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## Microfluidic device for quantifying bacterial chemotaxis in controlled and stationary chemical gradients

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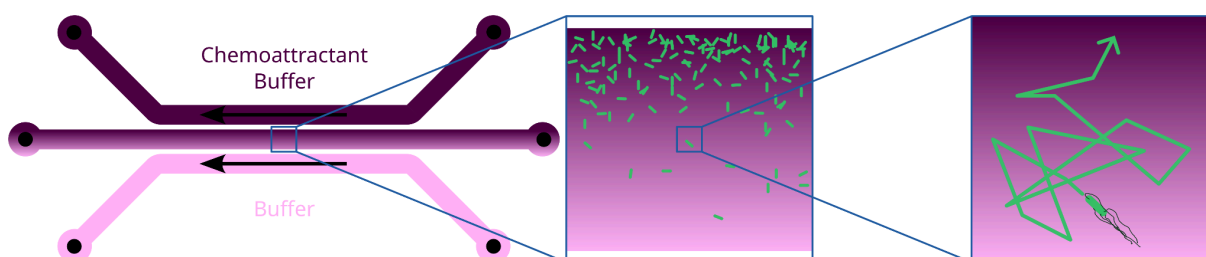
### [Abstract]

Chemotaxis refers to the ability of organisms to detect chemical gradients and bias their motion accordingly. Quantifying this bias is critical for many applications and this requires a device that can generate and maintain a constant concentration field over a long period allowing for the observation of bacterial responses. In 2010, Ahmed et al. introduced a method that combines microfluidics and hydrogel to facilitate the diffusion of chemical species and to set a linear gradient in a bacterial suspension in the absence of liquid flow. The device consists of three closely parallel channels: the two outermost channels contain chemical species at varying concentrations, forming a uniform, stationary and controlled gradient between them. Bacteria positioned in the central channel respond to this gradient by accumulating towards the high chemoattractant concentrations. Video-imaging and analysis give access to the key motility and chemotactic parameters of the studied bacterial species. This technique offers a significant advantage over other microfluidic techniques as it enables observations in a stationary gradient. Here, we outline a modified and improved protocol that allows for the renewal of the bacterial population, modification of the chemical environment, and the performance of new measurements using the same chip. To demonstrate its efficacy, the protocol was used to measure the response of a strain of *Escherichia coli* to gradients of  $\alpha$ -methyl-aspartate across the entire response range of the bacteria and for different gradients.

### [Key Features]

- The protocol is based on a previously proposed system (Ahmed et al., 2010a) that we improved for higher throughput.
- Setup allowing a rapid quantification of motility and chemotaxis responses. For instance, seventeen hours were required from the start of an *E. coli* culture to the measurements to obtain the chemotactic velocity under various chemical conditions.

### [Graphical Overview]



**Keywords:** Microfluidics, Chemotaxis, Bacteria, Flow-free, Microscopy, Image analysis

**[Background]**

Bacterial chemotaxis is an important area of research that spans multiple fields: (i) Microbiology: Understanding bacterial chemotaxis helps to explain fundamental aspects of bacterial behavior, signaling pathways, and cellular processes. (ii) Infectious Diseases: Insights into how pathogenic bacteria use chemotaxis to locate and infect host tissues can inform the development of new treatments and preventive measures. (iii) Environmental Microbiology: Chemotaxis is crucial for bacterial movement in natural environments, influencing nutrient cycling, bioremediation, and the functioning of microbial ecosystems. (iv) Ecology and Evolution: Chemotaxis affects microbial interactions, competition, and cooperation within communities, providing insights into microbial ecology and the evolutionary dynamics of these behaviors. (v) Agriculture: Understanding how soil bacteria use chemotaxis to interact with plant roots can improve agricultural practices, enhance crop growth, and lead to the development of biofertilizers or biopesticides.

It is accepted that the chemotactic drift takes place at a velocity  $\vec{v}_c$  dependent on the detected concentration  $c$  and local gradient  $\vec{\nabla}c$  of the chemoattractant according to the relation (Rivero et al, 1989):

$$\vec{v}_c = \chi(c) \vec{\nabla}c \quad (1)$$

where  $\chi(c)$  is the chemotactic susceptibility that depends on the local concentration  $c$  in the bacterial vicinity. Numerous assays have been used to determine the chemotactic response of bacteria. The Capillary Tube Assay is a classic method developed in the 1960s, which involves using a capillary tube filled with a chemoattractant solution. The tube is placed in a bacterial suspension, and the number of bacteria that enter the tube is counted. Some assays use the displacement of bacteria on soft agar plates containing the chemoattractant to measure the chemotactic velocity. Although these assays can provide quantitative insights into the chemotactic behavior of bacteria, they cannot determine  $v_c$ . To address this limitation, several microfluidic solutions have been developed (Ahmed et al, 2010b) but most of them are not able to control the stationarity of the gradient.

In contrast, our microfluidic device uses diffusion through agar to generate a stable and controlled concentration and gradient and allows to measure precisely  $v_c$  for this environment. Practically, the gradient is established between parallel channels separated by a distance of  $L$  and containing a concentration  $C_{\min}$  and  $C_{\max}$  respectively.

Its value is  $\nabla C = \frac{C_{\max} - C_{\min}}{L}$  and the average concentration of chemoattractant in which the bacteria swim is  $C = \frac{C_{\max} + C_{\min}}{2}$ . Bacteria are introduced into the device, and their movement in response to the gradient is observed using microscopy. Bacteria swim toward the chemoattractant, and gather on the channel wall closest to the source of the chemoattractant. The concentration of bacteria in this region decays exponentially, with a decay length :

$$\lambda = \frac{D}{v_c} \quad (2)$$

This results from the competition between the advection of bacteria towards the source of chemoattractant at velocity and diffusion, which is characterized by a diffusion coefficient  $D$  caused by bacterial swimming activity.

