as you will build an additional polyacrylamide layer with tracer beads on top. To our hands the coverslip can be easily removed using the "hooked" syringe (see Figure 2D). Note that others pre-treat the coverslip with Sigmacote to render it hydrophobic and thus more easily be able to remove it.[13]

i. Pipet 1.2 μL of 10% APS stock and 0.8 μL of TEMED to Solution 2 (with the tracer beads) to initiate polymerization and mix well by pipetting up and down.
j. Immediately after add 2.4 μL of Solution 2 to the center of each hydrogel built on the well of the 24-well dish but without touching the gels (Figure 2E).
k. Cover Solution 2 with 12-mm circular glass coverslips and gently press downwards using a pair of forceps to ensure the thickness of the second layer is minimal (Figure 2F).

Note: You can measure the thickness of the hydrogels experimentally through phase contrast microscopy by focusing on the upper surface of the glass coverslip and on the surface of the hydrogel. On our hands thickness is 30 +/- 5 μm and is mostly attributed to the first layer since tracer beads are all focused on one focal plane at the uppermost surface of the hydrogel.

l. Let Solution 2 polymerize for 20 min.

△ CRITICAL: If the solution polymerizes before you cover all wells of the multi-well plate, then use the second tube you prepared of Solution 2 and repeat previous steps.

m. Add 200 μL of water to each of the wells to facilitate subsequent removal of the glass coverslips using your hooked syringe needle and forceps (Figure 2G).
n. Add 1 mL of 50 mM HEPES pH 7.5 to each of the wells to keep the hydrogels hydrated.
o. UV-expose the hydrogels for 1 h in the tissue culture hood to allow sterilization making sure the lid of the multi-well plate is inverted too (Figure 2H).

⏸️ Pause point: You can store the plate with the hydrogels at 4°C indefinitely. However, it is advisable to place parafilm around the plate to avoid evaporation of the buffer and subsequent dehydration of the hydrogels.

10. Activation, protein coating and equilibration of hydrogels (Figures 2I–2K).
    a. Weigh 10 mg of sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (Sulfo-SANPAH) and place it in a 50 mL falcon tube.

Note: Sulfo-SANPAH powder should be stored at −20°C and solution should be made fresh.

    b. Add 100 μL DMSO and mix well so the Sulfo-SANPAH powder is dissolved.
    c. Add 10 mL 50 mM HEPES pH = 7.5 and mix well.
    d. Remove the HEPES buffer from the hydrogels and add 200 μL of the Sulfo-SANPAH solution on the hydrogels.
    e. Immediately expose them to UV (302 nm) for 10 min to activate them (Figure 2I).

Note: The lamp we used supplies a power of 8 Watts while the distance to the drawer where the plate with the hydrogels was placed is 5 cm. However, note that lamps "age" which leads to a reduction in resulting power.

⚠ CRITICAL: Doublecheck that UV wavelength is 302 nm. After UV exposure the solution should turn dark brown (see troubleshooting 1).

f. While UV-exposing the hydrogels, prepare 5 mL of 0.25 mg/mL solution of rat tail collagen I in 50 mM of HEPES pH=7.5.
g. Wash the hydrogels twice with 1 mL of 50 mM HEPES pH = 7.5.

Note: Repeat if needed to ensure that any excess crosslinker is removed.

h. Add 200 μL of the 0.25 mg/mL rat tail collagen I in 50 mM of HEPES on each hydrogel (Figure 2J).
i. Incubate the hydrogels, with the collagen solution on top for 16–18 h at 21°C–24°C.

Note: To prevent dehydration/evaporation, place the multi-well plates in a secondary containment and add laboratory cleaning tissues soaked in water in the inner periphery of the containment.

j. Next day wash once with PBS buffer to remove excess collagen.
k. Equilibrate hydrogels with cell culture media at 37°C for 30 min to 1 h (Figure 2K).

Note: You can use an epifluorescence or confocal microscope to measure the thickness of the hydrogels which you might need if you wish to perform TFM. To do so locate the z positions of the bottom (where the glass surface is) and top planes of the hydrogel (where the fluorescent beads' intensity is maximum). Then, subtract the z positions to determine the height. Also measure the depth of the tracer beads in reference to the upper surface of the gels, as this will affect the displacement of the beads and needs to be taken into account when performing TFM. On our hands the resulting thickness of the hydrogels is 30 μm +/- 5 μm and mainly results from the bottom layer without tracer beads. Even if a slightly higher volume of Solution I is added, the resulting thickness will be similar with this probably due to surface energy and adhesive forces between the glass and liquid solution. The second layer with the tracer beads should be thin enough so that beads are confined into a single focal plane as noted above.

Note: You can also perform atomic force microscopy (AFM) to confirm the exact stiffness of the hydrogels.

