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TITLE:

Combining Fluidic Devices with Microscopy and Flow Cytometry to Study Microbial Transport in Porous Media Across Spatial Scales

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SUMMARY:

Breakthrough curves (BTCs) are efficient tools to study the transport of bacteria in porous media. Here we introduce tools based on fluidic devices in combination with microscopy and flow-cytometric counting to obtain BTCs.

ABSTRACT:

Understanding the transport, dispersion, and deposition of microorganisms in porous media is a complex scientific task comprising topics as diverse as hydrodynamics, ecology, and environmental engineering. Modeling bacterial transport in porous environments at different spatial scales is critical to better predict the consequences of bacterial transport, yet current models often fail to upscale from laboratory to field conditions. Here, we introduce experimental tools to study bacterial transport in porous media at two spatial scales. The aim of these tools is to obtain macroscopic observables (such as breakthrough curves or deposition profiles) of bacteria injected into transparent porous matrices. At a small scale (10-1,000 µm), microfluidic devices are combined with optical videomicroscopy and image processing to obtain breakthrough curves and, at the same time, to track individual bacterial cells at the pore scale. At a larger scale, flow cytometry is combined with a self-made robotic dispenser to obtain breakthrough curves. We illustrate the utility of these tools to better understand how bacteria are transported in complex porous media such as the hyporheic zone of streams. As these tools provide simultaneous measurements across scales, they pave the way for mechanism-based models, critically important for upscaling. Application of these tools may not only contribute to the development of novel bioremediation applications but also shed new light on the ecological

strategies of microorganisms colonizing porous substrates.

INTRODUCTION:

Studies aiming to understand the transport of microbes through porous media have mainly been driven by concerns of contamination[1], the transmission of disease[2], and bioremediation[3]. In this regard, bacteria have mostly been treated as particles in transport models and processes such as filtration, straining[4], gravitational settling, or remobilization from biofilms. These have been identified as drivers of retention or transport of microbes. However, studying the transport of bacteria through porous landscapes can also inform us on the ecological strategies underpinning their success in these complex environments. This requires novel experiments and mathematical models operating at the single cell, population, or microbial community level.

Natural porous environments, such as those found in the hyporheic zone of streams and rivers, are densely colonized by diverse communities of biofilm-forming microbes[5]. Biofilms form structures that modify the flow and thus the transport and dispersal of bacteria in the liquid phase. The transport of bacteria at pore scale depends on the constrained space availability in the porous matrix[6] and motility-related dispersal may be an effective way to increase the individual fitness through reduced competition for resources in less densely populated areas. On the other hand, motile bacteria can also reach more isolated regions of the porous matrix and the extended exploration of such areas may provide ecological opportunities to motile populations. At larger spatial scales, biofilm growth diverts the flow paths also leading to (partial) clogging of pores and, thus, to the establishment of even more channelized and heterogeneous flow conditions. This has consequences for nutrient supply and dispersal capacity, frequency, and distance. Preferential flow, for instance, can generate so-called “fast-tracks” and motile bacteria can attain even higher velocities than the local flow along these tracks[7]. This is an effective way to increase the exploration of novel habitats.

A variety of tools avail themselves for the study of transport of motile and nonmotile bacteria and particles in porous media. Numerical models have great predictive capacities important for applications. However, they are often limited by inherent assumptions[8]. Laboratory-scale experiments[9,10] combined with breakthrough curve (BTC) modeling have provided significant insights in the importance of bacterial cell surface properties for sticking efficiency[11]. Typically, BTCs (i.e., the times series of particle concentration at a fixed location) are obtained via constant-rate releases and measurement of cell numbers at the outflow of the experimental device. In this context, BTCs reflect the advection-dispersion dynamics of bacteria in the porous matrix and can be extended by a sink term accounting for attachment. However, modeling of BTCs alone does not resolve the role of spatial organization of the porous substrate or biofilm for transport processes. Other macroscopic observables, like dispersivity or deposition profiles, have been proven to provide important information about the spatial distribution or the retained particles or growing communities. Microfluidics is a technology that allows studying transport in porous media by microscopy investigation[6,7,12]. However, experimental systems are typically constrained to a single length scale of resolution: the pore scale or the entire fluidic device scale.

Here, we introduce a suite of combined methods to study the transport of motile and nonmotile

bacteria in porous landscapes at different scales. We combine observations of bacterial transport at the pore scale with information at a larger scale by means of BTC analysis. Microfluidic devices built from soft lithography using polydimethylsiloxane (PDMS) are biocompatible, resistant to a range of chemicals, allow replicability at low costs, and provide excellent optical transparency as well as low autofluorescence critical for microscopic observation. Microfluidics based on PDMS has been previously used to study the transport of microbes in simple channels[13] or in more complex geometries[7]. However, typically microfluidics experiments focus on short-term horizons and epifluorescence microscopic observation of living cells is commonly restricted to genetically modified strains (e.g., GFP-tagged strains). Here we present tools to study bacterial transport using PDMS-based microfluidic devices in combination with microscopy and larger devices fabricated from poly(methyl methacrylate) (PMMA, also known as plexiglass) in combination with flow cytometry. PDMS and PMMA differ in gas permeability and surface properties, thus providing complementary opportunities to study bacterial transport. While the microfluidic device provides a more controlled environment, the larger device allows for experiments over extended periods of time or using natural bacterial communities. Microscopy counting at high temporal resolution in a dedicated area is used to obtain BTC in the PDMS-based microfluidic device. To obtain cell counts for BTC modeling from the PMMA-based device, we introduce a self-constructed automated liquid dispenser in combination with flow cytometry. In this setup, cells pass the fluidic device and are consecutively dispensed into 96 well plates. The temporal resolution is restricted by the minimum volume that can be accurately dispensed and thus the medium flow rate through the fluidic device. Fixative in the wells prevents growth and facilitates DNA staining for downstream flow-cytometric enumeration. To prevent bacterial growth during transport experiments we use a minimal medium (i.e., motility buffer).

Because protocols for the preparation of fluidic devices at different scales are readily available, we only briefly introduce the techniques to produce such devices and rather focus on the experimental procedures to record BTCs. Similarly, various routines exist for the flow-cytometric enumeration of microbes and users require expert knowledge to interpret results obtained by flow cytometry. We report the novel use of microfluidic devices in combination with microscopic imaging to record BTCs of fluorescently-tagged cells. At the pore scale, local velocities and trajectories are obtained by means of image processing. Further, we demonstrate the use of a PMMA-based fluidic device in combination with flow-cytometric counting to observe bacterial transport of motile and nonmotile cells in porous environments colonized by a native stream biofilm.

PROTOCOL:

1. Bacterial culture conditions

1.1. Working under a laminar flow hood, use 100 µL of a glycerol stock of GFP-tagged *Pseudomonas putida* KT2440 (1 x 10^7/mL, stored at -80 °C) to inoculate 5 mL of Luria-Bertani (LB) medium. Incubate at 30 °C while shaking at 250 rpm overnight.