Here the diffusion coefficient  $D$  is obtained by analyzing bacterial trajectories. It enables the quantitative determination of chemotactic velocity and its dependence on the average concentration and its gradient. The chemotactic coefficient can be determined from the relation (see Eq. (1)):

$$\chi(c) = \frac{L v_c}{c_{max} - c_{min}} \quad (3)$$

In its initial version, published by Ahmed et al. (2010a), the test required one microfluidic device per trial, making the method very time-consuming. Our protocol allows multiple trials to be run on the same chip, which may allow the method to be automated and enable continuous measurements. The method requires specialized equipment and expertises, which are presented here in detail. We illustrate the protocol using an *Escherichia coli* strain placed in  $\alpha$ -methyl-aspartate gradients.

## **Materials and Reagents**

### **Biological Materials**

1. *E. coli* RP437 (Parkinson, 1978) (CGSC: #12122 transformed with the plasmid pZA3R-YFP carrying a chloramphenicol resistance marker and a *yfp* gene) stored in 25% glycerol in a -80 °C freezer for long-term conservation

### **Reagents**

1. M9 Salts (MP Biomedicals, catalog number: SKU 113037012-CF)
2. Casamino Acids (VWR, catalog number: ICNA113060012)
3. D-(+)-Glucose (Sigma-Aldrich, catalog number: G7021-1KG)
4. MgSO<sub>4</sub>·7H<sub>2</sub>O (Sigma-Aldrich, catalog number: 63138-250G), dissolved in water at 1 M
5. KH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, catalog number: P5655-500G), dissolved in water at 1 M
6. K<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich, catalog number: P3786-500G), dissolved in water at 1 M
7. CaCl<sub>2</sub>·2H<sub>2</sub>O (Sigma-Aldrich, catalog number: C3306-100G), dissolved in water at 1 M
8. Sodium-lactate (Sigma-Aldrich, catalog number: L7022-10G), dissolved in water at 100 mM
9. EDTA (Sigma-Aldrich, catalog number: EDS-100G), dissolved in alkaline water at 10 mM
10. L-methionine (Sigma-Aldrich, catalog number: M5308-25G), dissolved in water at 100  $\mu$ M
11. Chloramphenicol (CAS n°: 56-75-7)
12. Bacto-Agar (BD-Difco, catalog number: 214010)
13. PDMS components (Ellsworth, Dow Sylgard 184, GMID: 1673921)
  - 13.a) Monomer
  - 13.b) Curing agent
14. Ethanol 95% (Fisher Chemical, catalog number: E/0500DF/P21)
15.  $\alpha$ -methyl-aspartate (MedChem, catalog number: HY-W142119)

### **Solutions**

1. M9G (see recipe)
2. Phosphate Buffer Solution (see recipe)
3. Motility Buffer (see recipe)
4. PDMS (see recipe)
5. Chloramphenicol stock solution (stored at -20 °C) (see recipe)

### Recipes

1. KHPO<sub>4</sub> buffer pH = 7.

Reagent	Final concentration	Amount
KH <sub>2</sub> PO <sub>4</sub>	38.5 mM	1.54 mL
K <sub>2</sub> HPO <sub>4</sub>	61.5 mM	2.46 mL
H <sub>2</sub> O	n/a	36 mL
Total	n/a	40 mL

2. M9G

Reagent	Final concentration	Amount
M9 Salts	11.28 g.L <sup>-1</sup>	5.64 g
D-(+)-Glucose	4 g.L <sup>-1</sup>	2 g
Casamino acids	1 g.L <sup>-1</sup>	500 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2 mM	1 mL
CaCl <sub>2</sub> ·2H <sub>2</sub> O	100 μM	50 μL
H <sub>2</sub> O	n/a	498.05 mL
Total	n/a	500 mL

3. Motility Buffer

Reagent	Final concentration	Amount
KHPO <sub>4</sub> buffer	150 mM	500 μL
Sodium lactate	10 mM	1 mL
EDTA	100 μM	100 μL
L-Methionine	1 μM	100 μL
H <sub>2</sub> O	n/a	8.88 mL
Total	n/a	10 mL

4. PDMS

Reagent	Final concentration	Amount
Monomer	90%	90 g
Curing agent	9%	9 g
Total	n/a	99 g

#### 5. Agarose gel

Reagent	Final concentration	Amount
Bacto-Agar	30 g.L <sup>-1</sup>	0.3 g
H <sub>2</sub> O mQ	n/a	10 mL
Total	n/a	10 mL

#### 6. 70% ethanol

Reagent	Final concentration	Amount
Ethanol (95 %)	70%	350 mL
H <sub>2</sub> O	n/a	125 mL
Total	n/a	475 mL

#### 7. Chloramphenicol stock solution (stored at -20 °C)

Reagent	Final concentration	Amount
Chloramphenicol	25 g.L <sup>-1</sup>	250 mg
70% Ethanol	n/a	10 mL
Total	n/a	10 mL

### **Laboratory Supplies**

1. Round-bottom double position 14 mL tubes (Falcon, reference: 352057)
2. 5 mL pipets (Costar, Stripette, product number: 4487)
3. Micropipet tips (Gilson, D1000, reference: F167104)
4. Scalpels (Swann Morton, code: 0501)
5. Tubing (Darwin, Tygon, SKU: LVF-KTU-13)
6. Safe-Lock Microtubes 2.0 mL (Eppendorf, catalog reference: 0030120094)
7. Microvalve (Cluzeau CIL, reference: P-782)
8. 2 mm diameter Sterile Disposable Biopsy punches (INTEGRA Miltex, product: 33-31)
9. 2.5 mL Syringes (Hamilton, Gastight 1002, reference: 81420)
10. Microscope glass slides 76 × 26 × 1 mm (Brand, article number: 474743)
11. Microscope glass slides 75.5 × 51.5 × 1.0 mm (Knittel, catalog number: VY11300051075.01)
12. Beaker 400 mL (Azlon, reference: BBBPB0400P)

### **Equipment**

1. Microscope (Leica, DMI 6000B, catalog number: 11888941)
  - Base (Leica, CTR6000, catalog number: 11888821)
  - Lens (Leica, HC PL FLUOTAR L 20x, catalog number: 11506243)
  - Fluorescence cube (Leica, Fluorescence Filter - Blue - I3, catalog number: 11513878)