EC seeding on hydrogels, time-lapse microscopy and exposure to *Bb*—Days 8–9

🕐 Timing: 2–4 h

Once hydrogels are equilibrated, ECs are seeded on them and incubated for 24 h to form a confluent cell monolayer (Figure 2L). The multi-well plate is then transferred into the insert stage of an epifluorescence microscope and time-lapse imaging recordings are conducted. Around 5 h post-initiation of the recordings live or heat inactivated *Bb* spirochetes are added and the recording continues for 24 h post-exposure. If TFM is performed, at the end of the recording sodium dodecyl sulfate (SDS) is added to acquire a reference image of the tracer beads in undeformed hydrogels where cells have been removed from the hydrogel surface.
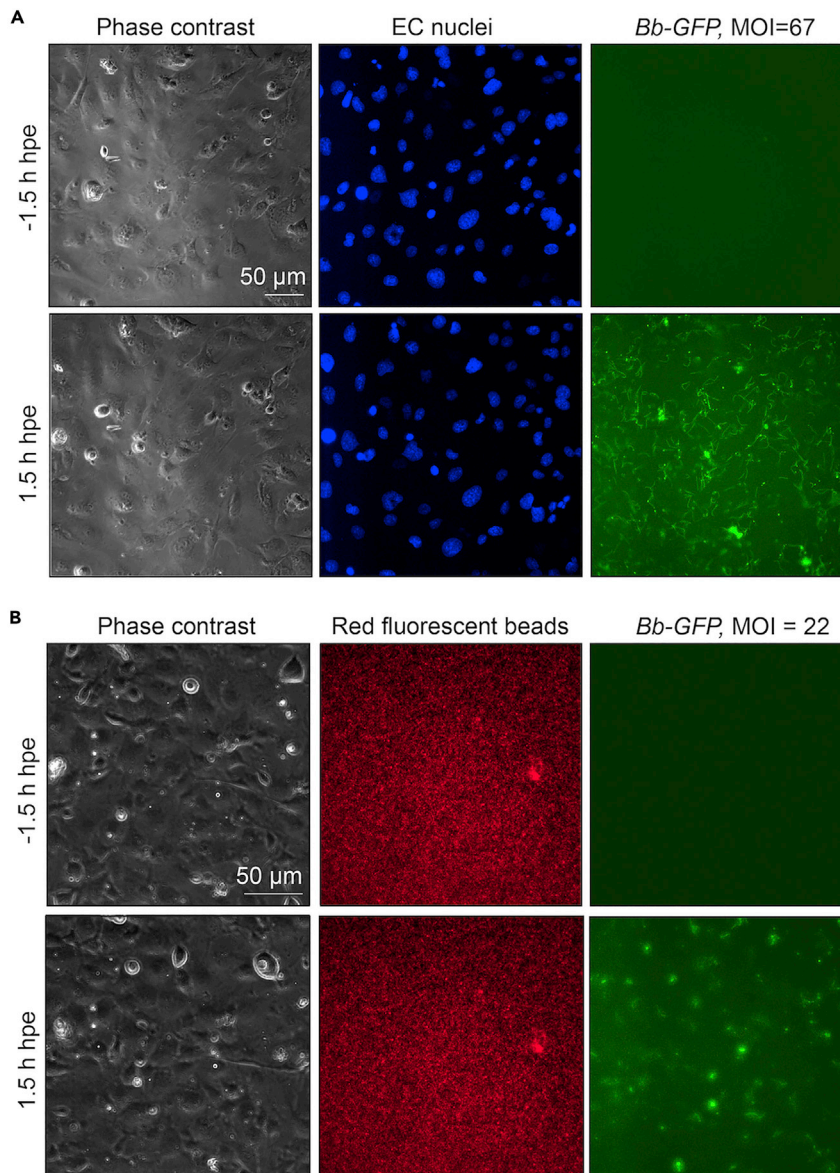
Figure 3. Multichannel images acquired prior and post-exposure to live or heat-inactivated *Bb*

(A) Columns show: phase contrast image of HMEC-1, Hoechst-stained HMEC-1 nuclei and *Bb-GFP* fluorescence before (upper row) and after (bottom row) *Bb-GFP* exposure. Times pre- and post-exposure (h post-exposure, hpe) are indicated. MOI is 67 bacteria/cell.

(B) Columns show: phase contrast image of HMEC-1, fluorescence of 0.2 μm red fluorescent tracer beads and *Bb-GFP* fluorescence before (upper row) and after (bottom row) *Bb-GFP* exposure. Times pre- and post-exposure (hpe) are indicated. MOI is 22 bacteria/cell.

11. Seeding of ECs on the hydrogels and staining of EC nuclei with Hoechst to characterize cell kinematics (Figure 3A).
    a. Detach the cells from their culture vessel using 0.25% trypsin/EDTA and seed them on the hydrogels.
        i. First wash the cells and their culture flask once with sterile PBS.
        ii. Add the appropriate amount of 0.05% trypsin/EDTA (2 mL on a 75-cm flask).
        iii. Incubate the flask at 37°C for 5–10 min to allow the detachment of the cells from their substrate.

  iv. Neutralize the trypsin by adding 10 mL of cell culture medium containing FBS.

  v. Pipet gently to break up the clumps of cells, and then place the solution into a conical centrifuge tube.

  vi. Take out 10 μL of the solution and very gently fill out the chamber underneath the cover-slip of a glass hemocytometer to count cells.

  vii. Pellet down the solution of cells contained in the conical centrifuge tube using centrifugation for 5 min at 200 × *g*.

  viii. During the 5 min waiting period, count the cells using the hemocytometer.

Alternatives: Use an automatic cell counter.

  ix. Remove the liquid out of the conical centrifuge tube while ensuring that the cell pellet is not disrupted.

  x. Resuspend the cells in the MCDB-131 full medium at a concentration of 4×10^5 cells/mL.

  xi. Seed the cells in suspension on the hydrogels by first removing the medium with which the hydrogels were incubated and then adding 1 mL of cell suspension on each hydrogel.

Note: If you do not plan to do TFM and just want to characterize changes in EC motility, you can place ECs on glass coverslips coated with collagen I. Just mix 1:100 collagen I solution (stock concentration is 3.1 mg/mL) in PBS and add 500 μL of the solution in each well of the multi-well plate. Incubate for 30 min at 37°C. After one wash with PBS, you can seed the glass coverslip with ECs as you would on hydrogels.

  xii. Let cells reside on the hydrogels for 24 h so they form a confluent monolayer.

b. Stain the EC nuclei with Hoechst and place live-microscopy medium on the wells.

  i. Prior to the initiation of the microscopy recording prepare a solution of 1 μg/mL Hoechst in cell culture media.

  ii. Remove the media from the cells and add 1 mL of Hoechst containing media.

  iii. Place the multi-well plate in the incubator for 10 min.

⚠ CRITICAL: Depending on your EC type you might need to adjust the concentration of Hoechst or reduce the incubation time or both. For example, for staining the nuclei of primary HUVECs we determined that a 100-fold lower concentration of Hoechst followed by a 5 min incubation are optimal for this cell type (see troubleshooting 2).

  iv. Aspirate out the Hoechst containing medium and wash the cells once with PBS.

  v. Add 1 mL warm live-microscopy medium by supplementing Leibovitz's L-15 with 10% fetal bovine serum, 10 ng/mL of EGF, and 1 μg/mL hydrocortisone as indicated above.

12. Initiation of acquisition of time-lapse images of cells and tracer beads.

a. Turn on your epifluorescence (or confocal microscope) and make sure the microscope incubator's temperature is set at 37°C for at least 30 min prior to the start of the microscopy recording.

b. Place the multi-well plate on the appropriate insert on the stage of your microscope.

c. Find the focal plane where the tracer beads are located in each well and make sure they look evenly dispersed in the field of view to be imaged (see troubleshooting 3).

Note: Especially if you are beginner in TFM, you might want to consider inspecting the hydrogels immediately after they are built and prior to activation with Sulfo-SANPAH. Hydrogels where beads' are not evenly distributed can be excluded from further processing.

d. Ensure the cells form a confluent monolayer and mark the specific position.

    e. Depending on how many replicate wells you want to track and how many conditions you desire to test mark different positions across the 24-well plate.