1.2. The next day, resuspend 100 µL of the overnight culture in 5 mL of LB medium and incubate

at 30 °C shaking at 250 rpm for 5 h. Add a 1 mL aliquot into a 2 mL tube, allow to cool to room temperature (RT) for ~15 min and centrifuge (2,300 x *g* for 5 min).

1.3. Remove the supernatant and add 1 mL of motility buffer (10 mM potassium phosphate, 0.1 mM EDTA, supplemented with 1% w/v glucose, pH = 7.0) to the pellet. Vortex briefly to homogenize the sample. Dilute to reach the desired cell concentration (e.g., 5 x 10^5/mL).

NOTE: For experiments involving natural communities, such as those derived from streams, prepare a nonselective cultivation medium. For instance, use sterile-filtered and autoclaved stream water or an artificial stream water medium amended with a complex carbon source (e.g., diluted LB medium).

2. Preparation of a microfluidic device in polydimethylsiloxane

2.1. Design the desired porous geometry by means of computer-aided drafting (CAD) software[14], which consists of a matrix of circles (i.e., the impermeable obstacle to flow), described by radius size and center coordinates.

NOTE: An example of a porous geometry with randomized grain and pore sizes is provided in **Figure 1A**. An observation channel without obstacles close to the outlet facilitates the acquisition of BTCs.

2.2. Based on the chosen geometry, prepare a mold using standard SU-8-photolithography[14].

NOTE: Alternatively, molds can also be ordered from a dedicated microfabrication facility. In order to obtain heterogeneous fluid flow in the horizontal plane, it is important to design the thickness of the microfluidics chamber on the same order of magnitude as the average pore throat size. However, make sure that the dimensions of the microfluidic device are suitable for observation under the microscope (e.g., work on microscope slides).

2.3. Prepare 50 mL of PDMS by adding 10% of cross linker (dimethyl, methylhydrogen siloxane copolymer) to 90% of elastomer (PDMS) using a syringe without a needle. Work under clean conditions and avoid dust as much as possible. Mix the two reagents in a clean disposable container and apply a vacuum (100 mbar) for 30 min to remove dissolved air and bubbles from the viscous PDMS.

2.4. Place the mold into a Petri dish (100 mm in diameter, 15 mm high). Pour the PDMS onto the mold to the desired height (e.g., 2–5 mm). Cover the Petri dish and keep it at 60 °C for 4 h (overnight for thicker layers) to cure.

NOTE: For visualization purposes, light should be able to pass through the PDMS. Thus, a thin layer between 2–5 mm is desirable. Thicker layers (>5 mm) reduce transparency and thinner ones are subject to deformation during application.

2.5. Allow the microfluidic device to cool to RT. Once it is cooled, carefully remove the desired portion of PDMS with a scalpel.

NOTE: Strong pressure on molds results in fractures. Do not touch the PDMS with bare hands, as fingerprints will impair optical transparency.

2.6. Temporarily seal the bottom of the PDMS channel (where the desired geometry has been engraved) with tape. With a 0.5 mm diameter biopsy puncher, pierce channels to create an inlet and an outlet fitting the 0.5 mm (inner diameter) tubing.

NOTE: The soft nature of PDMS will ensure tightness once the tubing is inserted. Inlet and outlet channels cannot be made after the PDMS has been sealed to glass (see step 2.7).

2.7. Seal the microfluidic channel via oxygen plasma bonding using the high frequency generator (plasma bonder, **Table of Materials**). For this, clean a silicate glass slide (25 mm x 75 mm) with ethanol and let it dry. Remove the tape from the PDMS channel and place the channel with the porous side facing up. Treat the glass slide and PDMS surfaces with plasma for about 45 s at RT.

2.8. Place the pretreated PDMS channel onto the pretreated glass slide and heat at 100 °C for 30 min on a hot plate. Remove the microfluidic device from the hot plate and cool it to RT. Apply vacuum for 30 min to remove air from PDMS, which is almost impermeable to fluids but permeable to gas.

2.9. Prepare 100 mL of motility buffer and inject 1 mL into the microfluidic device using a syringe pump operating at 10 µL/min.

NOTE: Because the PDMS is undersaturated in gas (due to the previous vacuum step), bubbles will disappear within ~30 min.

3. Preparation of a fluidic device in poly (methyl methacrylate)

3.1. Design the desired geometry with the CAD software. If applicable, make sure that the dimensions are suitable for observation under the microscope (e.g., dimensions of a standard 96 well plate in combination with an appropriate microscope stage holder).

NOTE: The fluidic device is composed of a base (127 x 127 x 12 mm) and a lid (127 x 127 x 12 mm), both made of PMMA. An example technical drawing is supplied in **Figure 1A**.

3.2. To produce the base containing the pore compartment, remove 0.5 mm from the base PMMA layer and mill a groove (1.1 x 1.1 mm) for a rubber O-ring by means of high precision micromilling (**Table of Materials**). Drill 12 threaded holes (M5).

NOTE: The dimensions of the fluidic device may need to be adjusted to fit the microscope stage and focal distance. A technical drawing is supplied in **Supplementary Figure 1**.

3.3. Drill two threaded holes (type 1/4-28 UNF) for an inlet and outlet into the top part of the fluidic device and 12 holes (5.5 mm diameter) for screws. This will serve as the lid of the fluidic device.

NOTE: Expertise in micromilling is advisable. The authors use support from a specialized workshop.

3.4. In order to clean and sterilize the fluidic device prior to and after each use, soak both parts of the fluidic device in 7% HCl and rinse 3x with deionized water. Then screw the base and lid together using the 12 threaded holes.

4. Setup of the automated dispenser

NOTE: Commercially available liquid dispensers are costly and often do not offer the flexibility to dispense directly from the outflow of the fluidic device. Therefore, assembling a cheap and flexible robotic dispenser system from an XY plotter robot (**Table of Materials**) is recommended.

4.1. In order to dispense the outflow from the fluidic device into 96 well plates, mount the robotic dispenser onto a PMMA plate with milled cavities of 85.8 x 128 mm and a depth of 1 mm to hold 96 well plates.

4.2. Attach the outflow tube of the fluidic device to the robotic arm of the dispenser.

4.3. Download bCNC from github: https://github.com/vlachoudis/bCNC and follow the instructions to install the program.

4.4. Download dispenser.py from the supporting material of this article.

NOTE: This Python code provides a plugin to bCNC for a simple robotic dispenser layout.

4.5. Connect the robotic dispenser to the computer running bCNC and identify the correct COM port. In bCNC, click the home button to return the robotic dispenser to the home position.

NOTE: Homing returns the robotic dispenser to a known position (X = 0, Y = 0) and therefore improves the accuracy of the dispenser.

4.6. Prior to the experiment, prepare a sufficient number of 96 well plates, with wells containing an appropriate amount of fixative (e.g., final concentration 3.7% formaldehyde).

