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TITLE:

Generating Controlled, Dynamic Chemical Landscapes to Study Microbial Behavior

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SUMMARY:

A protocol for the generation of dynamic chemical landscapes by photolysis within microfluidic and millifluidic setups is presented. This methodology is suitable to study diverse biological processes, including the motile behavior, nutrient uptake, or adaptation to chemicals of microorganisms, both at the single cell and population level.

ABSTRACT:

We demonstrate a method for the generation of controlled, dynamic chemical pulses—where localized chemoattractant becomes suddenly available at the microscale—to create micro-environments for microbial chemotaxis experiments. To create chemical pulses, we developed a system to introduce amino acid sources near-instantaneously by photolysis of caged amino acids within a polydimethylsiloxane (PDMS) microfluidic chamber containing a bacterial suspension.

We applied this method to the chemotactic bacterium, *Vibrio ordalii*, which can actively climb these dynamic chemical gradients while being tracked by video microscopy. Amino acids, rendered biologically inert ('caged') by chemical modification with a photoremovable protecting group, are uniformly present in the suspension but not available for consumption until their sudden release, which occurs at user-defined points in time and space by means of a near-UV-A focused LED beam. The number of molecules released in the pulse can be determined by a calibration relationship between exposure time and uncaging fraction, where the absorption spectrum after photolysis is characterized by using UV-Vis spectroscopy. A nanoporous polycarbonate (PCTE) membrane can be integrated into the microfluidic device to allow the continuous removal by flow of the uncaged compounds and the spent media. A strong, irreversible bond between the PCTE membrane and the PDMS microfluidic structure is achieved by coating the membrane with a solution of 3-aminopropyltriethoxysilane (APTES) followed by plasma activation of the surfaces to be bonded. A computer-controlled system can generate user-defined sequences of pulses at different locations and with different intensities, so as to create resource landscapes with prescribed spatial and temporal variability. In each chemical landscape, the dynamics of bacterial movement at the individual scale and their accumulation at the population level can be obtained, thereby allowing the quantification of chemotactic performance and its effects on bacterial aggregations in ecologically relevant environments.

INTRODUCTION:

Microbes rely on chemotaxis, the process of detecting chemical gradients and modifying motility in response[1], to navigate chemical landscapes, approach nutrient sources and hosts, and escape noxious substances. These microscale processes determine the macroscale kinetics of interactions between microbes and their environment[2,3]. Recent advances in microfluidics and microfabrication technologies, including soft lithography[4], have revolutionized our ability to create controlled microenvironments in which to study the interactions of microbes. For example, past experiments have studied bacterial chemotaxis by generating highly controlled, stable gradients of intermediate to high nutrient concentrations[5,6]. However, in natural environments, microscale chemical gradients can be short-lived—dissipated by molecular diffusion—and background conditions are often highly dilute[7]. To directly measure the chemotactic response of microbial populations first exposed to unsteady chemical environments, we devised and here describe methods to combine microfluidic technology with photolysis, thereby mimicking gradients that wild bacteria encounter in nature.

Uncaging technology employs light sensitive probes that functionally encapsulate biomolecules in an inactive form. Irradiation releases the caged molecule, allowing the targeted perturbation of a biological process[8]. Due to the rapid and precise control of cellular chemistry that the uncaging affords[9], photolysis of caged compounds has traditionally been employed by biologists, physiologists and neuroscientists to study the activation of genes[10], ion channels[11], and neurons[12]. More recently, scientists have leveraged the significant advantages of photolysis to study chemotaxis[13], to determine the flagella switching dynamics of individual bacterial cells exposed to a stepwise chemoattractant stimulus[14,15], and to investigate motility patterns of single sperm cells in three-dimensional (3D) gradients[16].

In our approach, we implement photolysis of caged amino acids within microfluidic devices to study the behavioral response of a bacterial population to controlled chemical pulses, which become near-instantaneously available through photorelease. The use of a low-magnification (4x) objective (NA = 0.13, depth of focus approximately 40 µm) allows both the observation of the population-level aggregative response of thousands of bacteria over a large field of view (3.2 mm x 3.2 mm), and the measurement of motion at the single-cell level. We present two applications of this method: 1) the release of a single chemical pulse to study bacterial accumulation−dissipation dynamics starting from uniform conditions, and 2*i*) the release of multiple pulses to characterize the bacterial accumulation dynamics under time-varying, spatially heterogeneous chemoattractant conditions. This method has been tested on the marine bacteria *Vibrio ordalii* performing chemotaxis toward the amino acid glutamate[17], but the method is broadly applicable to different combinations of species and chemoattractants, as well as to biological processes beyond chemotaxis (e.g., nutrient uptake, antibiotic exposure, quorum sensing). This approach promises to help elucidate the ecology and behavior of microorganisms in realistic environments and to uncover the hidden trade-offs that individual bacteria face when navigating ephemeral dynamic gradients.

PROTOCOL:

1. Fabrication of the microfluidic device for the single chemical-pulse experiment

1.1. Design the channel using computer-aided design (CAD) software and print it onto a transparency film to create the photo mask (**Figure 1A**).

1.2. Fabricate the master by soft lithography (under clean-room conditions).

1.2.1. Clean a silicon wafer (4 inches) in quick succession with acetone, methanol and isopropanol, then dry using nitrogen. Bake the wafer in the oven at 130 °C for 5 min.

1.2.2. Place the wafer at the center of a spin-coater and pour SU-8 photoresist onto the wafer. Ramp the speed of the spin-coater up to 500 rpm over 5 s, and keep at 500 rpm for 10 s. Ramp up to the final speed over 10 s and maintain at this speed for 30 s.

NOTE: The exact value of the final speed depends on the targeted coating thickness and the SU-8 used.

1.2.3. After the spin-coating process, bake the wafer at 65 °C and then at 95 °C. Let the wafer cool at room temperature (RT) for at least 5 min.

NOTE: The baking time depends on the targeted thickness and type of photoresist used. As a general rule, for every 100 µm layer the wafer should be baked for 5 min at 65 °C and 45 min at 95 °C.

1.2.4. Place the photo mask onto the wafer to ensure that just the region of interest is exposed

and polymerized. Expose to UV light for the time recommended in the SU-8 manual.

NOTE: Here, with exposure energy of 200 mJ cm^{-2} at a wavelength of 350 nm, the wafer was exposed for 150 s.

1.2.5. Bake the wafer at 65 °C and 95 °C for the time recommended in the SU-8 manual.

NOTE: As a general rule, for every 100 µm layer the wafer should be baked for 5 min at 65 °C and 45 min at 95 °C.

1.2.6. Immerse the wafer in a beaker filled with polymethyl methacrylate (PMMA) developer in order to obtain the master. Gently shake the beaker to ensure that the unpolymerized photoresist is washed out. Bake the master at 200 °C to further cross-link the SU-8 layer.

1.3. Prepare a polydimethylsiloxane (PDMS) mixture by combining the elastomer with its curing agent (**Table of Materials**) at a 10:1 ratio in a beaker (here, 40 mL). Mix vigorously until the liquid is homogeneous.

NOTE: The PDMS mixture will look opaque because bubbles are generated during the mixing process.