Note: We typically mark two positions per well so that a total of 48 positions are marked but that also depends on the conditions tested.

⚠ CRITICAL: Make sure your microscope's autofocus system is on to avoid potential drift in the normal (z-direction) over time. If drift occurs in the x-y direction it can be corrected, but there is no way to correct for drift in the z-direction.

    f. In the acquisition settings select the time interval to be 10 min (images acquired every 10 min).

    g. Select multi-channel acquisition and use a DAPI filter cube to image the cell nuclei, a GFP cube to image the GFP-expressing bacteria which will be added and an mCherry filter cube to image the tracer beads (Figures 3A and 3B).

Note: The filter cubes you will use depend on the color of the tracer beads you will choose and on the type of fluorescent protein your bacteria express. In our case power was set to 10% and the exposure time to 100 ms, when using a 40 × 0.60 NA Plan Fluor air objective. The microscope filters excitation/emission) used for DAPI, mCherry and GFP are 350/460, 470/525 nm and 530/645 nm, respectively. Note that we performed separate recordings to follow the nuclei of ECs and different ones to follow the tracer beads (Figures 3A and 3B). However, one could also simultaneously follow the tracer beads and EC nuclei. However, it might be pertinent to acquire two z-stacks at each instance of time one at the place where the tracer beads' intensity is maximum and one where the nuclei intensity is maximum.

    h. Initiate the time-lapse image acquisition.

13. Exposure of ECs to *Bb-GFP* during microscopy recording.

    a. Approximately 5 h post-initiation of video-microscopy, spin down (5 min at 2,000 × *g*) the desired volume of live or heat-inactivated *Bb-GFP* depending on the MOI you want to achieve.

Note: If you want to achieve an MOI of 10 *Bb*/cell in each well and assuming your ECs have doubled in each well (i.e., you have 8×10^5 cells per well), one needs to add 8×10^6 *Bb*.

    b. Wash the pellet once with PBS to remove the BSK culture media and spin down the bacteria for 5 min at 2,000 × *g*.

    c. Resuspend the bacteria in such a volume of PBS so that in each well of your 24-well plate you only add 10 μL of bacterial suspension.

Note: For a MOI of 10 *Bb*/cell resuspend 8×10^6 *Bb* in 10 μL PBS.

    d. When image acquisition stops and while you still have 10 min until next time point, carefully remove the lid of the multi-well plate.

    e. Add 10 μL of bacterial suspension in PBS aiming to release the droplet at the center of the well without disturbing the plate.

Note: We did this because we wanted to record cells several hours prior to exposure and continue recording after exposure to pathogens too. Therefore, we wanted to avoid manipulation and displacement of the plate sitting in the microscope insert stage.

    f. Add 10 μL of PBS alone on the control wells (no bacteria, vehicle control).

    g. Place the lid back and allow the time-lapse imaging to continue for approximately 24 h.

Note: Be careful when removing/returning the lid, as this can disturb the focal plane along the z-axis which cannot be corrected for.

14. Acquisition of reference image of tracer beads.
    a. Once you end the microscopy recording, carefully remove the lid of your multi-well plate.
    b. Add 500 μL of 10% SDS in water in each of the wells.
    c. Through the phase contrast image make sure that the cells have detached from the surface of the hydrogel and thus, the elastic hydrogel has returned to its initial undeformed state.
    d. Take a fluorescence image of the tracer beads using the same power and exposure time as during time-lapse imaging. This will serve as the reference image of the beads in the undeformed hydrogel.

Characterization of EC kinematics and hydrogel displacements using particle image velocimetry—Days 9–10

⏱ Timing: 2–10 h

The steps herein describe how to characterize EC displacements based on the images of the host EC nuclei and the displacements of the tracer beads within the hydrogel to calculate cell traction and monolayer stresses. In both cases we use a particle image velocimetry (PIV)-like technique to find EC and extracellular matrix (ECM) displacements.[14] The codes with example data can be found at https://github.com/ebastoun. All scripts and functions are written in MATLAB (MathWorks).

15. First, use the images of the EC nuclei to characterize changes in EC kinematics before and after exposure to *Bb* (Figures 4A and 4B).
    a. Place the time-lapse images of the nuclei into a folder as individual 16-bit tiff files.
    b. Perform PIV by comparing subsequent frames by running the urapiv.m function.
        i. Use the appropriate window size and overlap depending on the objective you used and the size of your nuclei.

    Note: We followed HMEC-1 nuclei that are ~10 μm diameter, used a 40× objective and the resulting calibration factor of the images was fcal=0.18 μm/pixel. We thus used windows of 48 pixels with an overlap of 24 pixels for running the urapiv.m function.[14] The size of the windows/overlap depends on the size of the moving objects, the texture within the image and the magnitude of displacements the objects are undergoing from frame to frame. Too small windows will improve the resolution of the PIV but might introduce noise while too big windows might smoothen the resulting motions and will reduce resolution.

        ii. For each experiment run the run_PIV.m script which calls the urapiv.m function to calculate the displacement vectors and store them as .txt files.
        iii. After generating and storing the .txt files you can inspect the vector displacements (u_x and u_y) by running the script Correlation_WT.m to overlay the vector displacements with the image of cells (Figure 4A).
        iv. While running the Correlation_WT.m script you can also calculate the average magnitude of the displacements as a function of time.

    Note: If you divide by the time interval between frames you can calculate the mean speed of cells.