NOTE: For instance, at a flow rate of 0.2 mL/min, 100 µL are dispensed into each well every 30 s. Therefore, add 10 µL of 37% formaldehyde to each well to reach a final concentration of formaldehyde between 2–4%. Using eight 96 well plates will allow the experiment to continue for more than 6 h with a total of 768 data points. Note that GFP-tagged cells may lose their

fluorescent signal after fixation using formaldehyde.

5. Analyze bacterial transport using PDMS microfluidic devices

5.1. Place the PDMS microfluidic device previously saturated with motility buffer on the microscope stage. Use tape to fix the tubing to minimize disturbance of the flow during stage movement.

5.2. Move the microscope stage to the observation channel close to the outlet. Using bright field microscopy or phase contrast, focus on the center of the observation channel and adjust the magnification to visualize individual bacterial cells.

5.3. Switch the light path settings to fluorescence microscopy and adjust the camera exposure time to resolve individual bacterial cells (e.g., 100 ms), or so that the fluorescence signals of the cells are at least 3x stronger than the background noise.

5.4. Next, insert the inlet tubing into a 2 mL tube containing the bacterial suspension. Reverse the pump direction and start withdrawing the suspension at a flow rate of 1 µL/min. Scan the cross section of the entire observation channel recording a composite picture every 2 min over the entire duration of the experiment.

6. Basic image processing

NOTE: The goal of these basic image processing routines is to count bacterial cells in the recorded images. Optimal processing procedures depend on the technical specifications of the microscope and camera, as well as on the fluorescence properties of the bacterial strain used in the experiment and therefore need to be adjusted.

6.1. First, export images in .tiff format.

6.2. Import images to a desired software platform (e.g., MATLAB, ImageJ, R, or Python). Remove camera noise, which is a random variation of pixel intensity, and correct for optical aberration.

NOTE: This can be done by applying a Gaussian filter to each picture; the size of the filter depends on the quality of the camera sensor. Optical aberration can be removed by normalizing each picture by a reference image collected in the absence of the specimen with the same optical configuration, because it depends on the properties of the optical system.

6.3. Crop the images to a region of interest. Identify a threshold value (pixel intensity), so that values greater than the threshold include bacterial cells. Subtract the threshold value from each picture.

NOTE: In case the images are unevenly illuminated (because of optical aberration or fluorescent signal from dust) it may be useful to apply an adaptive threshold, which chooses a threshold value

based on local mean intensities.

6.4. Binarize the resulting image, so that bacterial cells take a value of 1, whereas background takes a value of 0. Remove clusters of pixels with an area smaller than the smallest bacterial cell size in pixels.

6.5. Sum the binarized image to obtain the total number of pixels of the remaining clusters. Divide the number of pixels by the average size of a bacterial cell in pixels to obtain an estimate of the number of cells. Knowing the depth of view and the area of investigation, transform counts into concentration (particles/mL).

6.6. To identify the concentration of the injected bacterial suspension, inject the bacterial suspension into the observation channel of a clean microfluidic device with a 1 mL syringe. Record the image and calculate the influent bacterial concentration (C_0) as described before (steps 6.1−6.5).

6.7. Visualize BTCs by normalizing the effluent bacterial concentration (C) with the influent bacterial concentration (C_0) and plot C/C_0 versus time.

7. Analyze bacterial transport at the pore scale

7.1. In order to analyze local velocities and trajectories of bacteria transported through the porous matrix, move the microscope stage to a region of interest and adjust the focus to the center of the microfluidic device. Set the microscope to bright field or phase contrast.

NOTE: Use fluorescence microscopy in case the fluorescence signal of the bacterial cells allows recording images at exposure times shorter than the average displacement time.

7.2. Record time-lapse images (video) at an exposure time that captures bacterial displacement (shorter than the average displacement over a number of pixels smaller than the object size), and that optimizes bacterial cell detection (e.g., exposure of 20 ms and images recorded every 50 ms). Record pictures over a sufficient amount of time in order to record enough to be statistically representative of the slowest trajectories (e.g., 3 min).

NOTE: Make sure the computer has enough disk space.

7.3. To remove background noise, subtract the average of all recorded images from each image. To do that, create a matrix whose result is the sum of the intensity of all the images for each pixel, and divide it by the number of images.

7.4. From the resulting matrix (A), determine the modulus (B) of the numerical gradient $\begin{bmatrix} g_x \\ g_y \end{bmatrix}$ and normalize it by its maximum value (*max*), as defined below.

$$\begin{bmatrix} g_x \\ g_y \end{bmatrix} = \begin{bmatrix} \frac{\partial A}{\partial x} \\ \frac{\partial A}{\partial x} \end{bmatrix}$$

$$B = \frac{\sqrt{{g_x}^2 + {g_y}^2}}{max}$$

7.5. Binarize the matrix B via intensity thresholding (see step 6.5) and record coordinates (X, Y in pixel or mm) and the time of image acquisition into a three-column file. Then apply a particle tracking script to process the recorded data and compute the trajectories.

NOTE: For instance, use the established protocol[15] and the freely available particle tracking code (http://site.physics.georgetown.edu/matlab/).

8. Study bacterial filtration by means of deposition profiles

8.1. To obtain deposition profiles of GFP-tagged *P. putida* KT2440 cells by fluorescence microscopy, record a composite image of the entire porous channel before (i.e., background) and after injection of bacterial suspension through the microfluidic device. Use an exposure time that allows acquiring bacterial fluorescent signal (e.g., 100 ms), without bleaching the signal.

8.2. Export images and import into desired software. Remove background from the images recorded after bacterial injection.

8.3. Integrate the total fluorescence signal of retained bacteria along the transversal sections of the porous channel. In order to compute the deposition profile, plot the integrated florescence signal versus the porous channel length.

9. Analyze bacterial transport using PMMA fluidic devices and flow cytometry

9.1. Connect the peristaltic pump with the inlet using 50 cm (1 mm inner diameter) tubing and the outflow with the automated dispenser using the same tubing (50 cm, see section 4).

NOTE: Use the pump to dispense cultivation media and to inject bacterial cells. Use Luer-lock connectors and three-way valves to shift between medium and bacterial suspension during the constant-rate release.

9.2. Pump cultivation medium in the fluidic device. Note the arrival of medium at the outlet tubing fixed to the robotic dispenser.

9.3. Simultaneously start injecting the bacterial suspension through the PMMA fluidic device at a flow rate of 0.2 mL/min and dispensing. Inject bacterial suspension equivalent to several pore

volumes (e.g., 30x the volume of the fluidic device). After injection, switch to sterile cultivation medium until the end of the experiment.

NOTE: The volume of the fluidic device is approximately 0.2 mL, thus at the proposed flow rate every minute one entire volume is exchanged.

9.4. Once a 96 well plate is completed, cover the plate and store at 4 °C. Analyze bacterial abundance via flow cytometry, following established protocols[16].

NOTE: For instance, add 25 µL of the green fluorescent nucleic acid stain SYTO13 (**Table of Materials**) at 0.025 mM in ultrapure water to each well. Incubate for 15 min in the dark and then analyze using a flow cytometer equipped with a 488 nm laser and detectors at 515 nm.