- Fluorescent light source (Leica, EL 6000, catalog number: 11504115)
  - Controller (Leica, IV/2013, catalog number: 11505180)
2. Digital CMOS camera (Hamamatsu, Orca-Flash4.0 V3, product number: C13440-20CU) mounted on the microscope
  3. Syringe pump (Cetoni)
    - Base module (Cetoni, Base 120, type: NEM-B100-01 F)
    - Three dosing units (Cetoni, Dosingmodule 14:1, type: NEM-B101-02 D)Less sophisticated pumps can be used, including self-made pumps, [see this link for example](#)
  4. Spectrophotometer (Eppendorf, D30, international reference: 6133 000 001)
  5. Pipette controller (Integra, PIPETBOY pro, article references: 156 403/156 401)
  6. Biological Safety Cabinet (Telstar, Bio II Advance 3, #: 523913)
  7. Incubator-shaker (Eppendorf, New Brunswick Innova 40R, catalog reference: M1299-0086)
  8. Autoclave (Advantage-Lab, model: AL02-01-100)
  9. Oven (Labnet, Mini incubator, catalog number: I5110(A)-230V)
  10. Void Pump (KNF Lab, Laboport N816.3 KN.18, catalog number: 03533752)
  11. Mini Centrifuge (Sigma, 1-14, item number: 10014)
  12. Stirrer hotplate (Fisher Scientific, catalog number: FB15001)
  13. Semi-micro balance (Sartorius, model: CP(A)225D)
  14. Precision balance (Sartorius, model: CP420S)
  15. Vacuum desiccator (Bel-art, catalog number: F42022-0000)
  16. Vortex Mini Mixer (Crystal LabPro, model: VM-03RUW)
  17. -80 °C ultra-low temperature freezer (New Brunswick, New Brunswick Innova U101, model: U101-86)
  18. Refrigerator: -20°C freezer on top (Proline, DD133, code: 7605749)

## **Software and Datasets**

1. NemeSys (v: 2016.06.14)
2. Leica Application SuiteX (v: 3.4.7)
3. Fiji (v: 1.52)
4. Python (v: 3.9)
5. Inkscape (v: 1.3.2)

## **Procedure**

- A. Procedure applied to obtain the suspensions of *E. coli* that we used to validate our protocol.
  1. Add 5 mL M9G medium and 5 µL chloramphenicol stock solution into a double-position tube and vortex.
  2. Inoculate the tube with a small piece of ice scraped from the -80 °C glycerol stock of the *E. coli* strain and incubate overnight at 30 °C and 240 rpm.
  3. When the OD<sub>600</sub> is near 0.1, transfer 1 mL of the culture into a microtube and centrifuge it for 5 min at

10000 rpm (=  $7378 \times g$ ). Remove the supernatant and re-suspend the bacterial pellets in 1 mL mQ water. Centrifuge the microtube again at 10000 rpm for 5 min. Remove the mQ water, and resuspend the bacteria in 1 mL motility buffer.

4. Measure the  $OD_{600}$  with the spectrophotometer and adjust the suspension to an  $OD_{600} = 0.08$  by adding the appropriate volume of motility buffer.

## B. Fabrication of the microfluidic chip

1. Negative imprint of channels in photosensitive resin on a wafer for PDMS molding.

The manufacturing of the wafer requires a photolithography platform that can be found in nanotechnology laboratories. We used the [C2N](#) (Centre des Nanosciences et des Nanotechnologies) facilities to realize the imprint. The height of the channels is set by the thickness of the SU8 resin spin-coated onto a 4-inches silicon wafer. The experiments reported here were carried out with 117  $\mu\text{m}$  high channels. This method can achieve smaller heights by adjusting the spin-coater rotation speed and resin viscosity. When the resin is exposed to UV light, it polymerizes, attaches to the surface of the wafer, and becomes solid. To fix the resin where the channels will be present, a photomask must be placed between the UV lamp and the wafer on which the resin is deposited. The photomask must be printed using a very high-resolution printer to achieve good spatial resolution. [Selba](#) produced our photomask with a 25400 dpi resolution. The channels printed on the photomask were designed on Inkscape, and the SVG file was provided to the company. The SVG file can be downloaded [here](#). Several geometries are available in the file and can be used to make the microfluidic cell. We have used the one where the channels are 600  $\mu\text{m}$  wide and 200  $\mu\text{m}$  apart.

2. Molding of the PDMS channels

- a. Mix PDMS monomer and curing agent with a spatula in a 100 mL beaker for 1 to 2 minutes. Remove the bubbles generated during mixing by placing the beaker in a vacuum chamber.
- b. Cover the bottom of a Petri dish with a plastic film and aluminum foil. This will make it easier to release the molding. Carefully place the wafer inside and pour the degassed PDMS onto it. If there are any remaining bubbles, use a scalpel to remove them. Place the Petri dish in an incubator for at least 3 hours at 60 °C allowing the polymerization of the PDMS.
- c. Demold the PDMS and cut out the area containing the channels. The side on which the channels are molded can be protected from dust by placing the piece of PDMS on a cleaned glass surface. Eventual dust deposited on the PDMS surface should be removed by pressing and removing a piece of tape on the surface of the PDMS.
- d. To connect the chip to the pump and the reservoirs, make 2 mm radius holes with a biopsy punch at the enlarged extremities of the channels. Then, flexible tubings are inserted into the holes. The three output tubings, at one side of the channels, are connected to the pump, the three inlet tubings, at the other side, are dipped in a 100 mL water filled Erlenmeyer.