        v. While running the Correlation_WT.m script you can calculate the correlation length of the movement of neighboring cells.[15]
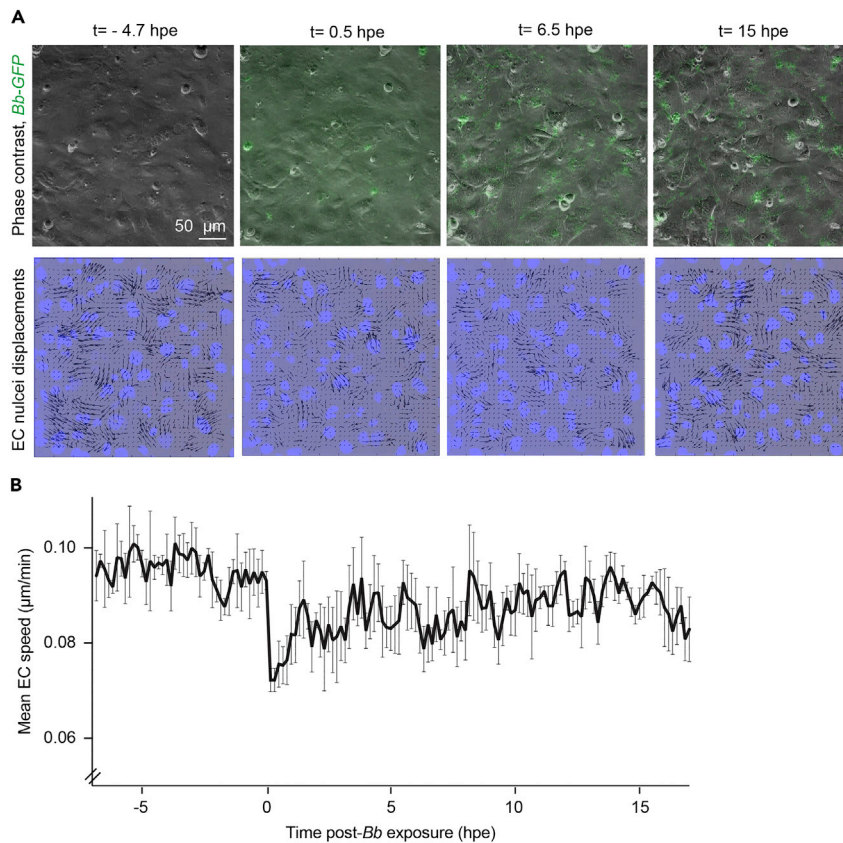
Figure 4. Analysis of HMEC-1 cell motility pre- and post-exposure to *Bb-GFP*
(A) Rows show representative: overlay of phase contrast image of HMEC-1 and *Bb-GFP* fluorescence (upper row) and overlay of Hoechst-stained HMEC-1 nuclei and HMEC-1 displacements (bottom row) determined through PIV. Columns show different time points pre- and post-*Bb* exposure. MOI is 200 bacteria per cell.
(B) Plot of mean HMEC-1 speed versus time (h) relative to the time point when host cells were exposed to *Bb* (mean+/-SEM, N=3 recordings).

    c. Save the output EC kinematical analysis as a .mat file and input various parameters (e.g., speed over time, correlation length) to Prism (GraphPad) if you wish to do statistical analysis of various independent experiments (Figure 4B).

    Note: You can also do the statistical analysis in MATLAB.

16. Measure the ECM displacements to perform TFM and monolayer stress microscopy (MSM).
    a. Place the time-lapse images of the fluorescent tracer beads into a folder as individual 16-bit tiff files.
    b. Add the reference image tiff file there too.

    Note: If you acquired 100 frames named beads001.tiff etc., then the reference image can be saved as beads101.tiff.

    c. Using the urapiv.m file perform PIV by comparing each frame with the reference frame you acquired at the end of the recording after the addition of SDS (see troubleshooting 4).
      i. Use the appropriate window size and overlap depending on the objective you used and the size of the tracer beads.

Note: We used a 40× objective (fcal=0.18 μm/pixel) to follow 0.2 μm tracer beads and windows of 32 pixels with an overlap of 16 pixels for running the urapiv.m function.

  ii. Run the urapiv.m function as before, with the exception that in this case you compare the image of the tracer beads at each time interval with the reference image of the undeformed beads.
  iii. After the beads displacements are saved as .txt files you can use custom-built scripts in MATLAB (MathWorks)[2] or other TFM published codes to calculate the resulting traction stresses exerted by cells and the strain energy (mechanical work) imparted by cells over time[3] (Figure 5A).

Note: There are various sources you can find those codes to perform TFM with detailed documentation. Some examples are shown here: https://github.com/jknotbohm, https://github.com/DanuserLab/TFM.

  iv. Save the strain energy overtime as a .mat file and input to Prism if you wish to do statistical analysis of various independent experiments (see troubleshooting 5) (Figure 5B).
  d. Based on the calculated traction stresses you can infer also monolayer stresses built within the cellular monolayer using MSM[16] (Figures 6A and 6B).

Note: For our custom-built scripts in MATLAB (MathWorks) please refer to: https://github.com/ebastoun/Monolayer-Stress-Microscopy. Note that in order to physically interpret the stresses that cells are exposed to, we make use of the principal directions where the magnitude of stresses does not depend on the choice of the coordinate system, and there is no shear stress component.[17,18] The principal stress I (σI) is related to the maximum tensile stresses in each point of the monolayer, whereas the principal stress II (σII) is related to the maximum compressive stresses in each point of the monolayer. An extended explanation of these mechanical variables can be found elsewhere.[1]

EXPECTED OUTCOMES

With the current protocol, users will be able to culture *Bb-GFP*, generate heat-inactivated *Bb-GFP*, and expose either of those to ECs in monolayer which reside on soft physiologically-relevant deformable matrices embedded with fluorescent tracer beads. Time-lapse multi-channel microscopy imaging prior to and over the time following exposure will be used to monitor the displacements of Hoechst-stained EC nuclei concurrently with the displacements of the tracer beads. This will enable users to follow alterations of the EC motion during exposure to *Bb* as well as alterations in the displacements that ECs impart on their underlying matrix through the engagement of their focal adhesions and cytoskeleton. Moreover, using image processing and previously developed codes to conduct TFM and MSM users will learn how EC motility changes before and after exposure and how the host cell traction stresses and monolayer stresses built within the cell monolayer are dynamically modulated due to exposure to live or heat-inactivated pathogens.