9.5. Prior to BTC analysis, consider the dilution of the sample due to the addition of fixative and stain. Correct the bacterial abundance by a factor of 1.35 to account for fixative and stain.

REPRESENTATIVE RESULTS:

To illustrate the functionality of the presented workflow, we performed experiments using genetically modified *Pseudomonas putida* KT2440, a gram negative motile bacterium important for bioremediation and biotechnology. Genetically modified versions of this strain that express GFP are commercially available. A nonmotile strain of *P. putida* KT2440 that lacks the relevant structural and regulatory genes for motility is also available. Using both motile and nonmotile GFP-tagged *P. putida* KT2440, we performed sequential experiments in PDMS microfluidic devices with a random array of pillars (**Figure 1B**) and recorded BTCs (**Figure 2A**). BTCs were normalized to the concentration of injected cells (C_0). Simultaneously, bacterial trajectories at the pore scale were visualized via image processing and particle tracking as described above (**Figure 2B**).

Next, we performed experiments with large-scale fluidic devices milled from PMMA (**Figure 1A**). Motile and nonmotile *P. putida* KT2440 (non-fluorescent) were injected into a regularly spaced porous matrix and BTCs were obtained using the liquid dispenser and flow cytometry counting as described above (**Figure 3A**). Strikingly, in a porous environment devoid of biofilm, motile and nonmotile *P. putida* KT2440 showed a markedly different transport behavior. In a porous matrix colonized for 48 h with a complex stream biofilm community, these differences in BTC between motile and nonmotile *P. putida* KT2440 vanished (**Figure 3B**).

FIGURE LEGENDS:

Figure 1: Fluidic devices to study microbial transport in porous media. (**A**) Illustration of a fluidic device milled from PMMA. The cross section shows the arrangement of the pillars within the fluidic device. The insert shows a porous matrix with a regularly spaced grid of pillars and the respective velocity flow field. See **Supplemental Figure 1** for detailed technical drawings. (**B**) The PDMS device is mounted onto a microscopy glass slide. Shown are the inflow and outflow connected to the medium reservoir and the syringe pump, respectively. The observation

chamber for microscopic counting is placed as a separate chamber without a porous matrix onto the same microscope slide. The insert shows a porous matrix with a random array of pillars in diameter and spacing.

Figure 2: Bacterial transport at channel and pore scale in the PDMS fluidic device. (**A**) BTCs of motile and nonmotile *P. putida* KT2440 (GFP-tagged) obtained with a PDMS microfluidic device and microscopic counting. (**B**) Trajectories of nonmotile cells at the pore scale. Colors are chosen to enhance differentiation of trajectories.

Figure 3: Bacterial transport at channel and pore scale in the PMMA fluidic device. (**A**) BTCs of motile and nonmotile *P. putida* KT2440 (non-tagged) obtained using a PMMA fluidic device and flow cytometry counting. (**B**) The fluidic device was colonized by a natural stream community for 2 days.

Supplementary Figure 1: Technical drawings of the PMMA fluidic device. The device is composed of a base unit containing the porous matrix and a lid unit featuring the holes for the inlet and outlet. The device is sealed using 12 screws and an O-ring.

DISCUSSION:

Here we suggest two means to study the transport of microbes through porous systems at the single cell and population level. While the study of transport phenomena using BTC modeling has provided valuable insights into the spread of pathogens or contaminants at the ecosystem scales, difficulties to scale from laboratory experiments to field conditions still exist. The tools described here allow researchers to experimentally resolve the spatial and temporal scales in order to better understand the ecological strategies of microbes relevant for transport in porous environments. Experimenters may use or modify these systems to study microbial traits other than motility, such as chemotaxis or quorum sensing, or modify the geometry or other habitat characteristics of the porous matrix. Moreover, using these systems the bacterial transport behavior can be readily coupled to deposition profiles, which provide important insights into colonization patterns and are critical to understand how biofilms modify local flow fields. We anticipate that a better understanding of microbial strategies to disperse and colonize porous media will improve model predictions and thus contribute to the management of pathogen spread or contaminant containment. Further modifications of the system may also contribute to the development of novel filtration devices or biotechnology tools in which cells need to be physically separated.

We recommend PMMA-based devices for large and long-term experiments and PDMS-based devices for smaller, shorter term experiments or when high temporal resolution is critical. It has to be kept in mind that the two materials have different properties. For instance, PDMS is permeable to gases like oxygen, while PMMA is gas tight. This difference might be used to study gas consumption in PMMA, while PDMS might be more suitable for experiments where oxygen limitations related to bacterial respiration are undesired.

In general, the protocols described here are easily reproducible and data obtained using these

tools consistently reveal differences in the transport of motile and nonmotile bacteria. The self-made liquid dispenser may be replaced by a commercially available alternative. However, for reasons of versatility and cost-effectiveness we recommend the one described here. Critical steps in the protocol mainly concern the handling of the fluidic devices and experience with image processing. The quality of data obtained through image analysis critically depends on image quality (mainly determined by focus and exposure time) and an appropriate thresholding strategy. Data quality obtained by flow-cytometric counting critically depends on effective fixing and staining of the cells and expertise in the interpretation of flow cytometry results.

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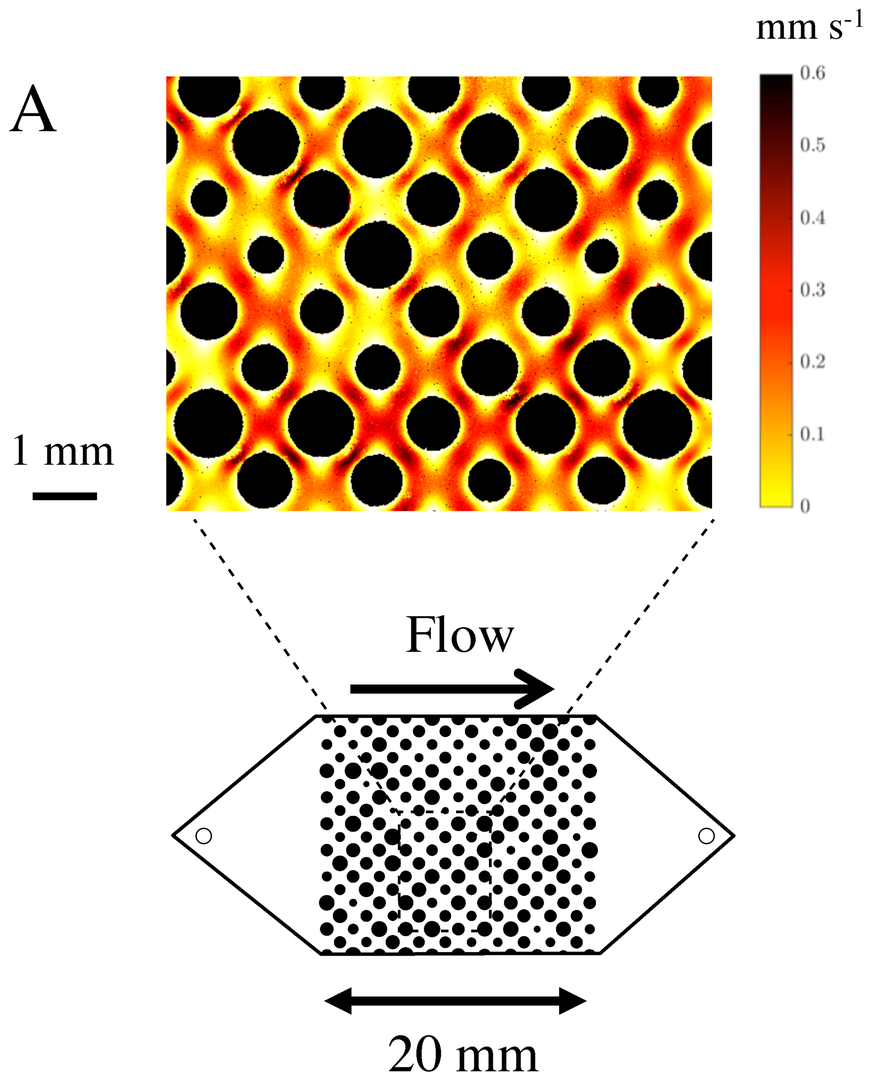
DISCLOSURES:

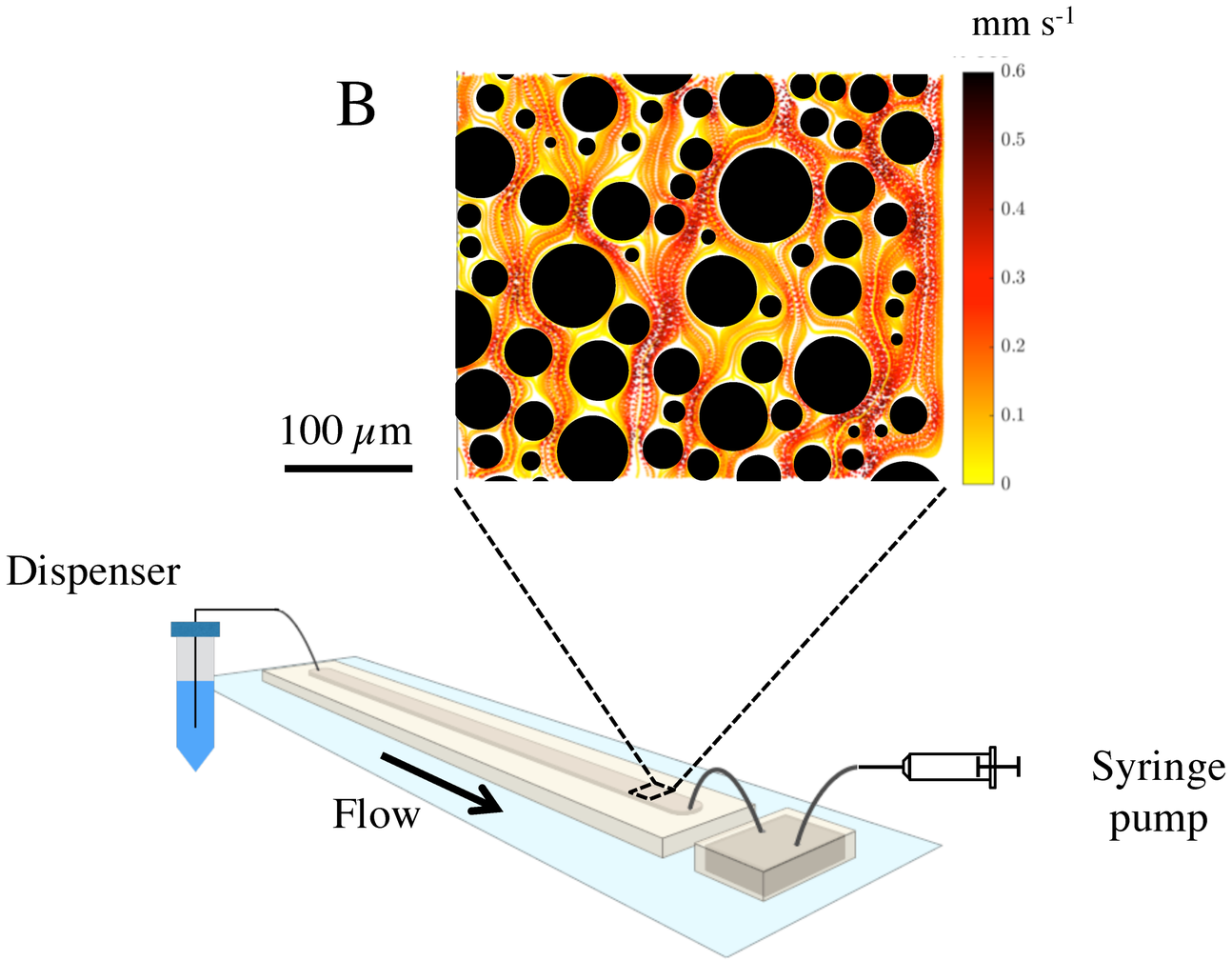
The authors have nothing to disclose.

REFERENCES:

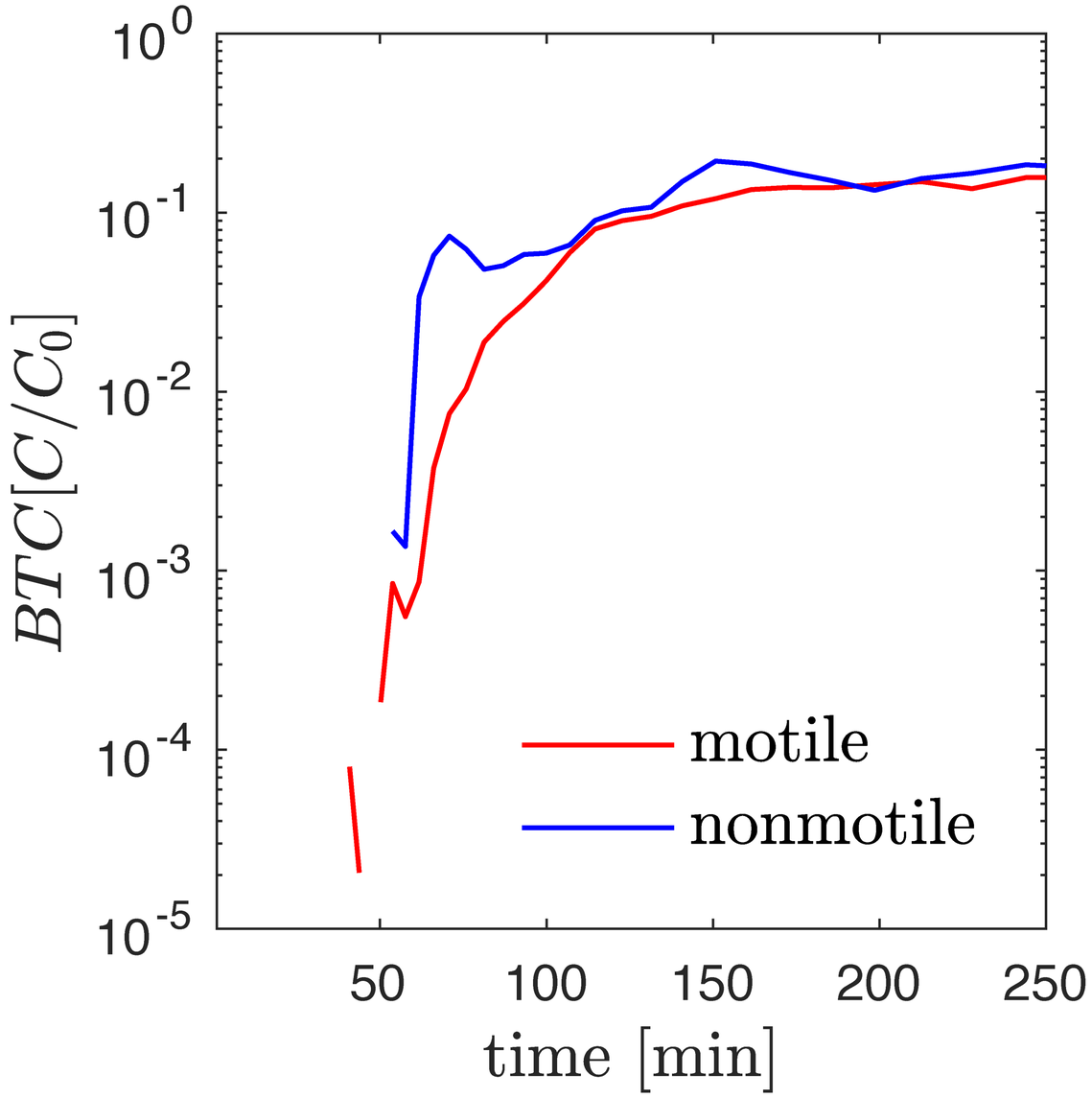
1. Stevik, K., Aa, K., Ausland, G., Fredrik Hanssen, J. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: a review. *Water Research*. **38** (6), 1355–1367 (2004).
2. Ribet, D., Cossart, P. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection*. **17** (3), 173–183 (2015).
3. Ginn, T. R., Wood, B. D., Nelson, K. E., Scheibe, T. D., Murphy, E. M., Clement, T. P. Processes in microbial transport in the natural subsurface. *Advances in Water Resources*. **25** (8), 1017–1042 (2002).
4. Foppen, J. W., Van, M. H., Schijven, J. Measuring and modelling straining of Escherichia coli in saturated porous media. *Journal of Contaminant Hydrology*. **93** (1–4), 236–254 (2007).
5. Battin, T. J., Besemer, K., Bengtsson, M. M., Romani, A. M., Packmann, A. I. The ecology and biogeochemistry of stream biofilms. *Nature Reviews Microbiology*. **14** (4), 251–263 (2016).
6. Bhattacharjee, T., Datta, S. S. Bacterial hopping and trapping in porous media. *Nature Communications*. **10** (1), 2075 (2019).
7. Creppy, A., Clément, E., Douarche, C., D'Angelo, M. V., Auradou, H. Effect of motility on the transport of bacteria populations through a porous medium. *Physical Review Fluids*. **4** (1), 013102 (2019).
8. Tufenkji, N. Modeling microbial transport in porous media: Traditional approaches and recent developments. *Advances in Water Resources*. **30** (6–7), 1455–1469 (2007).
9. Camesano, T. A., Logan, B. E. Influence of Fluid Velocity and Cell Concentration on the Transport of Motile and Nonmotile Bacteria in Porous Media. *Environmental Science & Technology*. **32** (11), 1699–1708 (1998).
10. Lutterodt, G., Basnet, M., Foppen, J. W. A., Uhlenbrook, S. The effect of surface characteristics on the transport of multiple Escherichia coli isolates in large scale columns of quartz sand. *Water Research*. **43** (3), 595–604 (2009).
11. Bozorg, A., Gates, I. D., Sen, A. Impact of biofilm on bacterial transport and deposition in porous media. *Journal of Contaminant Hydrology*. **183** (Supplement C), 109–120 (2015).

12. Long, T., Ford, R. M. Enhanced Transverse Migration of Bacteria by Chemotaxis in a Porous T-Sensor. *Environmental Science & Technology*. **43** (5), 1546–1552 (2009).
13. Rusconi, R., Garren, M., Stocker, R. Microfluidics Expanding the Frontiers of Microbial Ecology. *Annual Review of Biophysics*. **43** (1), 65–91 (2014).
14. Xia, Y., Whitesides, G. M. Soft Lithography. *Annual Review of Materials Science*. **28** (1), 153–184 (1998).
15. Crocker, J. C., Grier, D. G. Methods of Digital Video Microscopy for Colloidal Studies. *Journal of Colloid and Interface Science*. **179** (1), 298–310 (1996).
16. del Giorgio, P. A., Bird, D. F., Prairie, Y. T., Planas, D. Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13. *Limnology and Oceanography*. **41** (4), 783–789 (1996).





A



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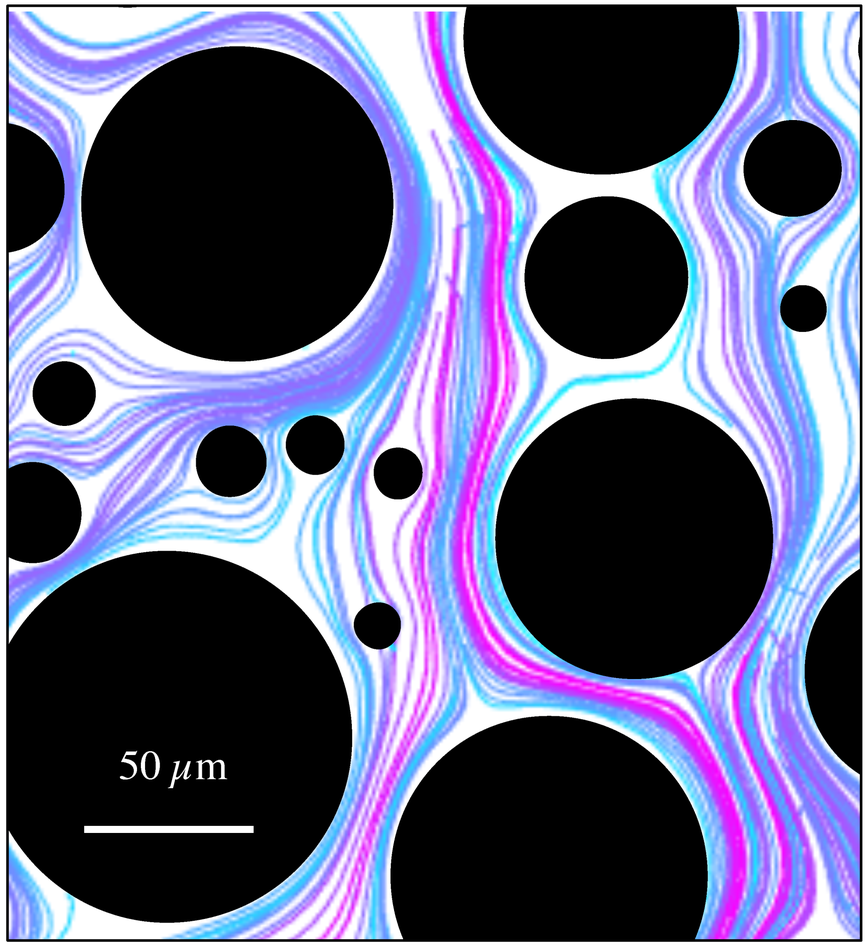