3. Preparation of the agarose layer

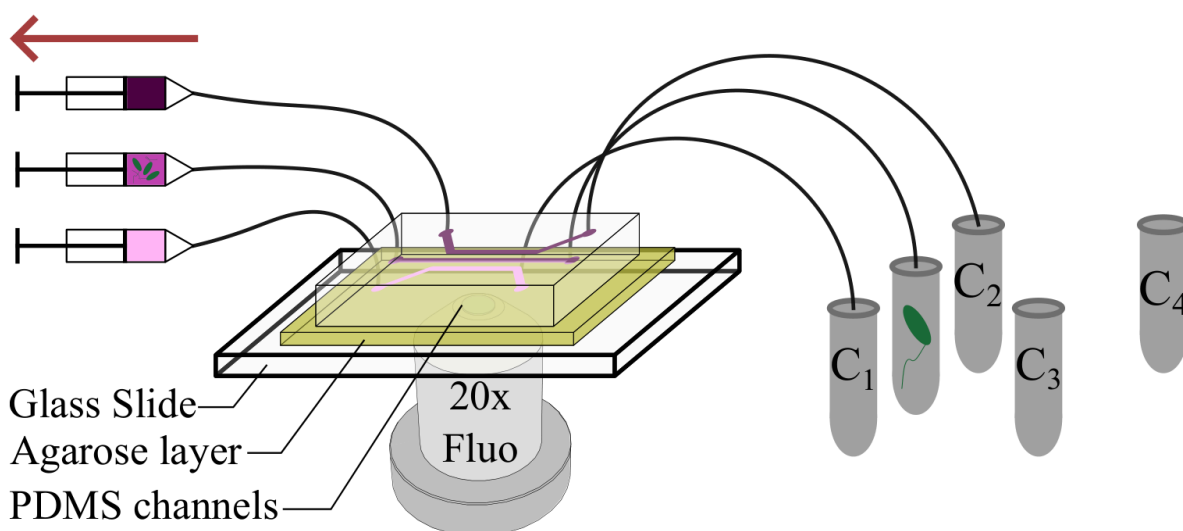
- a. Place a 15 mL Falcon tube containing 10 mL of 3  $\text{g}\cdot\text{L}^{-1}$  agarose in a boiling water bath until fully dissolved.
- b. To obtain a thin flat surface of an agarose gel that will be used subsequently to contact the PDMS



- layer, use the following procedure (see SI [Movie 1](#)) : (i) Make a flat surface out of PDMS: pour PDMS into a Petri dish, then degas and place in the incubator as above before cutting a 6x6 cm piece. (ii) Place two 75 × 26 mm microscope slides on the PDMS surface. Make sure they are separated by 5 cm, with their long sides as parallel as possible. They act as spacers, and their thickness determines the final thickness of the agarose layer. Use glass slides 1 mm thick; this thickness can be increased by layering multiple microscope slides. (iii) Pour a few milliliters of molten agarose on the surface, then cover it with a 75 × 50 mm microscope slide. This operation is done by sliding the microscope slide slowly over the spacers to allow the drops to spread and evenly fill the space between both spacers. Then, place the ensemble in the fridge for 10 min.
- c. This operation produces a gel surface of agarose, which needs to be transferred onto a microscope slide. First, take off the microscope slide placed on top of the agarose. Then, scrutinize the agarose layer to ensure the absence of imperfections. Slide the agarose from the PDMS surface onto a new 75 × 50 mm microscope slide. SI [Movie 2](#) shows the procedure.
4. Procedure for making the microfluidic chip with the PDMS mold and the agarose surface (Fig. 1)
    - a. First, fill the tubings with water. To do so, use the pump or create a gravity flow with the Erlenmeyer. It is important to avoid leaving bubbles in the tubes. They may prevent the proper fluid flow in the channels of the microfluidic chip.
    - b. Wet the agarose layer with a few drops of water, then place the PDMS molding with the channels facing down on the top of the agarose layer. Press gently to avoid bubbles remaining trapped between the PDMS and the agarose. The assembly can then be positioned on the microscope's moving stage.
    - c. Withdraw water with the pump set at a 10  $\mu\text{L}\cdot\text{min}^{-1}$  flow rate to remove the water layer between the PDMS mold and the agarose. If the water layer is very thick, use a syringe to suck the water manually by placing the syringe on the side of the chip. Once the water layer is removed, the PDMS and agarose parts are in contact, and water flows equally through all three channels. Visualize the flow in bright field mode by focusing the microscope on the channels at 10x magnification and taking advantage of particle impurities present in the liquids. There should be no particle movement at the contact areas between the PDMS and agarose.
    - d. The chip is ready for the experiment. Replace the Erlenmeyer filled with water by the microtubes containing the chemoattractant and the bacteria. This is done by quickly removing the tubings from the Erlenmeyer and dipping them into the microtubes. Set the flow rate to 2  $\mu\text{L}\cdot\text{min}^{-1}$  to purge the water from the tubings and fill them with the liquids.
    - e. When the liquids arrive in the microfluidic chip (this takes 10 min for 50 cm long tubings), reduce the flow to 1  $\mu\text{L}\cdot\text{min}^{-1}$ . Wait for the concentration gradient to form between the two outer channels. The gradient is established as the chemoattractant diffuses through the agarose. We advise waiting for 30 minutes until the gradient is stationary.
    - f. Adjust the microscope to view the central channel of the chip and focus on the fluid in the channel. Stop the flow in the central channel when the number of visible bacteria is constant. This takes less than 5 minutes.

C. Setting the microscope to observe bacteria:

1. Set the acquisition software to acquire with a  $2 \times 2$  binning (This increases the image's brightness and eases the image treatment). Use a 20x magnification objective so that the field of view encompasses the entire width of the central channel. Make the focus at the bottom of the channel. We found that 10 frames per second (fps) for 20 s is sufficient to perform the statistical analysis of the trajectories.
2. Start acquisition 20 minutes after step B.4.f. of the protocol
3. Save the films as a LIF project (Leica Image File)
4. New gradients can be generated by changing the microtubes A and C by microtubes containing new chemical concentrations. Repeat the protocol from point B.d. It is recommended to start with low concentrations and to increase them from one experiment to another to avoid a bias due to residual presence of chemoattractant.



*Figure 1: Schematic diagram of the microfluidic device: Microtubes  $C_1$  and  $C_2$  contain a chemoattractant solution at two different concentrations  $C_1$  and  $C_2$ . The central microtube contains the bacterial suspension. Microtubes  $C_3$  and  $C_4$  contain another pair of concentrations that can be studied after measurement with the first pair of microtubes. Other chemical conditions can be studied consecutively using the same microfluidic chip to study the chemotactic response of the bacteria systematically.*

**Data Analysis**

The goal of the data analysis is to extract the accumulation distance value  $\lambda$  and the diffusion coefficient of the bacteria  $D$  from the tracks. Based on these two quantities, the chemotactic velocity will be calculated using Eq. (2). Section D explains how to extract the trajectories of the bacteria from the movies, while Section E provides insight into the method used to determine the diffusion coefficient and the accumulation distance.