QUANTIFICATION AND STATISTICAL ANALYSIS

All image processing was performed using custom-built scripts in MATLAB (MathWorks). The codes to perform PIV on the time-lapse images of Hoechst-stained cell nuclei are available at our GitHub repository together with instructions on how to run them (https://github.com/ebastoun/Correlation_length_of_movement_of_epithelial_cells_in_monolayer). Sample data are also provided to allow users to test the codes. We also used the urapiv.m function to calculate displacements of hydrogels due to EC exerting stresses onto them. TFM codes have been previously explained and published elsewhere.[2,3,4] The codes for MSM are uploaded in our gitlab repository together with instructions on how to run them https://github.com/ebastoun/Monolayer-Stress-Microscopy.[16]
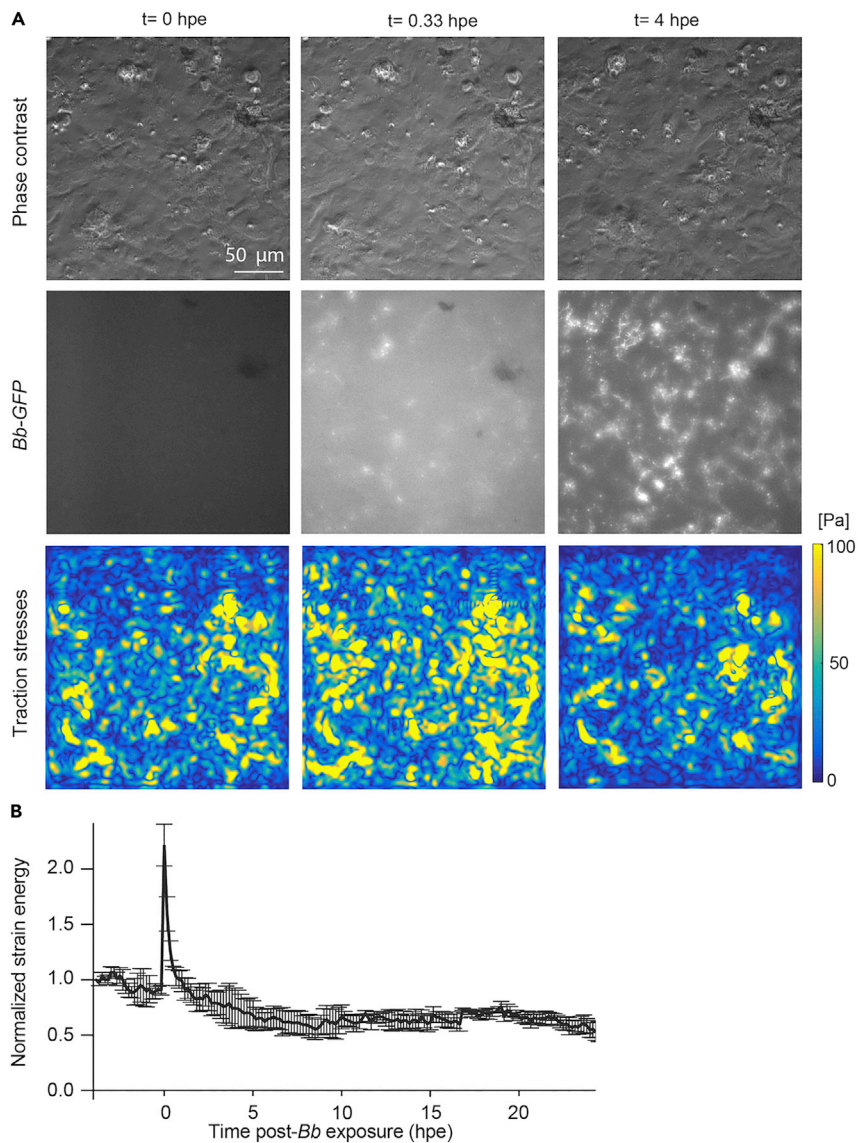
Figure 5. Traction stresses and strain energy of HMEC-1 pre- and post-exposure to *Bb-GFP*

(A) Columns show representative: phase contrast image of HMEC-1; *Bb-GFP* fluorescence; and cellular traction stresses map (3rd column, Pa) for HMEC-1 in monolayer at different time points (rows) pre- and post-exposure to *Bb-GFP*. MOI is 200 bacteria per cell and TFM was performed for cells residing on 3 kPa ECM.

(B) Normalized strain energy (mechanical work) imparted by HMEC-1 during a TFM recording pre- and post-exposure to *Bb-GFP* (mean+/-SEM, N=3 independent experiments). Strain energy has been normalized with respect to the first value at the beginning of each recording. Time (h) is represented relative to the time at which *Bb* was added.

Statistical parameters and significance are reported in the corresponding Figures and the Figure Legends. Boxplots and plots represent mean+/-SEM as indicated. Statistical analysis was performed in GraphPad PRISM 8.

LIMITATIONS

The protocol described herein concerns a particular EC type (HMEC-1) exposed to a specific extracellular bacterial pathogen (*Bb*). Its application in the context of different infection settings, e.g., different host cell types and bacterial or viral pathogens, requires optimization of different parameters. For infection protocols tailored to intracellular bacterial pathogens *Listeria monocytogenes*
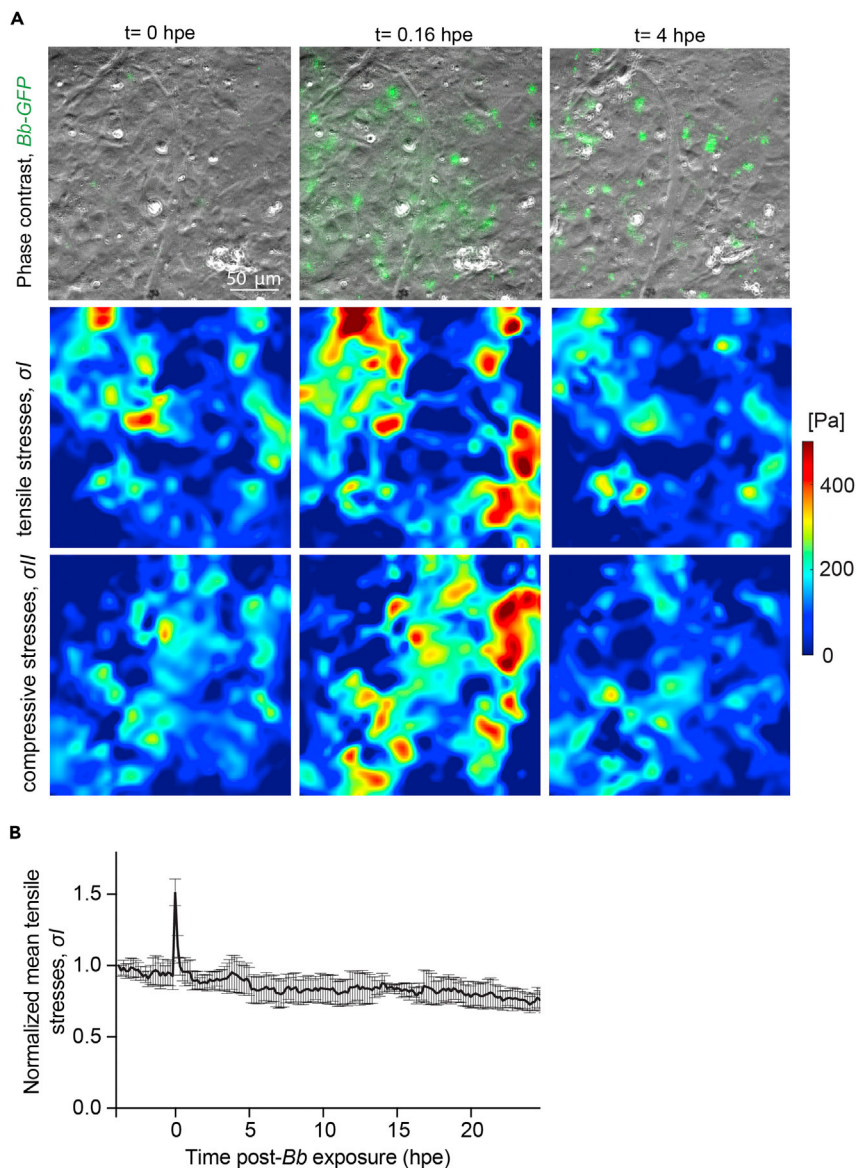
Figure 6. HMEC-1 monolayer stresses pre- and post-exposure to *Bb-GFP*