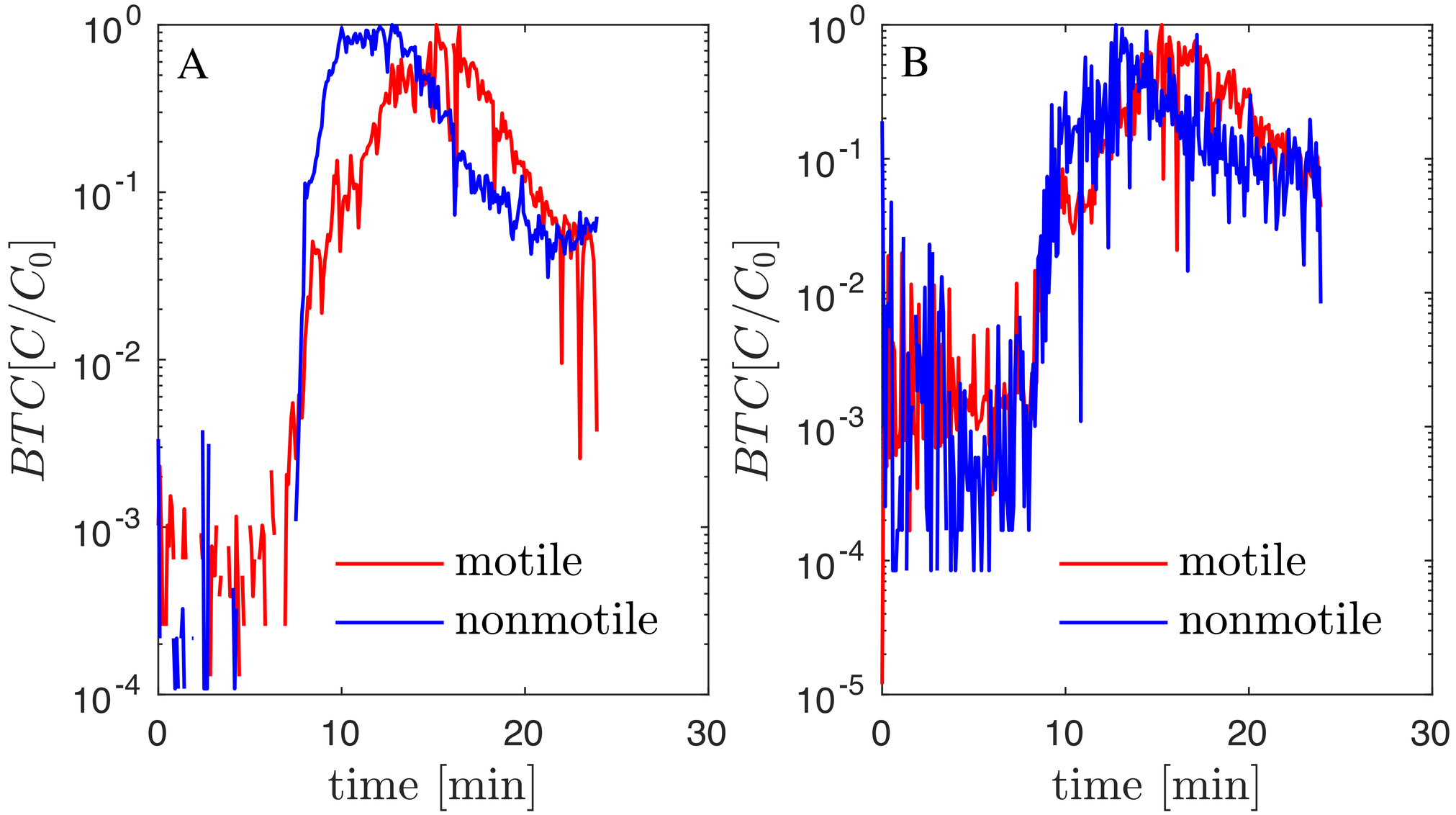

Name of Material/Equipment	Company	Catalog Number
EDTA	Sigma	
Elastomer Sylgard 184	Dowsil	101697
Flow cytometer NovoCyte	Acea	
Glucose	Sigma	
LB broth	BD	
Liquid dispenser, XY Plotter Robot Kit	makeblock	
Microscope Axio Imager	Zeiss	
Microscope AxioZoom v16	Zeiss	
Microscope slides, 75 mm × 25 mm	Corning	
Minipuls 3 peristaltic pump	Gilson	
Plasma bonder Corona SB	BlackHole Lab	
Potassium phosphate	Sigma	
Syringe pump New Era NE 4000	New Era	
Syto 13 Green Fluorescent Nucleic Acid Stain	Molecular Probes, Invitrogen	
Tygon tubing	Ismatec	
WF31SA universal milling machine	Mikron	

Comments/Description

https://www.makeblock.com/project/xy-plotter-robot-kit

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Dear Editor,
Many thanks for the feedback on our manuscript. We have address all issues in the revised manuscript. Please see our specific responses below (in red). We also added a detailed technical drawing for the PMMA device as Supporting Figure (and adapted Fig. 1 accordingly).

Yours sincerely,
Hannes Peter

2. Please note that the highlighted protocol text will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks or menu selections for software actions, numerical values for settings, etc.). There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have substantially revised the software-related sections of the manuscript to provide as much detail as possible. However, many of the basic image processing routines depend on the specific microscopy/camera system used. For instance, cleaning images from background depends on the numerical noise of the camera, whereas image cropping depends on the illumination quality of the microscope. Hence, we opted against providing annotated code to perform these steps. Also, there are numerous software solutions (e.g. ImageJ, code written in Python, R, C or Matlab) and these basic operations can be performed by any of these. We therefore provide guidelines for such basic image processing but recommend users to explore alternative software solutions.

3. As we can only film 2.75 pages of the protocol, please review and shorten the highlighted portion to 2.75 pages. Note that the highlighted content should contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please note that the editor has removed highlighting of sections 3 and 4 to condense the highlighted content.