D. Process followed to obtain the positions and trajectories of the bacteria

1. With Fiji, open the LIF project with the Bio-Format Importer and select the movies.
2. Tracking is then carried out using the trackmate plug-in (Thivenez et al., 2017). Tracking determines the positions of bacteria and filters out motile and non-motile bacteria. We have identified three crucial

stages in this operation:

- a. It is necessary to give a dimension to the objects to be detected (Fig. 2a). This parameter is set at 4 microns. A threshold for the quality factor is also needed. This threshold is used to eliminate false detections. Generally speaking, the distribution of quality factors is bimodal, and the threshold lies between the two distributions (Fig. 2a).
- b. The second important stage is the re-construction of the trajectories, which involves identifying the particles between two successive images. Set the tracker to 4 microns (the distance around which the algorithm searches for the bacteria in the next image) to guarantee a good trajectory reconstruction (Fig. 2b).
- c. Before saving the trajectories to a file, a filter is applied to the length of the trajectories. Only trajectories of more than 10 positions are saved (Fig. 2c). This corresponds to trajectories longer than 1 second.
- d. Save the track table as a CSV file

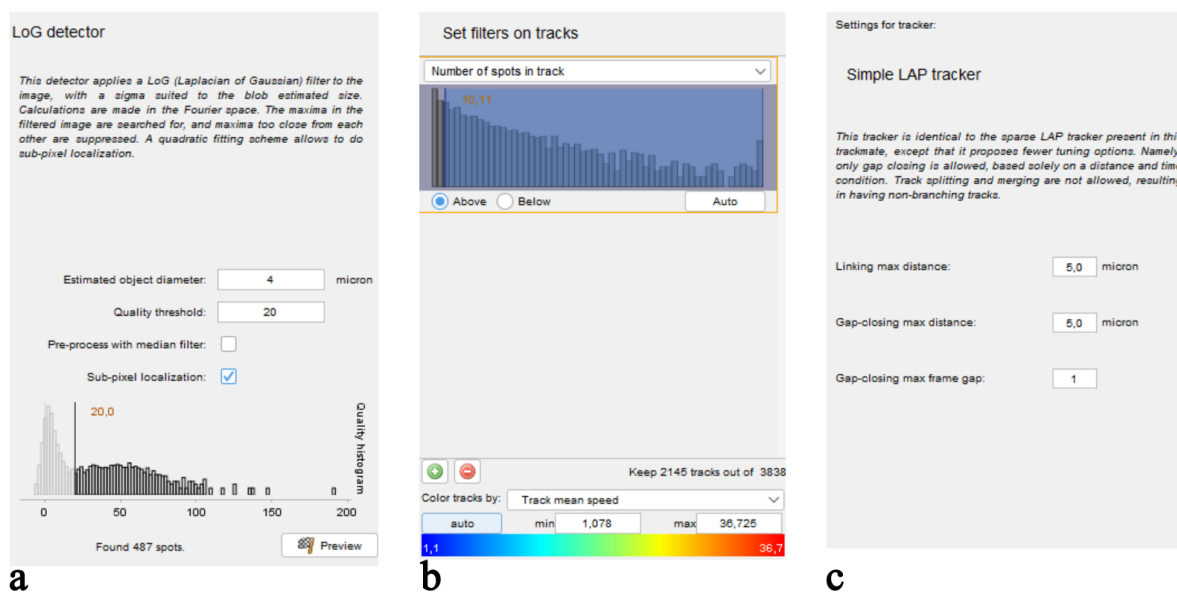


Fig. 2: Screenshots taken during film analysis to determine bacterial trajectories using trackmate plugging. (a) Window in which you can enter the size and quality factor for detecting bacteria on each of the images. (b) window that opens when you need to enter the parameters that will be used to link the particles detected between two successive images and thus reconstruct its trajectory. (c) Step at which filters can be applied to the trajectories. We have chosen to keep only trajectories containing more than 10 positions.

- E. Suppression of the non-motile bacteria from the tracks, determination of the accumulation length and diffusion coefficient from the trajectories, and determination of the chemotactic velocity
  1. Treat the CSV files with a tabulator. We recommend using the Pandas library in Python. It is also possible to use Microsoft Excel.
  2. The CSV file lists the trajectories by index  $i$  and gives the coordinates of the bacteria determined at time  $t$  for each trajectory  $(x_i(t), y_i(t))$ . From the position, (i) determine the velocity components  $v_{ix}(t)$  along the channel direction and  $v_{iy}(t)$  in the direction normal to the channel of the bacteria of index  $i$  at

time  $t$  by applying the formula:  $v_{ix}(t) = \frac{x_i(t+\delta t) - x_i(t)}{\delta t}$  and  $v_{iy}(t) = \frac{y_i(t+\delta t) - y_i(t)}{\delta t}$  where  $\delta t$  is the time

taken to capture two successive images. (ii) determine the average velocity  $v_i = \langle \|\vec{v}_i(t)\| \rangle_t$  of each

trajectory  $i$  where:  $\|\vec{v}_i(t)\| = \sqrt{v_{ix}(t)^2 + v_{iy}(t)^2}$ . (iii) calculate the average velocity  $v_y$  component along the  $y$  direction by averaging the velocity component  $v_{iy}(t)$  over time and the trajectory index  $i$ .

3. Non-motile bacteria are in the liquid, while others stick to the surface and are recorded during the image acquisition. As this sub-population shows no chemotaxis, it is necessary to remove these bacteria from the list of trajectories obtained previously before conducting a statistical analysis of the trajectories. This is done by removing the trajectories  $i$  with an average velocity  $v_i$  higher than a threshold of 5  $\mu\text{m}\cdot\text{s}^{-1}$ .

4. Determination the diffusion coefficient from the average value of  $v_y$  and the persistence time  $\tau$  of the bacteria trajectories.