CAUTION: In order to prevent skin coming into contact with potentially hazardous chemicals or biological material, always wear a lab coat and disposable plastic gloves throughout the protocol and follow any specific safety protocols according to the Material Safety Data Sheet (MSDS).

1.4. Degas the PDMS mixture in a vacuum chamber for 45 min at RT. To expedite the process, periodically release the vacuum in order to burst the bubbles that form at the interface.

NOTE: The degassing process must be performed within 1 h to prevent the PDMS mixture from beginning to cure.

1.5. Remove dust from the surface of the master with a pressurized cleaner, then pour the degassed PDMS mixture onto the master and bake in an oven at 80 °C for 2 h.

NOTE: Alternatively, baking overnight (or for at least 12 h) at 60 °C would achieve the same hardened PDMS.

1.6. Cut the PDMS with a blade around the microstructures (at a distance of approximately 5 mm) and then carefully peel the PDMS from the master.

1.7. Punch holes to serve as inlet and outlet of the microchannel (here, one inlet and one outlet, see **Figure 1A**). Ensure that nothing touches the bottom face of the PDMS where the features are located.

NOTE: The protocol can be paused here. The microchannel should be sealed with adhesive tape to prevent accumulation of dust and other particles during storage.

1.8. Bond the PDMS microchannel to a glass slide.

1.8.1. Thoroughly clean a glass slide with soap, isopropanol and deionized water. Let the glass slide dry or use compressed air to speed up the process.

1.8.2. Remove dust particles by dabbing with adhesive tape. Bond the PDMS microchannel on the clean glass slide, immediately after treating both surfaces with plasma (by using either a corona system or a plasma oxygen chamber) for 2 min.

1.8.3. Place the microfluidic device to heat on a hot plate at 80 °C for at least 1 h to strengthen the chemical bond with the glass.

2. Fabrication of the 3D-printed millifluidic device for the experiment with multiple pulses

2.1. Design the 3D shape using 3D-design software and print the master for the PDMS mold with a high-resolution 3D printer (**Figure 1B**).

NOTE: See **Table of Materials** for the 3D printer and mold material used to create the master.

2.2. Repeat steps 1.3−1.4, then clean the surface of the master by dabbing with adhesive tape. Put the master on a scale, then, while avoiding the central region of the master that will be occupied by the membrane, pour on the exact quantity of uncured PDMS mixture (here, 23.4 g) in order to obtain the desired height of PDMS by matching the height of the master (here, 0.5 mm). Remove any remaining bubbles with the help of compressed air.

2.3. Place the PDMS cast on the master in an oven at 45 °C to bake for at least 12 h.

2.4. Bond the 3D PDMS mold to a Petri dish.

2.4.1. Gently peel off the hardened PDMS layer and punch inlet and outlet holes for the injection of the bacterial suspension (**Figure 1C**).

2.4.2. Activate both the surface of a Petri dish (90 mm x 15 mm) and the PDMS mold with oxygen plasma for 2 min. Bond the PDMS mold on the Petri dish. Gently press the mold to the Petri dish, but do not press where the features are located as this can collapse the interrogation chamber.

2.4.3. Place the Petri dish bonded to the PDMS 3D mold in an oven at 45 °C for at least 12 h to strengthen the chemical bond.

NOTE: The protocol can be paused here.

2.5. Membrane surface functionalization[18]

2.5.1. Activate the nanoporous polycarbonate (PCTE) membrane in an oxygen plasma chamber for 1 min at RT.

CAUTION: Avoid using a corona system for the plasma activation because it will damage the membrane.

2.5.2. Under a chemical hood, dilute a commercial solution of 3-aminopropyltriethoxysilane (APTES) in deionized water to 1% by volume (here, 40 mL), by using a polypropylene tube.

CAUTION: The APTES solution is acutely toxic (MSDS health hazard score 3) and should be handled with extreme care exclusively under a chemical hood with gloves. Change gloves immediately after handling APTES solution. APTES fumes are destructive to the mucous membranes and the upper respiratory tract. The target organs of APTES are nerves, liver, and kidney. If a fume hood is not available, a face shield and full-face respirator must be implemented.

2.5.3. Transfer the diluted APTES solution in a Petri dish and immerse the activated membrane in the APTES solution for 20 min.

2.5.4. Remove the membrane from the APTES solution with tweezers and place it on a cleanroom wipe to dry.

2.6. For the fabrication of the PDMS microfluidic channels that will lie on the membrane and allow washing of the bacterial arena, repeat steps 1.1–1.7.

2.7. Bond the PDMS washing channels to the functionalized membrane.

2.7.1. Activate both the PDMS washing channel and the PCTE membrane with an oxygen plasma chamber for 2 min.

NOTE: The membrane, which will be sandwiched between two PDMS layers, should be smaller than the PDMS washing channel.

2.7.2. Immediately after the plasma treatment, bring the functionalized membrane and the PDMS washing channel into contact by gently pressing the PDMS washing channel onto the membrane.

NOTE: Do not apply excessive pressure at this stage, because it might cause (irreversible) attachment of the membrane to the channel's roof, blocking the channel.

2.8. Sandwich the membrane between two PDMS layers (**Figure 1E**).

2.8.1. Activate both the bonded laminate PDMS washing channel–PCTE membrane and the 3D PDMS mold previously bonded to the Petri dish with an oxygen plasma chamber for 2 min. Bring them into contact and press together.

2.8.2. Place the Petri dish bonded to the sandwich structure in an oven at 45 °C for at least 12 h to strengthen the chemical bond.

NOTE: The protocol can be paused here.

3. Cell culture

3.1. Grow a population of *V. ordalii* (strain 12B09 or 12B09pGFP) overnight for 20 h in 2216 medium[19] on an orbital shaker (600 rpm) at 30 °C and harvest cells in late-exponential phase. For isolates harboring pGFP, add spectinomycin (50 µg mL^{-1}) to maintain the plasmid.

3.2. Centrifuge a 1 mL aliquot of cells at 2500 x *g* for 3 min, remove the supernatant and re-suspend the cells in 1 mL of filtered artificial seawater. Repeat this step.

3.3. Starve the population of *V. ordalii* on an orbital shaker (600 rpm) at 30 °C for 3 h.

3.4. Prepare a stock artificial seawater solution of 10 mM (here, 1 mL) of 4-methoxy-7-nitroindolinyl-caged-*L*-glutamate (MNI-caged-*L*-glutamate) and store at -20 °C.

NOTE: Protect the MNI-caged-*L*-glutamate solution from ambient light to prevent photolysis.

3.5. Dilute the cells 50x by re-suspending the starved cells in an artificial seawater solution of 1 mM MNI-caged-*L*-glutamate.

NOTE: This will ensure a final bacterial concentration lower than 5×10^7 mL^{-1} (the exact value depends on the initial concentration of cells in late exponential, which is typically ~10^9 mL^{-1}). Bacterial concentration was estimated using a spectrophotometer at optical density (OD) of 600 nm.

4. Calibration of the uncaging protocol

4.1. Place a droplet (20 µL) of an artificial seawater solution of MNI-caged-*L*-glutamate[20] at a concentration C_0 = 10 mM on a glass slide. Encapsulate the droplet by covering it with a circular PDMS chamber (diameter d = 5 mm, height h = 250 µm).