(A) Rows show representative: overlay of phase contrast image of HMEC-1 and *Bb-GFP* fluorescence (green); monolayer tensile stresses (σ_I); and compressive stresses (σ_{II}), for HMEC-1 in monolayer at different time points (columns) pre- and post-exposure to *Bb-GFP*. MOI is 200 bacteria per cell and cells reside on 3 kPa ECM.

(B) Normalized mean monolayer tensile stresses (σ_I) as a function of time post-exposure (mean+/-SEM, N=3 independent experiments). Mean σ_I has been normalized with respect to the first value at the beginning of each recording. Time (h) is represented relative to the time at which *Bb* was added.

or Rickettsia parkeri, please refer to our previous papers.[19,20] Moreover, in this particular protocol the investigation of the changes in motility of *Bb*-exposed ECs was performed for ECs residing on glass while traction forces were measured for ECs residing on soft 3 kPa hydrogels. This is due in that in our original paper, we initially placed ECs on collagen I-coated glass coverslips and followed them through microscopy prior- and post-exposure to *Bb* to evidence that shortly after exposure to pathogens ECs lower their migration speed but EC speed is recovered at later times.[1] Triggered by the observation that EC kinematics displayed time-dependent alterations post-exposure to *Bb*, we sought to investigate whether those are due to changes in cellular force transduction and thus performed TFM by placing ECs on soft 3 kPa hydrogels. We found that they do. Future studies could

address whether ECM stiffness and/or ECM-coating play a role in modulating the observed alterations in EC kinematics and dynamics upon pathogen exposure. The ECM stiffness for TFM was chosen so that it represents physiological values of subendothelial stiffness that ECs experience in small arteries.[7,21,22] However, during aging, hypertension, and atherosclerosis there are increases in subendothelial stiffness[23,24] and it could be interesting to study how increasing ECM might alter the behavior of ECs during exposure to pathogens and how that differs potentially for ECs residing on conventional glass coverslips. Finally, to estimate changes in inter- and intra-cellular stresses here we used MSM.[18,25] MSM though represents an indirect way of measuring cell monolayer tension, similar to other indirect ways such as laser ablation[26] and relies on several simplifications in terms of boundary conditions, mechanical properties, and the dimensionality of the system.[17,18] 2D MSM assumes that the intra- and inter- cellular stress components along the normal direction to the plane of the monolayer are negligible, and moreover, MSM considers perfect cell-cell and cell-ECM adhesions. All together, these previous assumptions allow to consider the simplification of the plane stress problem, where the thickness of the monolayer is assumed to be small, and it remains uniform and constant. Under this simplification, the analysis of stresses is focused on the plane of the monolayer. Therefore, MSM is based on both assumptions and does not provide an exact readout of intercellular forces which would allow to directly assess changes in barrier integrity. Continuing work on this aspect, including usage of FRET-based force sensors, could potentially facilitate a more absolute quantitation of the intercellular forces between neighboring ECs and how those directly relate to changes in barrier integrity.[27,28]

TROUBLESHOOTING

Problem 1
ECs do not attach well on the hydrogel and I cannot get a confluent monolayer.

Potential solution
First, this could be due to insufficient collagen I coating. Double check that during exposure of the hydrogels to UV, the UV wavelength is indeed 302 nm. If this is not the case, locate a transilluminator box that allows exposure to 302 nm. Alternatively, you can try doubling the concentration of the Sulfo-SANPAH to test whether that enhances collagen I coating. Make sure to wash out the remaining Sulfo-SANPAH and to proceed fast after activation with adding the collagen I solution. Finally, it could be that 3 kPa for your specific EC type is too soft and cells just do not firmly attach due to low stiffness. To assess whether that is the case build slightly stiffer hydrogels (e.g., 5 kPa).

Problem 2
I use a different EC type and Hoechst staining results in EC toxicity.

Potential solution
Depending on your EC type Hoechst or the concentration of Hoechst you use might be toxic for your ECs. First, test different conditions of Hoechst staining and assess whether they work for your EC type. Either lower the concentration of Hoechst or reduce the incubation time or both. If this does not work, you can use different EC nuclear dyes like SiR-DNA offered by Cytoskeleton. Moreover, one can also attempt to perform PIV-based kinematic analysis directly on the phase-contrast images. However, imaging the cell nuclei can be valuable in allowing one to conduct additional cell kinematical analysis like individual cellular tracking, correlation of movement of neighboring cells[29] and tracking of cellular numbers over the course of long-term live-cell imaging.

Problem 3
During the TFM image acquisition, I observe large displacements of the tracer beads to the extent that the beads move out of the focal plane imaged.

Potential solution

To perform TFM, cells must be able to displace the tracer beads embedded on the hydrogel on which cells reside as they transduce traction stresses onto it. HMEC-1 residing on 3 kPa hydrogels will definitely be able to impart deformations on the hydrogel and to our experience those are not large enough to move the beads out of the focal plane. However, if you use a higher magnification objective it is possible that this could happen. Also, if a different EC type is used that is more contractile or if cells are sub-confluent, deformations might be higher and that could lead to significant z-displacement of the beads due to cells imparting large normal deformations. In that case 2D TFM cannot be applied since the fluorescent beads will move in and out of the focal plane imaged. In that case one can either use a spinning disk confocal microscope and perform 3D TFM[30] or alternatively increase the stiffness of the hydrogel.