- a. The persistence time measures the time over which the orientation of a bacterium's trajectory remains identical (Lovely and Dahlquist, 1975). Over longer times, the trajectory is equivalent to that of a random walker. This time is obtained from the velocity correlation function calculated as (Bouvard et al, 2022) :

$$C_y(\Delta t) = \langle v_{iy}(t) \cdot v_{iy}(t + \Delta t) \rangle_{i,t} \quad (4)$$

For particles randomly exploring their environment, the correlation function decays like  $C_y(\Delta t) = v_y^2 e^{-\frac{\Delta t}{\tau}}$  (5), where  $\tau$  is the correlation time we look for and  $v_y$  is the average velocity of all the particles along the  $y$  axis estimated in E.2.

- b. When the correlation function is divided by  $v_y^2$ , the only remaining fitting parameter of Eq. (5) is  $\tau$  obtained by an exponential fit of the data in the range  $0.5 \text{ s} < \Delta t < 3 \text{ s}$ .
- c. The diffusion coefficient is  $D = v_y^2 \tau$  (Lovely and Dahlquist, 1975)

5. Determination of accumulation characteristic length  $\lambda$  from the positions of the bacteria in the channel

- a. Segment the image along the  $y$ -axis into 20  $\mu\text{m}$  wide bins and measure the bacterial density by counting the number of spots inside each bin to finally get the density distribution.

- b. Fit the distribution with the exponential function:  $b(y) = b_0 e^{-\frac{y-y_0}{\lambda}}$  where the upper boundary location  $y_0$ , the highest bacterial density  $b_0$ , and the characteristic accumulation length  $\lambda$  are the fitting parameters.

6. The chemotactic velocity  $v_c$  is obtained from Eq. (2) using the diffusion coefficient obtained in E.4 and the accumulation length obtained in E.5. The chemotactic velocity is measured for an average concentration  $C = \frac{c_1 + c_2}{2}$  and a gradient  $\nabla C = \frac{c_1 - c_2}{L}$  where  $L = 1 \text{ mm}$  is the distance between the channels containing the chemoattractant.

- The chemotactic susceptibility  $\chi(C)$  is obtained from Eq. (3) by dividing  $v_c$  by  $\nabla C$ . The variation of  $\chi(C)$  with  $C$  can be obtained by performing experiments with various couples  $(C, \nabla C)$ .

### Validation of Protocol

We applied this protocol by placing a suspension of *E. coli* in the central channel between two channels containing 200  $\mu\text{M}$ , and 0  $\mu\text{M}$  of  $\alpha$ -methyl-aspartate in the motility buffer. We waited 30 minutes for the gradient to be established before starting to acquire the films. For this experiment, we then have  $C = 100 \mu\text{M}$  and  $\nabla C = 200 \mu\text{M}\cdot\text{mm}^{-1}$ . Fig. 3 shows the first image of three films recorded during the experiment. In the first image, the bacteria, which appear as white dots, are evenly distributed across the image.

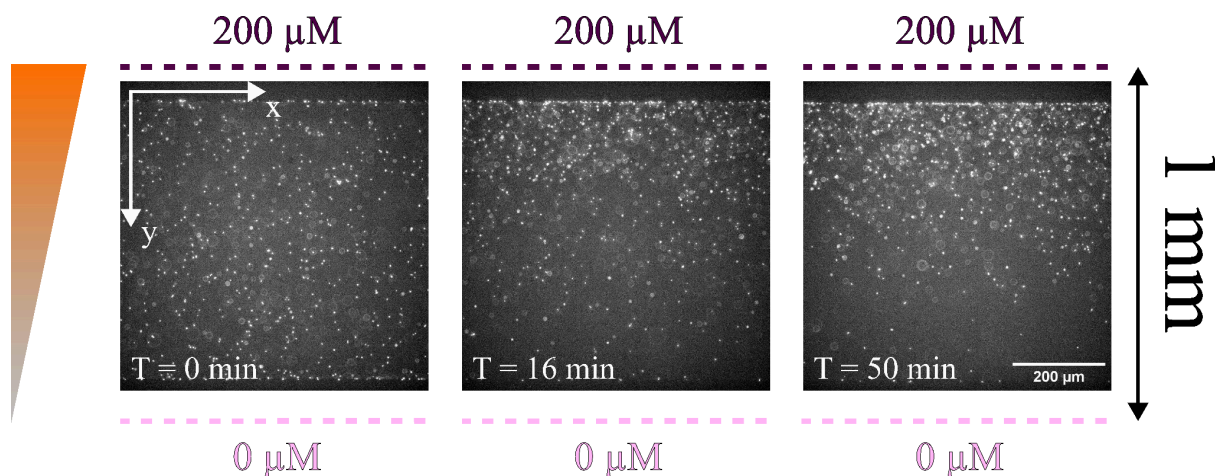


Figure 3: Images acquired at three different times (0, 16 and 50 min). The images show the central channels where the bacteria suspension was injected. The observation is done with a 20x objective. The bacteria appear as white dots. Over time, we can see that the bacteria have moved upwards and are accumulating on the side closest to the channel containing the chemoattractant.

After a few minutes, the bacteria are more numerous at the top of the image, on the side where the chemoattractant is present. This is shown quantitatively in Fig 4a where we plotted the profiles  $b(y)$  obtained after the image treatment. The adjustment of each profile by an exponential function gives a decay length that we plot as a function of the time at which the films were recorded in Fig 4c. The decay length changes very little over time, indicating that the bacterial concentration profile reaches its stationary state within a few minutes and no longer evolves over time. The average decay length measured is  $\lambda = 130 \pm 10 \mu\text{m}$  (Fig 4c).

Next, each film was processed to determine the velocity correlation function  $C_y(\Delta t)$  (See Fig 4b). Fitting it gives us the correlation time  $\tau$ , which combined with the average velocity of the bacteria along to  $y$   $v_y$  gives us the diffusion coefficient  $D = v_y^2 \tau$ . The diffusion coefficient as a function of time is shown in Fig. 4d. The diffusion coefficient shows little variation over time and we obtained  $D = 260 \pm 30 \mu\text{m}^2\cdot\text{s}^{-1}$ . The accumulation length  $\lambda$  and diffusion coefficient  $D$  are then used to determine the chemotactic velocity  $v_c$  for each film. This is shown as a function of time in Fig. 4e. The chemotactic velocity also shows little variation with time and we found:  $v_c = 1.9 \pm 0.4 \mu\text{m}\cdot\text{s}^{-1}$ .