4.2. Place the glass slide on a microscope stage and expose the entire chamber for 20 ms to an LED beam at a wavelength of 395 nm (power 295 mW) by using a 4x objective (numerical aperture [NA] = 0.13).

4.3. Open the PDMS chamber and extract a droplet (1 µL) for analysis with a UV-Vis

spectrophotometer.

4.4. Repeat steps 4.1−4.3 for different durations of LED illumination of 0.1 s, 0.5 s, 2.5 s, 25 s, 250 s (or until saturation of the chemical reaction) and 0 s (to obtain the background). Replicate the procedure steps 4.1−4.4 at least 3x (here, 4x).

4.5. From the absorption spectrum, extract the value at 405 nm, which represent the peak in the absorption spectrum of the cage molecule[21]. To determine the rate k of the chemical uncaging reaction by the LED exposure[14], use the following first-order kinetics equation for the numerical fit of the experimental data[17]

$$C(t) = C_0 (1 - e^{-kt}), \quad \text{(Eq. 1)}$$

where $C(t)$ is the value of the absorption spectrum of the solution at 405 nm (after background removal) with an uncaging time of t seconds. For small uncaging time t such that $kt \ll 1$, Eq. 1 can be simplified to the linear formulation

$$C(t) = C_0 kt. \quad \text{(Eq. 2)}$$

5. Single chemical-pulse experiment

5.1. Maintain the microfluidic channel under vacuum for 20 min to reduce the gas concentration within the PDMS so that bubbles are less likely to form during the filling process.

5.2. Extract the channel from the vacuum pump and immediately introduce the dilute bacterial suspension in 1 mM MNI-caged-*L*-glutamate in artificial seawater into the microchannel gently using a micropipette to avoid flagellar damage by mechanical shearing forces (here, the entire filling process took 5-10 s). After filling the channel with the bacterial suspension, suck the solution in excess with paper towel, and seal inlet and outlet with PDMS plugs by gently pressing them into the holes.

NOTE: In this way, fluid flow in the chamber is prevented.

5.3. Place the microchannel onto a microscope stage and move the stage to set the field of view in mid-channel. Set up the microscope to perform imaging in phase contrast (4x objective) at a frame rate of 12 fps.

NOTE: This value can be varied but should not be less than 10 fps to allow reconstruction of the bacterial trajectories through image analysis (see protocol section 7).

5.4. Set the pinhole of the LED beam at the minimum aperture. By setting the exposure time of the camera to 5 s, record a few frames to obtain precise measurements of the spatial location and size of the LED beam.

NOTE: The LED light source connects to the microscope's epi-fluorescence illuminator via liquid light guide connection. The LED beam does not affect video capturing, because video acquisition and LED stimulation are commanded independently via software.

5.5. Perform continuous imaging for a total duration of 10 min (20 min for the largest chemical pulse), and simultaneously activate the focused LED beam at 395 nm for the desired duration (in this experiment, t = 20 ms, 100 ms, 500 ms) at a user-defined time point (here, 10 s after the start of the video acquisition) via microscope software. To obtain multiple replicates of the same process, move the stage to record the bacterial response over different positions of the microfluidic channel. Replicate this procedure for each pulse size, using a new microchannel.

NOTE: The uncaging process generates an axisymmetric cylindrical pulse that diffuses radially outwards in the imaging plane.

5.6. Conduct separate experiments without chemical uncaging at higher magnification (20x objective, NA = 0.45) at a higher frame rate (50 fps) to record the unbiased swimming motion of the bacteria.

6. Multiple chemical-pulse experiment

6.1. Maintain the millifluidic chamber under vacuum for 20 min.

6.2. Place the Petri dish containing the millifluidic chamber onto a microscope stage. Fill the chamber below the membrane with the dilute bacterial suspension (here, the entire filling process took 10−15 s) of GFP-fluorescent *V. ordalii* in 1 mM MNI-caged-*L*-glutamate solution in artificial seawater. After filling the channel with the bacterial suspension, suck the solution in excess with paper towel, and seal inlet and outlet with PDMS plugs by gently pressing them into the holes.

NOTE: In this way, fluid flow in the chamber is prevented. To allow better visualization of bacteria in this setup where the imaging occurs through the nanoporous membrane, it is strongly recommended to use fluorescent strains instead of the wild type (as used in the single chemical-pulse experiment).

6.3. Fill a syringe with an artificial seawater solution of 1 mM MNI-caged-*L*-glutamate and attach tubing to the inlet and outlet of the washing channel above the membrane.

6.4. Connect the tubing to a waste reservoir and ensure that the tubing is entirely submerged in the fluid waste reservoir to avoid pressure oscillations.

6.5. Set the appropriate flow rate on the syringe pump (here, 50 µL min^{-1}) to obtain the desired mean flow rate in the washing channel, which depends on the channel geometry.

6.6. Start the syringe pump to establish the flow in the washing channel above the membrane.

6.7. Run the software controlling the LED beam and microscope stage to generate user-defined sequences of pulses at different locations and with different intensities.

NOTE: The continuous flow of the artificial seawater solution of 1 mM MNI-caged-*L*-glutamate in the washing channel above the membrane replenishes the caged compound solution and washes the spent medium from the bacterial arena.

6.8. Record video using a 4x objective at regular time intervals over a period of several hours and over multiple contiguous locations to cover a large surface (here, 1 cm x 1 cm, **Figure 1F**).

7. Image analysis and data analysis

7.1. Reconstruction of the bacterial trajectories

7.1.1. From the movies recorded with the 4x objective, reconstruct the bacterial trajectories using tracking software[17].

NOTE: These trajectories represent the 2D projections of the 3D bacterial motion in the microchannel.

7.1.2. From the reconstructed bacterial trajectories, determine the radial drift velocity of each swimming individual by projecting its swimming speed over the direction towards the center of the chemical pulse (**Figure 2D**). For the visualization of the spatio-temporal dynamics of the radial drift velocity, use a binning grid with spatial size 75 µm x 75 µm and temporal window of 5−10 s interval (**Figure 2D,E**).

NOTE: Because of the cylindrical symmetry of the process, data can be analyzed in polar coordinates and averaged over the angular dimension (**Figure 3**).

7.1.3. From the reconstructed bacterial trajectories, determine the spatio-temporal dynamics of the distribution of bacteria as they respond to the chemical pulses (**Figure 2E**).

7.1.4. From the higher resolution movies recorded with the 20x objective during the single-pulse experiments, extract the distribution of swimming speed and run time for the bacterial population as they respond to the chemical pulse (**Figure 4**).

7.2. Estimate the diffusion coefficient of bacteria by considering the dissipation dynamics of the bacterial population after chemotaxis is completed[17] (here, for $t > 300$ s; **Figure 3**).

7.3. Estimate the diffusion coefficient of glutamate by applying the Stokes-Einstein equation

$$D_C = k_B T/(6\pi\eta r_G), \qquad \text{(Eq. 3)}$$

where the amino acid molecules are assumed to be spherical particles with hydrodynamic radius[22] r_G, k_B is the Boltzmann constant (1.38 x 10^{-23} m^2 kg s^{-2} K^{-1}), T is the temperature (296 K), and η is the dynamic viscosity of the artificial seawater (salinity 36 g kg^{-1}) at 23 °C (10^{-3} Pa s)[23].