Problem 4

The displacements fields of the tracer beads that result after I run PIV on the TFM recordings appear rather noisy.

Potential solution

First check whether there is significant x, y or z drift. If there is x,y drift that can be corrected using image registration. However, if there is significant z drift there is nothing one can do, and the experiments need to be repeated. To avoid z drift, make sure when you remove the lid of the multi-well pate (in order to add SDS and detach the cells from the hydrogel) that you do not displace the plate. Moreover, if slight displacement is introduced in the reference image you can actually compare the position that you are imaging with the image of the tracer beads you acquired during the recording. You can correct for the slight drift which can be in x,y or even the z axis, by adjusting the focus and/or centering the image appropriately. Usually, there are some features in the texture of your tracer beads' image, like bead aggregates that are extremely bright, which can help you in performing this adjustment.

Problem 5

There is a large variation in the magnitude of cellular traction stresses from one experiment to another.

Potential solution

One potential reason for the discrepancies can be variations in cell confluency from experiment to experiment, despite seeding similar cell numbers. One way to ensure that monolayers in different experiments have similar cell numbers in the field of views images, is by staining the EC nuclei with a nuclear stain (e.g., Hoechst) and by acquiring multiple images of the cell nuclei in different fields of view. The host cell nuclei can be segmented and counted, and one can check whether indeed the density of cells is similar in the different fields of view. Another reason, that could lead to variations in the magnitude of cellular traction stresses from one experiment to another, could result from whether the beads imaged are indeed at the uppermost surface of the hydrogel or slightly below. To address this issue first endure that the upper hydrogel layer is thin and confined into a single plane. If that is the case, you should be in focus when imaging, irrespective of whether you are imaging the tracer beads' fluorescence or looking at the phase contrast image of ECs' basal surface. If the upper layer containing the tracer beads is slightly thicker (which might occur sometimes) you might be imaging deeper into the hydrogel (thus, estimating lower forces because these beads will also get displaced but to a lesser extent than beads right at the surface). Some of the TFM codes available allow you to take into account both the finite thickness of the substrate and the minute difference in height between the plane of the cells and the plane where the fluorescence of the tracer beads is maximal (in case those do not coincide).[31]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Effie E. Bastounis (effie.bastounis@uni-tuebingen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The codes generated during this study to measure EC displacements are available at https://github.com/ebastoun/Correlation_length_of_movement_of_epithelial_cells_in_monolayer (https://doi.org/10.5281/zenodo.7155531). Codes to perform TFM in cellular monolayers have been published elsewhere.[2,3,4] The codes generated during this study to measure monolayer stresses are available at https://github.com/ebastoun/Monolayer-Stress-Microscopy (https://doi.org/10.5281/zenodo.7155523).

AUTHOR CONTRIBUTIONS

E.E.B. designed and optimized the protocol for construction of polyacrylamide hydrogels on multi-well plates and for the performance of infection assays. E.E.B. performed EC kinematical analysis. E.E.B. and M.M. performed the TFM/MSM experiments. R.A.-Y. implemented the code and software to compute MSM. M.M. designed and prepared the graphical abstract and the figures presented herein under E.E.B.'s supervision. E.E.B. wrote the manuscript. P.K., M.C.T., R.A.-Y., and M.M. revised and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Yuste, R.A., Muenkel, M., Axarlis, K., Gómez Benito, M.J., Reuss, A., Blacker, G., Tal, M.C., Kraiczy, P., and Bastounis, E.E. (2022). Borrelia burgdorferi modulates the physical forces and immunity signaling in endothelial cells. iScience 25, 104793. https://doi.org/10.1016/j.isci.2022.104793.

2. Lamason, R.L., Bastounis, E., Kafai, N.M., Serrano, R., Del Álamo, J.C., Theriot, J.A., and Welch, M.D. (2016). Rickettsia Sca4 reduces vinculin-mediated intercellular tension to promote spread. Cell 167, 670–683.e10. https://doi.org/10.1016/j.cell.2016.09.023.

3. Saraswathibhatla, A., Galles, E.E., and Notbohm, J. (2020). Spatiotemporal force and motion in collective cell migration. Sci. Data 7, 197. https://doi.org/10.1038/s41597-020-0540-5.

4. Teo, J.L., Lim, C.T., Yap, A.S., and Saw, T.B. (2020). A biologist's guide to traction force microscopy using polydimethylsiloxane substrate for two-dimensional cell cultures. STAR Protoc. 1, 100098. https://doi.org/10.1016/j.xpro.2020.100098.

5. Dunham-Ems, S.M., Caimano, M.J., Pal, U., Wolgemuth, C.W., Eggers, C.H., Balic, A., and Radolf, J.D. (2009). Live imaging reveals a biphasic mode of dissemination of Borrelia burgdorferi within ticks. J. Clin. Invest. 119, 3652–3665. https://doi.org/10.1172/JCI39401.

6. Moriarty, T.J., Norman, M.U., Colarusso, P., Bankhead, T., Kubes, P., and Chaconas, G. (2008). Real-time high resolution 3D imaging of the lyme disease spirochete adhering to and escaping from the vasculature of a living host. PLoS Pathog. 4, e1000090. https://doi.org/10.1371/journal.ppat.1000090.

7. Bastounis, E.E., Yeh, Y.-T., and Theriot, J.A. (2018). Matrix stiffness modulates infection of endothelial cells by Listeria monocytogenes via expression of cell surface vimentin. Mol. Biol. Cell 29, 1571–1589. https://doi.org/10.1091/mbc.E18-04-0228.

8. Bastounis, E.E., Yeh, Y.T., and Theriot, J.A. (2019). Subendothelial stiffness alters endothelial cell traction force generation while exerting a minimal effect on the transcriptome. Sci. Rep. 9, 18209. https://doi.org/10.1038/s41598-019-54336-2.

9. Reed, S.C.O., Lamason, R.L., Risca, V.I., Abernathy, E., and Welch, M.D. (2014). Rickettsia actin-based motility occurs in distinct phases mediated by different actin nucleators. Curr. Biol. 24, 98–103. https://doi.org/10.1016/j.cub.2013.11.025.

10. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

11. Faralla, C., Bastounis, E.E., Ortega, F.E., Light, S.H., Rizzuto, G., Gao, L., Marciano, D.K., Nocadello, S., Anderson, W.F., Robbins, J.R., et al. (2018). Listeria monocytogenes InlP interacts with afadin and facilitates basement membrane crossing. PLoS Pathog. 14, e1007094. https://doi.org/10.1371/journal.ppat.1007094.