The experiment was continued with different concentrations  $C_1$  and  $C_2$  in the channels, respectively which makes it possible to explore other pairs of parameters  $(C, \nabla C)$  and their influence on the chemotactic velocity. Fig. 5 shows in a log-log representation the chemotactic susceptibility  $\chi(C) = \frac{v_c}{\nabla C}$  as a function of  $C$ . Each point corresponds to one experiment. Outside the window of  $C$  represented in Fig. 5, no chemotactic responses were detected. The central part of the data are adjusted by a line indicating that over this region we have:  $\chi(c) \propto \frac{\chi_0}{C}$  with  $\chi_0 = 490 \pm 40 \mu\text{m}^2 \cdot \text{s}^{-1} \cdot \mu\text{M}^{-1}$ .

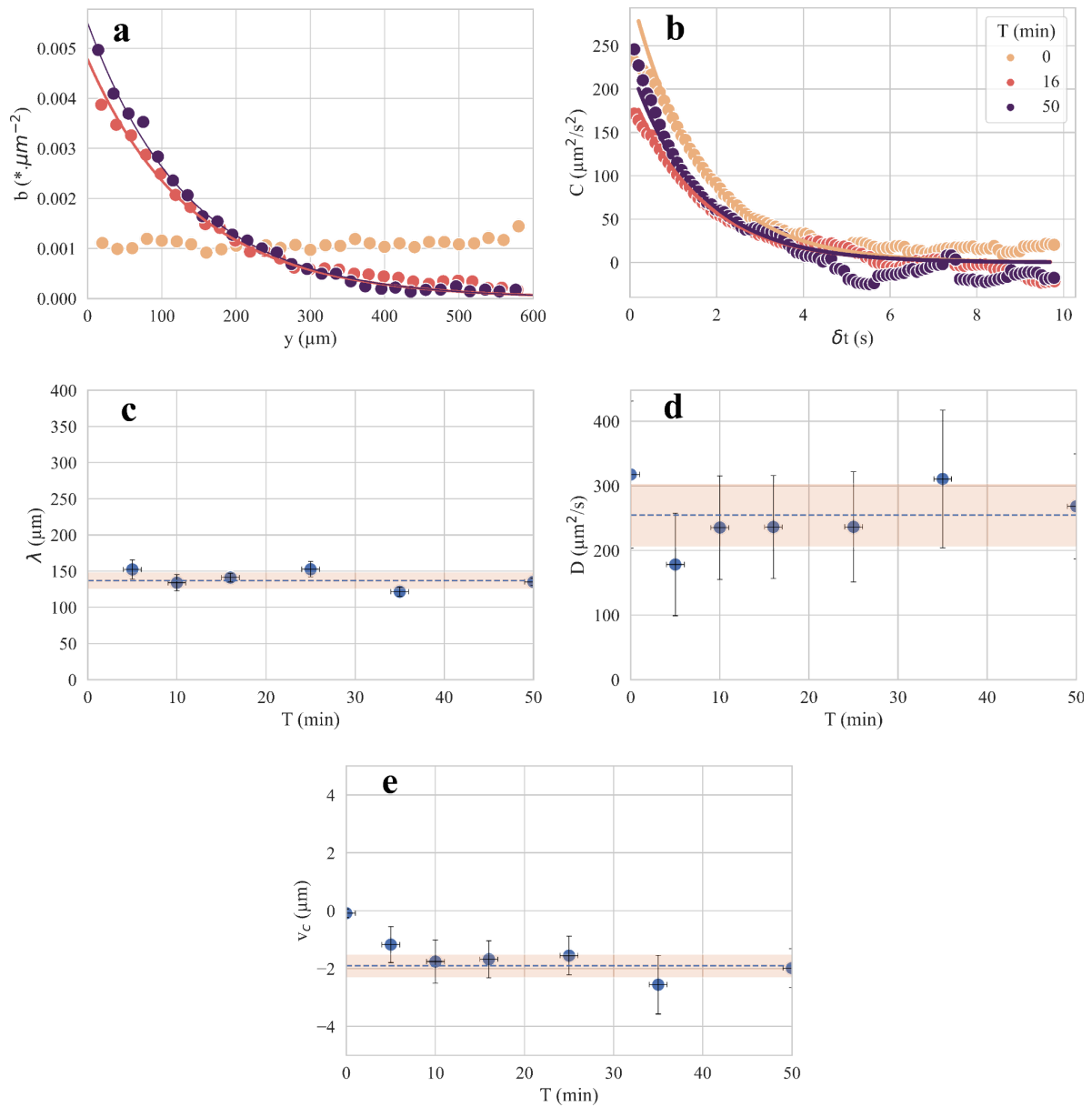


Figure 4: (a) Bacterial concentration profiles  $b(y)$  and (b) velocity correlation function  $C_y(\Delta t)$  measured at three different times during one experiment. The solid lines are exponential fit that give in a) the decay length  $\lambda$  and b) the persistence time  $\tau$ . (c) Time evolution of the decay length  $\lambda$ . (d) Time evolution of the

diffusion  $D = v_y^2 \tau$ . (e) Chemotactic velocity  $v_c$  obtained by Eq. (2) that combines the decay length  $\lambda$  and the diffusion coefficient  $D$ . The data are from an experiment with  $C = 100 \mu\text{M}$  and  $\nabla C = 200 \mu\text{M}.\text{mm}^{-1}$ .