REPRESENTATIVE RESULTS:

We used the microfluidic and millifluidic devices (**Figure 1**) to study bacterial accumulation profiles under dynamic nutrient conditions. Bacterial trajectories were extracted from recorded videos acquired by phase contrast microscopy of the accumulation-dissipation dynamics of a bacterial population following a chemical pulse released by photolysis (**Figure 2** and **Figure 3**). By averaging millions of trajectories, the spatiotemporal dynamics of the radial drift velocity and bacterial concentration were obtained. Statistics describing the swimming in the absence of a chemical gradient were obtained in separate experiments with higher spatial and temporal resolution (**Figure 4**).

FIGURES LEGENDS:

Figure 1: Microfluidic and millifluidic devices for bacterial experiments under dynamic nutrient conditions. (**A**) Photomask of the microfluidic channel used to observe the bacterial accumulation to a single chemical pulse. (**B**) Photomask of the microfluidics channel used to wash the millifluidic bacterial chamber for the multi pulse experiments. The small dots are micropillars (200 µm in size) that help the bonding of the membrane to the PDMS, and at the same time help prevent the membrane from buckling or collapsing in the center. (**C**) Design of the 3D printed master used to create the bacterial chamber. (**D**) The PDMS layer with the patterning of the bacterial chamber. (**E**) The complete millifluidic device (top view, PCTE membrane in white). (**F**) Schematic of the millifluidic device for the generation of dynamic nutrient conditions (side view). Optics ray diagram is shown on the bottom of the device, where a violet beam (395 nm) performs the uncaging of the chemoattractant, whereas an (independent) blue beam (470 nm) is used for the observation of fluorescent bacteria (harboring pGFP) through a sCMOS camera, which captures the bacterial density (bottom centre). The device is placed on a motorized stage (bottom right), which can be moved in the *x*-*y* plane to release chemical pulses at user-defined positions (controlled via NIS software, bottom left).

Figure 2: Representative bacterial responses to a chemical pulse. (**A,B**) Maximum intensity projection showing bacterial position (white) over a 0.5 s interval, shown (**A**) immediately following, and (**B**) 40 s after the pulse release (objective: 4x, NA = 0.13, Ph 2; video recording at 12 fps). (**C**) Bacterial trajectories (black) shown 60 s after the pulse release. The shaded region represents the chemical pulse released by photolysis at t = 0 s in the middle of the field of view (black cross), which subsequently diffuses. Trajectories were reconstructed using custom in-house software. (**D,E**) Temporal dynamics of the radial drift velocity (**D**) and of the bacterial concentration (**E**) following a pulse release in the center of the field of view. In panel D, negative values of the drift velocity (in blue color) correspond to directed chemotactic motion towards the center of the pulse. This figure has been modified after Brumley et al.[17].

Figure 3: Spatio-temporal profiles of the radial drift velocity and bacterial concentration following a chemical pulse release. (**A,B**) The radial drift velocity (**A**) and the bacterial

concentration (**B**) as a function of time and distance from the center of a 35 µM glutamate pulse. In panel A, negative values of the drift velocity (in blue color) correspond to directed chemotactic motion towards the center of the pulse. Values were calculated by averaging over all trajectories. The black dashed line at t = 250 s roughly delimits the period of active chemotaxis (blue shading in panel A, region I), after which the cells diffuse isotropically outward (green shading in panel A, region II). (**C**) Bacterial concentration (rescaled over the background B_0) as a function of distance at t = 10, 30, 60 s after the creation of a diffusing chemical pulse. Bacteria aggregate close to the site of the pulse due to chemotaxis. (**D**) Bacterial concentration takes ~20 min to relax to the background B_0 by bacterial diffusion. The inset shows the fit of the diffusive spreading with a diffusion coefficient of D_B = 165 $\mu m^2 s^{-1}$. Panels A, C, and D have been modified from Brumley et al.[17].

Figure 4: Swimming statistics for a bacterial population in the absence of chemical gradients. (**A**) Trajectories represent the two-dimensional projections of the three-dimensional bacterial motion in the microchannel. Bacterial trajectories are extracted using a custom in-house tracking script. Here the blue cross indicates the start of the track, and red and green symbols indicate reorientation events. Data were recorded at 50 fps with a 20x objective, NA 0.45. (**B**) Probability distribution of measured bacterial swimming speed (black) together with a gamma distribution fit (blue). Because the depth of focus when imaging with a 20x objective (NA 0.45) is only a few microns[24], the recorded trajectories are essentially planar and measurements of the swimming speed are not biased by the projection. (**C**) Probability distribution of the time between successive reorientations. These data were required to effectively calibrate an individual based model that takes into account the reorientation pattern, the distribution of swimming speed, and the reorientation statistics of the organisms. This figure has been modified from Brumley et al.[17].

DISCUSSION:

This method allows researchers to study bacterial chemotaxis under controlled, dynamic gradients in micro- and millifluidic devices, enabling reproducible data acquisition. The near-instantaneous creation of chemical pulses at the microscale by photolysis aims to reproduce the types of nutrient pulses that bacteria encounter in the wild from a range of sources, for example, the diffusive spreading of plumes behind sinking marine particles[25], or the nutrient spreading from lysed phytoplankton cells[26].

We presented two applications of this method: 1) the release of a single chemical pulse to study the bacterial accumulation−dissipation dynamics starting from uniform conditions, and 2) the release of multiple pulses to characterize the bacterial accumulation profiles under non-equilibrium nutrient conditions. The first application is particularly suited to characterize the behavioral responses of microbes when first encountering a nutrient source. Under such conditions, in which the concentration of chemoattractant molecules is extremely low, the early phase of chemotaxis is dominated by the stochastic binding−unbinding events of chemoattractant molecules to the chemoreceptors[17]. Our method, by rapidly and precisely releasing a known mass of chemoattractant in a zero-nutrient background, offers significant advantages over previous approaches to characterize the bacterial response under dynamic conditions[27–29]. Advantages include knowing the full distribution of chemoattractant at all

locations and at all times (since its diffusivity is known), and completely avoiding the generation of fluid flow that is inherently associated with other devices, such as the microinjector[27] or three-inlet geometries[30].

In the second application, the chemical pulses occur in a large, quasi-2D domain according to a user-defined sequence in space and time that is fully customizable via software, which can be used to impose random sequences or particular patterns. Importantly, this method provides a powerful link between the high-resolution behavioral dynamics of bacterial chemotaxis and nutrient uptake over timescales of seconds and long-term dynamics, such as growth and potentially evolution. The bacterial arena is considerably larger (2 cm x 2 cm) than the spatial range of chemical interaction of the bacteria with a single pulse (from hundreds of micrometers for the smallest pulses to a few millimeters for the largest pulses). Key to the maintenance of a low chemical background (much lower than the concentration of the chemical pulses at their release) is the inclusion of the nanoporous PCTE membrane sandwiched between the two PDMS layers[18]. By applying a fluid flow in the microfluidic channel placed at the top of the device, a continuous wash-out of the uncaged compounds and spent medium in the bacterial arena is realized by means of molecular diffusion through the nanoporous membrane, without creating flow in the test section of the device where bacteria are located (**Figure 1**).