12. Trepat, X., Wasserman, M.R., Angelini, T.E., Millet, E., Weitz, D.A., Butler, J.P., and Fredberg, J.J. (2009). Physical forces during collective cell migration. Nat. Phys. 5, 426–430. https://doi.org/10.1038/nphys1269.

13. Subramani, R., Izquierdo-Alvarez, A., Bhattacharya, P., Meerts, M., Moldenaers, P., Ramon, H., and Van Oosterwyck, H. (2020). The influence of swelling on elastic properties of polyacrylamide hydrogels. Front. Mater. 7. https://doi.org/10.3389/fmats.2020.00212.

14. Gui, L., and Wereley, S.T. (2002). A correlation-based continuous window-shift technique to reduce the peak-locking effect in digital PIV image evaluation. Exp. Fluids 32, 506–517. https://doi.org/10.1007/s00348-001-0396-1.

15. Angelini, T.E., Hannezo, E., Trepat, X., Fredberg, J.J., and Weitz, D.A. (2010). Cell migration driven by cooperative substrate deformation patterns. Phys. Rev. Lett. 104, 168104. https://doi.org/10.1103/PhysRevLett.104.168104.

16. Aparicio-Yuste, R., Muenkel, M., Clark, A.G., Gómez-Benito, M.J., and Bastounis, E.E. (2022). A stiff extracellular matrix favors the mechanical cell competition that leads to extrusion of bacterially-infected epithelial cells. Front. Cell Dev. Biol. 10, 912318. https://doi.org/10.3389/fcell.2022.912318.

17. Tambe, D.T., Hardin, C.C., Angelini, T.E., Rajendran, K., Park, C.Y., Serra-Picamal, X., Zhou, E.H., Zaman, M.H., Butler, J.P., Weitz, D.A., et al. (2011). Collective cell guidance by cooperative intercellular forces. Nat. Mater. 10, 469–475. https://doi.org/10.1038/nmat3025.

18. Tambe, D.T., Croutelle, U., Trepat, X., Park, C.Y., Kim, J.H., Millet, E., Butler, J.P., and Fredberg, J.J. (2013). Monolayer stress microscopy: limitations, artifacts, and accuracy of recovered intercellular stresses. PLoS One 8, e55172. https://doi.org/10.1371/journal.pone.0055172.

19. Bastounis, E.E., Serrano-Alcalde, F., Radhakrishnan, P., Engström, P., Gómez-Benito, M.J., Oswald, M.S., Yeh, Y.-T., Smith, J.G., Welch, M.D., García-Aznar, J.M., and Theriot, J.A. (2021). Mechanical competition triggered by innate immune signaling drives the collective extrusion of bacterially infected epithelial cells. Dev. Cell 56, 443–460.e11. https://doi.org/10.1016/j.devcel.2021.01.012.

20. Bastounis, E.E., Radhakrishnan, P., Prinz, C.K., and Theriot, J.A. (2021). Volume measurement and biophysical characterization of mounds in epithelial monolayers after intracellular bacterial infection. STAR Protoc. 2, 100551. https://doi.org/10.1016/j.xpro.2021.100551.

21. Zieman, S.J., Melenovsky, V., and Kass, D.A. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. Arterioscler. Thromb. Vasc. Biol. 25, 932–943. https://doi.org/10.1161/01.atv.0000160548.78317.29.

22. Stroka, K.M., and Aranda-Espinoza, H. (2011). Endothelial cell substrate stiffness influences neutrophil transmigration via myosin light chain kinase-dependent cell contraction. Blood 118, 1632–1640.

23. Kohn, J.C., Lampi, M.C., and Reinhart-King, C.A. (2015). Age-related vascular stiffening: causes and consequences. Front. Genet. 6, 112. https://doi.org/10.3389/fgene.2015.00112.

24. Huynh, J., Nishimura, N., Rana, K., Peloquin, J.M., Califano, J.P., Montague, C.R., King, M.R., Schaffer, C.B., and Reinhart-King, C.A. (2011). Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. Sci. Transl. Med. 3, 112ra122. https://doi.org/10.1126/scitranslmed.3002761.

25. Gómez-González, M., Latorre, E., Arroyo, M., and Trepat, X. (2020). Measuring mechanical stress in living tissues. Nat. Rev. Phys. 2, 300–317. https://doi.org/10.1038/s42254-020-0184-6.

26. Bastounis, E.E., Radhakrishnan, P., Prinz, C.K., and Theriot, J.A. (2022). Mechanical forces govern interactions of host cells with intracellular bacterial pathogens. Microbiol. Mol. Biol. Rev. 86, e0009420. https://doi.org/10.1128/mmbr.00094-20.

27. Eder, D., Basler, K., and Aegerter, C.M. (2017). Challenging FRET-based E-Cadherin force measurements in Drosophila. Sci. Rep. 7, 13692. https://doi.org/10.1038/s41598-017-14136-y.

28. Sim, J.Y., Moeller, J., Hart, K.C., Ramallo, D., Vogel, V., Dunn, A.R., Nelson, W.J., and Pruitt, B.L. (2015). Spatial distribution of cell–cell and cell–ECM adhesions regulates force balance while maintaining E-cadherin molecular tension in cell pairs. Mol. Biol. Cell 26, 2456–2465. https://doi.org/10.1091/mbc.E14-12-1618.

29. Hayer, A., Shao, L., Chung, M., Joubert, L.-M., Yang, H.W., Tsai, F.-C., Bisaria, A., Betzig, E., and Meyer, T. (2016). Engulfed cadherin fingers are polarized junctional structures between collectively migrating endothelial cells. Nat. Cell Biol. 18, 1311–1323. https://doi.org/10.1038/ncb3438.

30. del Álamo, J.C., Meili, R., Álvarez-González, B., Alonso-Latorre, B., Bastounis, E., Firtel, R., and Lasheras, J.C. (2013). Three-dimensional quantification of cellular traction forces and mechanosensing of thin substrata by fourier traction force microscopy. PLoS One 8, e69850. https://doi.org/10.1371/journal.pone.0069850.

31. del Álamo, J.C., Meili, R., Alonso-Latorre, B., Rodríguez-Rodríguez, J., Aliseda, A., Firtel, R.A., and Lasheras, J.C. (2007). Spatio-temporal analysis of eukaryotic cell motility by improved force cytometry. Proc. Natl. Acad. Sci. USA 104, 13343–13348. https://doi.org/10.1073/pnas.0705815104.