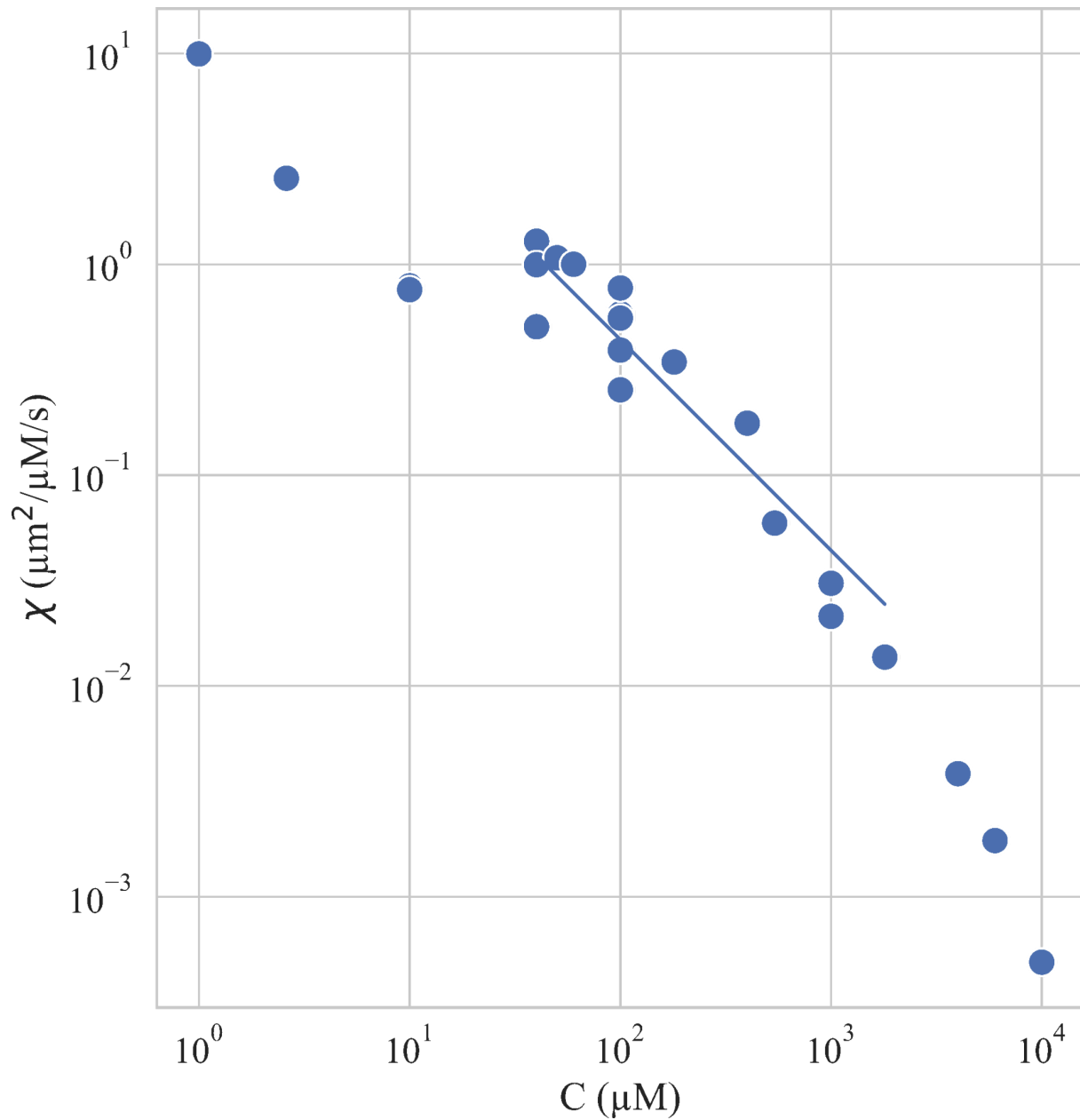


Fig. 5: Evolution of the chemotactic coefficient  $\chi(C)$  as a function of the average concentration  $C$ . For concentrations below 1 and above  $10^4 \mu\text{M}$  no chemotactic response was detected. Solid line:  $\chi(c) = \frac{\chi_0}{c}$  with  $\chi_0 = 490 \pm 40 \mu\text{m}^2.\text{s}^{-1}.\mu\text{M}^{-1}$

### General Notes and Troubleshooting

#### General Notes

- Any remaining bubbles in the channels might cause residual flow that will bias the experiment. Special care is needed to avoid them during the different steps.
- The settings are for fluorescent strains of bacteria. If the bacteria are not fluorescent, an alternative is to use phase contrast image acquisition. The tracking might in this case require a treatment to eliminate artifacts in the image.
- The bacteria density profile does not evolve anymore after 5 to 20 minutes, depending on the experiment. We recommend waiting for 20 minutes before starting the image acquisition.
- The acquisition frequency has to be adjusted so that the typical displacement of a bacteria between two successive images is of the order of 2 pixels. For a characteristic swimming velocity of bacteria  $\sim 10 \mu\text{m}\cdot\text{s}^{-1}$ , an acquisition frequency of  $0.1 \text{ s}^{-1}$  fits this requirement.
- Films can be acquired at different heights above the agar. We did not observe any effect of this parameter on the estimation of  $\chi(C)$ .
- If you like to reuse the PDMS mold then:
  - Before dismounting the microfluidic cell, flush water into the device.
  - After dismounting, clean the PDMS with ethanol, dry it, and remove the dust on the surface with tape.
  - Finally, stick some tape on the PDMS mold. The mold can then be kept and eventually reused.
- Swimming velocity or diffusion coefficient measurements are also a good way of identifying batch-to-batch variation in bacterial suspension.

## **Troubleshooting**

Problem: The liquid PDMS foams out of the container.

Cause: Bubbles develop in the vacuum chamber and form a foam.

Solution: Break the vacuum regularly.

Problem: The agarose layer breaks when manipulated.

Cause: The layer is still too warm.

Solution: Make sure the plate is cold enough. It should have some condensation on it.

Problem: Flow in the central channel.

Cause 1: Agarose delamination on the extrema after 3 hours under the microscope.

Solution 1: Wet the agarose by pouring water drops around the chip.

Cause 2: Motion and vibration of the tubings due to air conditioning.

Solution 2: Put a valve between the microtube containing bacteria and the chip.

Cause 3: Deformation of the PDMS mold due to the tubing.

Solution 3: Don't insert the tubing too deep into the PDMS mold. The molding should be  $\sim 1$  cm thick so that it can hold the tubing and still remains flat.

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### **Competing interests**

The authors have no conflict of interest to report.

### **Ethical considerations**

Not concerned.

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### **Supplementary information**

Movie 1: How to pour the liquid agarose into a 1 mm thick layer.

Movie 2: How to cut the agarose layer