By modulating the focused LED beam in time, amplitude, size, and geometry, photorelease technology endows the experimenter with great flexibility to generate different types of chemical environments. At the same time, while the tests presented here were performed under quiescent conditions, our method can be further expanded to test bacterial chemotaxis under different flow configurations. By faithfully reconstructing the bacterial accumulation dynamics through video microscopy, our method generates large quantities of high quality data that can be used to estimate the statistics of bacterial behavior and the potential nutrient uptake by bacterial cells. Our experimental microfluidic approach, mimicking nutrient landscapes that bacteria might face under natural environmental conditions, allows the systematic study of the foraging behavior of microbial species that are essential in the cycling of nutrients at the macroscopic scale[2,3]. As such, the type of data generated through this methodology is useful to effectively calibrate population uptake rates and better derive nutrient kinetics in mesoscale ecosystem models.

The fabrication of the PDMS molds to make the large bacterial arena was performed using a commercial 3D printing service. However, similar results can be achieved using a high-end 3D printer in house, with a resolution of 50−100 µm required to resolve the smallest features of the microchannel designs. A smooth surface of the 3D-printed material for the PDMS mold is required to achieve a good bonding between the cast PDMS and the other surfaces of the device (i.e., glass, polystyrene, PDMS). For our application, the use of a polystyrene Petri dish (90 mm x 15 mm) as the lower surface of the 3D arena presents two advantages over the use of a glass slide as commonly employed in microfluidics studies: first, it considerably reduces the attachment of bacterial cells compared to a glass surface (although attachment might depend on the particular surface properties of the microbe under consideration); second, it provides secondary containment, which can prevent leakage of media over microscopy equipment in the case of spills. The PDMS mold curing process typically occurs at a high temperature (70−80 °C),

but in this application the experimenter must bake the PDMS mold at a considerably lower temperature (45 °C in the current case, see **Table of Materials**), below the heat deflection and the melting temperature of the material used for the 3D printing of the master. The lower baking temperature considerably lengthens the curing process (overnight), but does not change the mechanical and chemical properties of the PDMS.

Although our method has been applied to one particular combination of bacteria and chemoattractant, the methodology is suitable to test diverse biological processes, including nutrient uptake or antibiotic exposure, and can be applied to model systems of different species and chemoattractants, given that a myriad of molecules have been (or can be) caged[8]. One potential limitation arises from the costs of commercially available caged compounds, but these costs are comparable to those incurred when using the molecular probes typically employed in the life sciences for cell viability, counting, or intracellular staining. Notwithstanding this potential limitation, the proposed methodology may find broad applications across biophysical and biomedical sciences, to characterize early responses and adaptation dynamics of microbial populations at single cell resolution to dynamic chemical gradients.

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DISCLOSURES:

The authors have nothing to disclose.

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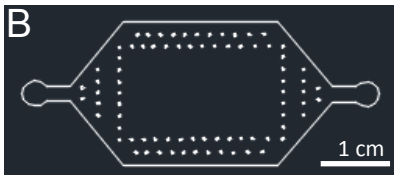
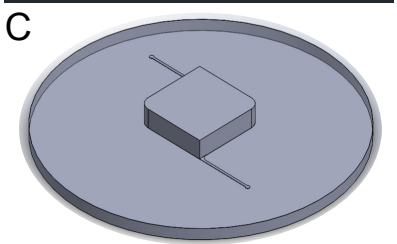
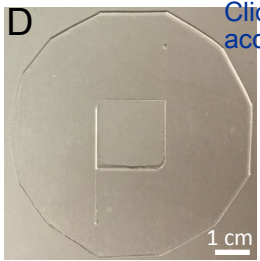
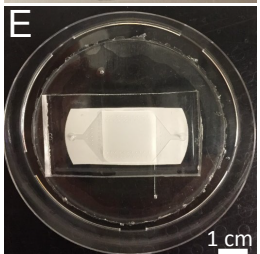
Fig. 1