Thank you for condensing the highlighted content. We have carefully selected the highlighted text for the script.

4. Please address specific comments marked in the attached manuscript.

We addressed all specific comments.

5. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove any ™/®/© symbols and sort the materials alphabetically by material name.

Corrected.

1. Preparation of a microfluidic device in polydimethylsiloxane

1.1. Design the desired porous geometry which consists of a matrix of circles.

1.2. Based on the chosen geometry, prepare a mold using standard SU-8-photolithography[14].

1.3. Prepare 50 mL of PDMS by adding 10% of cross linker to 90% of elastomer. Mix the two reagents and apply a vacuum (100 mbar) for 30 min to remove dissolved air and bubbles.

1.4. Place the mold into a Petri dish. Pour the PDMS onto the mold to the desired height (e.g., 2–5 mm). Cover the Petri dish and keep it at 60 °C for 4 h (overnight for thicker layers) to cure.

1.5. Allow the microfluidic device to cool to RT. remove the PDMS with a scalpel.

1.6. Temporarily seal the bottom of the PDMS channel with tape. With a 0.5 mm diameter biopsy puncher, pierce channels to create an inlet and an outlet.

1.7. Remove the tape from the PDMS channel and place the channel with the porous side facing up. Treat the glass slide and PDMS surfaces with plasma for about 45 s at RT.

1.8. Place the pretreated PDMS channel onto the pretreated glass slide and heat at 100 °C for 30 min cool it to RT. Apply vacuum for 30 min to remove air from PDMS.

2. Preparation of a fluidic device in poly (methyl methacrylate)

2.1. To produce the base containing the pore compartment, remove 0.5 mm from the base PMMA layer and mill a groove (1.1 x 1.1 mm) for a rubber O-ring by means of high precision micromilling (**Table of Materials**). Drill 12 threaded holes (M5).

2.2. Drill two threaded holes (type 1/4-28 UNF) for an inlet and outlet into the top part of the fluidic device and 12 holes (5.5 mm diameter) for screws. This will serve as the lid of the fluidic device.

2.3. Then screw the base and lid together using the 12 threaded holes.

3. Setup of the automated dispenser

3.1. mount the robotic dispenser onto a PMMA plate to hold 96 well plates.

3.2. Connect the robotic dispenser to the computer running bCNC and identify the correct COM port. In bCNC, click the home button to return the robotic dispenser to the home position.

4. Analyze bacterial transport using PDMS microfluidic devices

4.1. Place the PDMS microfluidic device previously saturated with motility buffer on the microscope stage.

4.2. Using bright field microscopy or phase contrast, focus on the center of the observation

channel and adjust the magnification to visualize individual bacterial cells.

4.3. Switch the light path settings to fluorescence microscopy and adjust the camera exposure time to resolve individual bacterial cells (e.g., 100 ms).

4.4. Next, insert the inlet tubing into a 2 mL tube containing the bacterial suspension. Reverse the pump direction and start withdrawing the suspension at a flow rate of 1 µL/min. Scan the cross section of the entire observation channel recording a composite picture every 2 min.

5. Basic image processing

5.1. Import images to a desired software platform (e.g., MATLAB, ImageJ, R, or Python). Remove camera noise, which is a random variation of pixel intensity, and correct for optical aberration.

5.2. Crop the images to a region of interest. Identify a threshold, so that values greater than the threshold include bacterial cells. Subtract the threshold value from each picture.

5.3. Binarize the resulting image, so that bacterial cells take a value of 1, whereas background takes a value of 0. Remove clusters of pixels with an area smaller than the smallest bacterial cell size in pixels.

5.4. Sum the binarized image to obtain the total number of pixels. Divide the number of pixels by the average size of a bacterial cell in pixels to obtain an estimate of the number of cells. Knowing the depth of view and the area of investigation, transform counts into concentration.

5.5. To identify the concentration of the injected bacterial suspension, inject the bacterial suspension into the observation channel of a clean microfluidic device with a 1 mL syringe. Record the image and calculate the influent bacterial concentration (C_0) as described before.

5.6. Visualize BTCs by normalizing the effluent bacterial concentration (C) with the influent bacterial concentration (C_0) and plot C/C_0 versus time.

6. Analyze bacterial transport at the pore scale

6.1. In order to analyze local velocities and trajectories of bacteria transported through the porous matrix, move the microscope stage to a region of interest and adjust the focus to the center of the microfluidic device. Set the microscope to bright field or phase contrast.

6.2. Record time-lapse images (video) at an exposure time that captures bacterial displacement. Record pictures over a sufficient amount of time (e.g., 3 min).

6.3. To remove background noise, subtract the average of all recorded images from each image. To do that, create a matrix whose result is the sum of the intensity of all the images for each pixel, and divide it by the number of images.

6.4. From the resulting matrix (A), determine the modulus (B) of the numerical gradient $\begin{bmatrix} g_x \\ g_y \end{bmatrix}$ and normalize it by its maximum value (*max*), as defined below.

$$\begin{bmatrix} \mathrm{g_x} \\ \mathrm{g_y} \end{bmatrix} = \begin{bmatrix} \frac{\partial \mathrm{A}}{\partial \mathrm{x}} \\ \frac{\partial \mathrm{A}}{\partial \mathrm{x}} \end{bmatrix}$$

$$\mathrm{B} = \frac{\sqrt{\mathrm{g_x}^2 + \mathrm{g_y}^2}}{max}$$

6.5. Binarize the matrix B via intensity thresholding (see step 6.5) and record coordinates (X, Y in pixel or mm) and the time of image acquisition into a three-column file. Then apply a particle tracking script to process the recorded data and compute the trajectories.

7. Study bacterial filtration by means of deposition profiles

7.1. To obtain deposition profiles, record a composite image of the entire porous channel before (i.e., background) and after injection of bacterial suspension through the microfluidic device.

7.2. Remove background from the images recorded after bacterial injection.

7.3. Integrate the total fluorescence signal of retained bacteria along the transversal sections of the porous channel. plot the integrated florescence signal versus the porous channel length.

8. Analyze bacterial transport using PMMA fluidic devices and flow cytometry

8.1. Connect the peristaltic pump with the inlet using 50 cm (1 mm inner diameter) tubing and the outflow with the automated dispenser using the same tubing (50 cm, see section 4).

8.2. Pump cultivation medium in the fluidic device. Note the arrival of medium at the outlet tubing fixed to the robotic dispenser.

8.3. Simultaneously start injecting the bacterial suspension through the PMMA fluidic device at a flow rate of 0.2 mL/min and dispensing. Inject bacterial suspension equivalent to several pore volumes (e.g., 30x the volume of the fluidic device). After injection, switch to sterile cultivation medium until the end of the experiment.

8.4. Once a 96 well plate is completed, cover the plate and store at 4 °C. Analyze bacterial abundance via flow cytometry, following established protocols[16].

8.5. Prior to BTC analysis, Correct the bacterial abundance by a factor of 1.35 to account for fixative and stain.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Figure 1.png