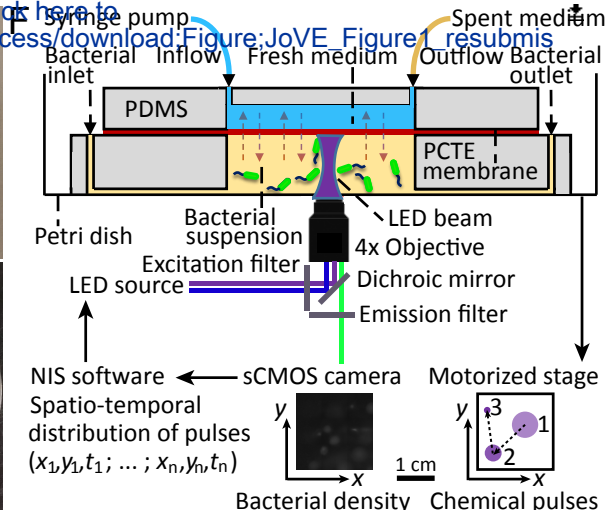


Figure2

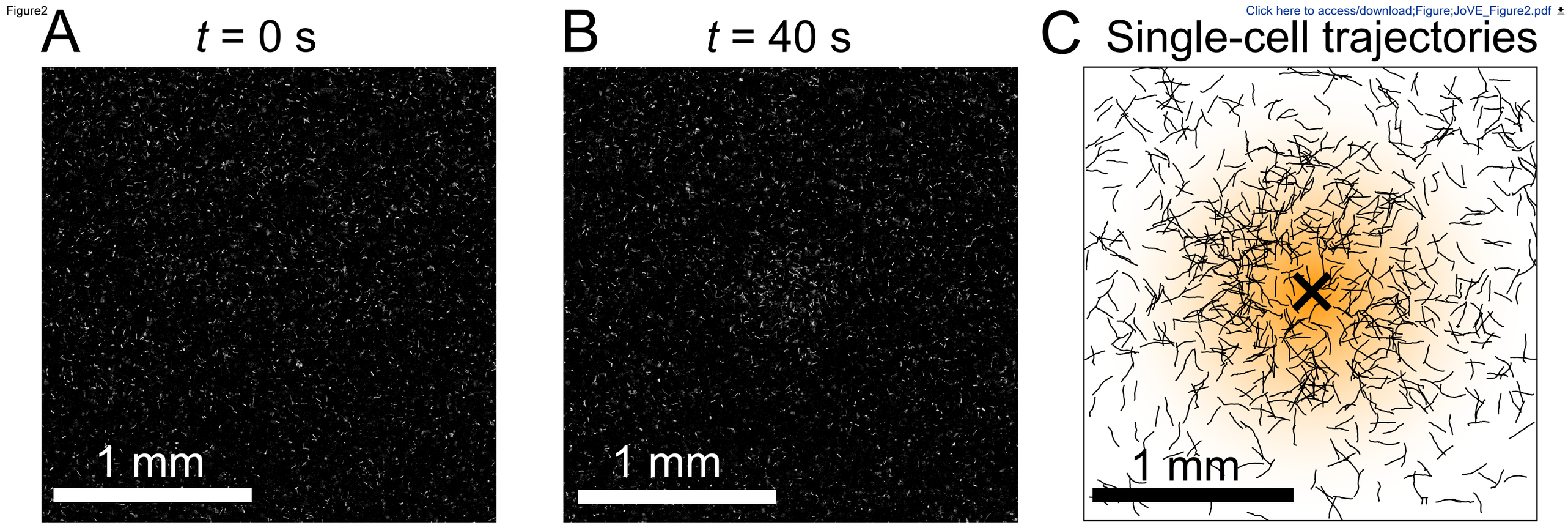
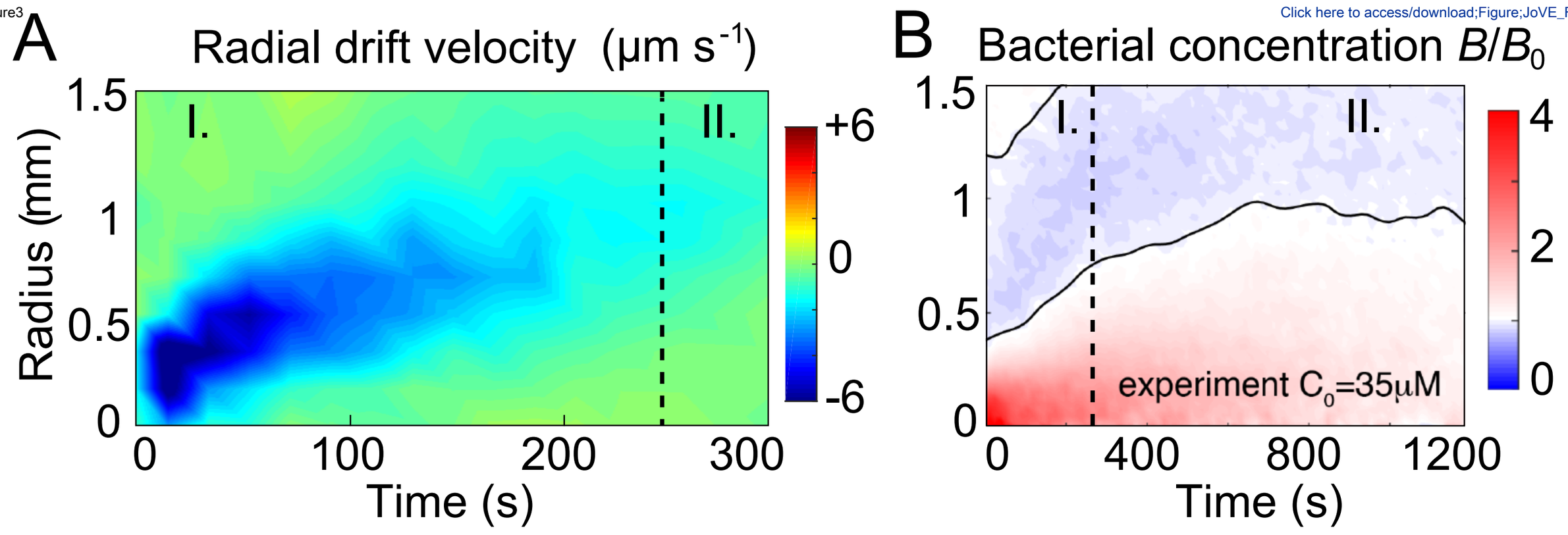


Figure3



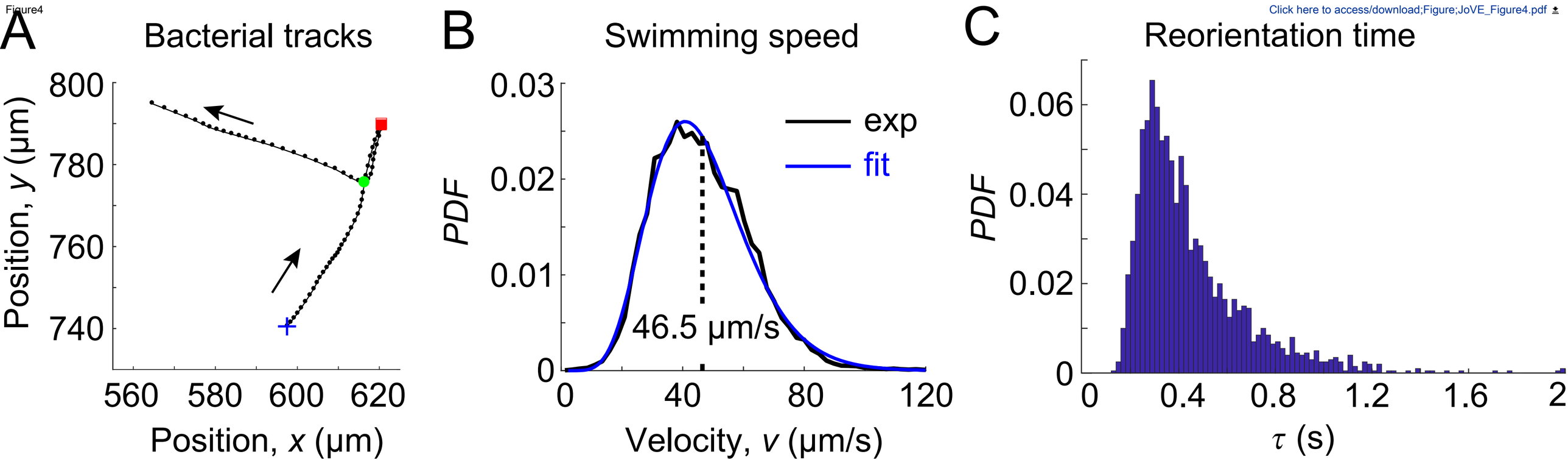


Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
(3-Aminopropyl) triethoxysilane (APTES)	Sigma-Aldrich	A3648	> 98% purity, highly toxic
CELLSTAR tube	Greiner Bio-One	210261	50 ml
Centrifuge	Eppendorf	5424R	to eliminate spent media from the bacterial culture
Digital Incubators Incu-Line	VWR-CH	390-0384	to bake 3D master
Duster	VWR-CH	16650-22	to clean the wafer and microchannels
Hot plate	VWR-CH	444-0601	to bond the microchannels
Isopropanol	Sigma-Aldrich	W292907	
LightSafe micro centrifuge tubes	Sigma-Aldrich	Z688312	1.5 ml
MATLAB	Mathworks		for image analysis and bacterial tracking
Microcentrifuge tube	Eppendorf	30120086	1.5 ml
Microscope glass slide	VWR-CH	631-1552	
Microscope Nikon Eclipse TiE	Nikon Instruments	MEA53100	with motorized stage
MNI-Glutamate	Tocris Bioscience	1490	>98 % purity, photosensitive
Mold printing equipment	Stratasys		Objet30 3D printer
Mold printing service	3D Printing Studios	Custom	https://www.3dprintingstudios.com/
Nanodrop One UV-Vis Spectrophotometer	Thermo Fisher Scientific	ND-ONE-W	to calibrate the uncaging
NIS Elements	Nikon Instruments		Microscope Imaging Software
Oven Venti-Line	VWR-CH	466-3516	to bake PDMS (with forced convection)
Photoresist SU-8-3050	MicroChem Corp.	SU8-3050	
Plasma chamber Zepto	Diener Electronic	ZEPTO-1	to functionalize the surfaces before bonding
Polycarbonate membrane	Sterlitech	PCT0447100	0.4 µm pore size, 19 % open area, 24 µm thickness
Polyethylene microtubing	Scientific Commodities	BB31695-PE/2	I.D. x O.D.: 0.015" x 0.043" / 0.38mm x 1.09mm
Polystyrene Petri dish	VWR-CH	25373-100	bottom surface (90 mm x 15 mm) to bond the millifluidic device
Scale	VWR-CH	611-2605	to weight PDMS mixture
sCMOS camera Andor Zyla	Oxford Instruments		for phase contrast and fluorescence microscopy (max 100 fps)
Sea salt	Instant Ocean	Product No. SS1-160p	
SolidWorks 2015	Dassault Systemes SolidWorks		Used to design the mold
Spectra X light engine	Lumencolor		for LED 395 nm
Sylgard 184	Dow Corning	110-41-155	PDMS Si Elastomer Kit; curing agent
Syringe (Luer-Lok)	B Braun Omnifix	4616308F	
Syringe Needle	Agani	A228	from 10 to 30 ml
Syringe Pump 11 Pico Plus Elite	Harvard Apparatus	70-4506	Terumo Agani 23 gauge 5/8 inch (16mm)
VeroGrey	Stratasys		Dual Syringe Pump
Vortex-Genie	Scientific Industries	SI-0236	Mold Material

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Article Title:	A METHOD FOR GENERATING CONTROLLED, CHEMICAL LANDSCAPES ...
Signature: *	Francesco Carrara
Date:	22.07.2019

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Zurich, September 26, 2019

Dear Editors,

Thank you for your time in handling our manuscript and thank you to the Reviewers for their positive reception of our work and their constructive comments. We have now addressed all comments, as detailed in the attached point-by-point response.

Specifically, we expanded the summary to include a general description of the method and its applications, as requested by the editorial comments. In addition to adding further details to each step, which will supplement the actions seen in the video, we also expanded our figures: we added an optic ray diagram as requested by Reviewer #2 and we added a schematic of the single pulse as requested by Reviewer #5.

We hope you will find that our revised manuscript suitably addresses the comments from the Editors and Reviewers. We remain grateful for their insights and time, and look forward to hearing from you in due course.

With best regards,

Francesco Carrara

(on behalf of all authors)

Response to Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editors for the suggestion. We took the opportunity to thoroughly proofread the manuscript, including a round of editorial revision with our lab editor. We did our best to avoid any errors in this version.

2. Summary: Please expand to include a general description of the method and its applications.

We expanded the summary as requested. This now reads: "A protocol for the generation of dynamic chemical landscapes by photolysis within microfluidic and millifluidic setups is presented. This methodology is suitable to study diverse biological processes, including the motile behavior, nutrient uptake, or adaptation to chemicals of microorganisms, both at the single cell and population level."

3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We thank the editor for providing additional information in helping clarifying the protocol. We have followed the suggestions and implemented the changes where needed throughout the manuscript.

4. 1.2.1: Please specify the size of the silicon wafer.

We used 4 inches wafers. We now give this information in the text.

5. 1.2.3, 1.2.5: Please specify the baking time and the targeted thickness.

We have now added this important information in the text.

6. 1.2.4: Please specify the UV exposure time.

The UV exposure time was calculated based on the light intensity. In our case with exposure energy of 200 mJ cm^{-2} at 350 nm, the wafer was exposed for 150 s. We have now provided this additional explanation.

7. 1.3: Please specify the curing agent used. What volume is used?

The curing agent is part of the kit Sylgard 184 by Dow Corning, and the exact chemical formulation for the curing agent is proprietary of Dow Corning. We added this information at step 1.3 to clarify this point. We also added the information on the volume used, which is 40 mL.

8. 1.7: How many holes are punched?

This information has been added.

9. 2.5.1: At what temperature?

This information has been added.

10. 2.5.2: What volume of the solution is needed? What container is used?

This information has been added.

11. 3.4: What volume of the solution is needed?

This information has been added.

12. 7.1.1: Please describe how to reconstruct the bacterial trajectories. Alternatively provide a relevant reference here.

We provided the relevant reference (Brumley, Carrara, et al., PNAS 2019) here, where the reconstruction process of the bacterial trajectories is explained.

13. 7.1.2: Please describe how spatio-temporal dynamics are determined.

We have now provided a better characterization of the the spatio-temporal dynamics.

14. 7.2: Please describe how to estimate the diffusion coefficient of bacteria.

We provided the relevant reference (Brumley, Carrara, et al., PNAS 2019) here, where the method for the estimate of the diffusion coefficient of bacteria is explained.

15. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Thank you for the explanation regarding the highlighting of text for video purposes. We have now identified 2.75 pages to be featured in the video.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

We have adopted these criteria.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have adopted these criteria.

18. When reviewing the highlighting length for the protocol, please watch out for repeated steps. Please ensure that the repeated step has been highlighted previously.

We have ensured that repeated steps are avoided.

19. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial

policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Thanks for the information. We have now provided the link to the editorial policy from PNAS website that refers to re-prints.

Response to Reviewer #1

The manuscript is well written and its subject matter may be of interest for investigators in the field of microbial cell behavior. I recommend publication of the manuscript.

We thank the Reviewer for their positive assessment of our manuscript.

Response to Reviewer #2

Manuscript Summary:
In the submitted manuscript entitled "A method for generating controlled, dynamic chemical landscapes to study microbial behavior", Francesco Carrara et al. demonstrate a microfluidic design using light patterns and photolysis of caged amino acids to generate desired chemical patterns in the microbes swimming environments. The dual-layers design with a permeable membrane allowing continuous removal of spent media. The use of low magnification objective allows large area observation of bacterial response to the stimuli. The describe method will be valuable not only for bacterial chemotaxis research but also more general bacterial behavior investigation.

Overall the manuscript is well written and the protocol is clear for the microfluidic device construction. I believe the read with little microfluidic device experience would be benefit from the protocol. However, some optical descriptions are needed for the general reader to understand the protocol.

We thank the Reviewer for their positive assessment of our manuscript, and for their comments, which we address in detail below.

Major Concerns:
1. Protocol 4.2. I am not sure if a LED beam through 4x objective can expose the entire chamber. The LED power is also an important reference for reader application.

We thank the Reviewer for their comment. We confirmed that the diameter of a LED beam through 4x objective is at least 5 mm (see Figure R1 below). We also added the information in the text on the power of the LED (295 mW).

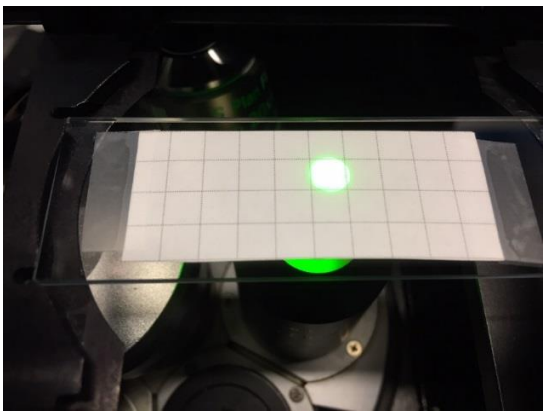

Figure R1: The picture shows the LED beam through 4x objective used for the calibration of the photolysis method. For getting a size reference, a piece of paper (the size of the square pattern is 5 mm) was placed on top of the glass slide sitting on the microscope stage, at the *z*-position at which the LED is focused. This confirms that the LED beam through 4x objective used for the calibration is at least 5 mm in diameter, as stated now in the text.

2. Protocol 4.5. The calibration curves are important and should be shown or referenced.

We have now provided the relevant reference (Brumley, Carrara, et al., PNAS 2019) here, where the calibration curves of the uncaging kinetics are shown.

3. Protocol 5.5. Line 305 said a focus LED beam… However, the Figure 1E shows a parallel beam? Which one is the optical setup in your experiments? I would suggest a clearer optics ray diagram for reader to understand.

Thanks for the comment. The beam shown in the figure was for illustration purposes only, but the Reviewer is right when stating that the beam is instead focused. We have now corrected this feature of the beam, by adding some curvature to the beam. We have now added an optic ray diagram in Figure 1E, which include the optical setup used in our experiments.

4. Figure 2 AB. Are these two images phase-contrast images? Or fluorescent? Please state clearly the imaging method and the image process.

These two images are phase-contrast images, captured with a Ph2 condenser annulus. We added this additional detail in the caption. As stated in the figure caption, these are maximum intensity projections showing bacterial positions (in white) over a 0.5 second interval.

Minor Concerns:
1. Protocol 3.5, Line 255. It would be better to mention the method you used to measure cell density (OD 600?).

Thanks for the suggestion. We have now added the method used to measure cell density (spectrophotometer at OD 600).

Response to Reviewer #3

Manuscript Summary:
The manuscript described a method to generate spatiotemporally controlled chemical fields in microfluidic/millifluidic devices to study bacteria chemotaxis. The chemoattractants were first 'caged' by chemical modification and can be released later by a LED beam. This method would be of interest to the researchers who would like to investigate the chemotaxis of vary biological organisms.

We thank the Reviewer for their positive assessment of our manuscript, and for their comments, which we address in detail below.

Major Concerns:
1.2.6. Why use PMMA developer but not SU-8 developer for the developing step?

We used a solvent-based developer recommended for SU8 - mr-Dev 600.

2.1 What type of 3D printer did you use, fused deposition modeling (FDM), stereolithography (SLA), or Digital Light Processing (DLP)? And what material was used? The material selection is important for PDMS molding. Some 3D printing materials can prevent PDMS from polymerization. Any post-processing was applied to the 3D printed master?

We thank the Reviewer for raising this point. We have now added this information in our manuscript. We used the Objet30 3D printer, which uses PolyJet technology. It works by jetting photopolymer materials in ultra-thin layers onto a build platform. Each photopolymer layer is cured by UV light immediately after it is jetted, producing fully cured models that can be handled and used immediately, without post-curing. Vero material, a rigid and opaque photopolymer, was used for our specific purpose. This material has already been tested in other studies, including Lambert et al. Nature Microbiology 2017, and it does not prevent PDMS from polymerization. No post-processing was applied to the 3D printed master.

5.2 Did you add bacterial suspension only to inlet, or to both inlet and outlet? How long did it take to fill the whole chamber? How did you prevent the fluid flow in the chamber? These questions should be addressed for step 6.2 as well.

Thank you for your comments. Following these suggestions, we have now provided additional explanations in the text.

How did you integrate the LED to the microscope? If the LED beam is introduced using the same microscope objective, does it affect the video capturing?

The LED light source connects to the microscope's epi-fluorescence illuminator via liquid light guide connection. The LED beam does not affect video capturing, because it is possible to independently command video acquisition and LED stimulation via software.

Minor Concerns:
1.8.2. I think air plasma treatment is good enough to bond PDMS to glass slide.

We thank for the comment. We added this additional note to the manuscript. However, we still recommend treating the surfaces with oxygen plasma for bonding PDMS to PDMS, and PDMS to polystyrene, which is more difficult that bonding PDMS to glass.

What are the small dots in Figure 1A?

The small dots are micropillars (200 µm in size) that help the bonding of the membrane to the PDMS, and at the same time help prevent the membrane from buckling or collapsing in the center. We have now added this additional detail in the figure caption.

Response to Reviewer #4

The authors describe a detailed protocol for the creation of chemical landscapes that can be used to study chemotaxis. These landscapes are controllable with high precision in space and time through the projection of light patterns, and therefore very useful for both the development of fundamental research and the direct applications. The protocol itself is written out in much detail, which will be helpful for students and experts alike to reproduce this type of advanced experiments.
Thus, I strongly recommend publication.

We thank the Reviewer for their positive assessment of our work.

I only have a few minor points:
1) The protocol described here is almost identical to the methods in PNAS 116 (22), 10792-10797, doi: 10.1073/pnas.1816621116 (2019). Also the figures are very similar. The authors and editorial board should check that copyrights are not infringed, just to make sure that (self) plagiarism is not an issue here. I am not a legal expert and I personally think that this publication should be fine. But I think I should at least point it out.

We thank the Reviewer for pointing this aspect out. We checked on the PNAS policy at https://www.pnas.org/page/about/rights-permissions, which allows PNAS authors for reprint of their own figures, when properly cited as we do.

2) The protocol involves a few steps with safety risks. For example, "the APTES solution is acutely toxic" but also other steps involve potential hazards. Can the authors write a bit more about where possible issues could arise and how these risks can be mitigated?

We thank the Reviewer for this comment. We have now provided additional information on how to avoid risks when handling hazardous chemicals. Specifically, we have now added a note at the beginning of the protocol about the compulsory use of a labcoat, disposable plastic gloves, and adherence to the individual Material Safety Data Sheet precautions.

3) In some steps it is not completely clear how much material is used, in terms of volume for a given experimental setup.

We now provided the volume of material used when needed.

4) Perhaps it is helpful to provide a price list or budget of the most important pieces of equipment and consumables?

We have not added a price list in the text because we think that the interested reader could easily find this information online based on the Table of materials, which is already provided in the main text. Moreover, we expect that prices are subject to geographical variation, and should be identified specifically for each user. However, we would be happy to provide rough estimates if the Editors deem it reasonable for completeness.

Otherwise I think this a very interesting paper and I look forward to seeing the video.

We thank the Reviewer again for his/her time. We look forward to producing the video of our method.

Response to Reviewer #5

Manuscript Summary:
The manuscript by Carrara et al. describes a novel method to create engineered dynamic chemical landscapes to study the motile behavior of bacteria exposed to these landscapes. The method is based on the use of micro- and millifluidic structures, caged chemoeffector compounds and structured UV illumination of the sample. The clever combination of these makes it possible to create precisely controlled chemical gradients or more complex patterns which may be used to study the chemotactic response to specific patterns, or mimic natural scenarios (nutrient plumes). Although the method is demonstrated by measuring the spatiotemporal distribution and velocity distribution of Vibrio ordalii bacteria, the concept and the method has potentially much broader implications (for example in studying microbial communication, stress response, adaptation, etc on the single cell and the population level). Therefore I think the method is well worth of publication in Jove.
The manuscript is written clearly, and gives enough details of the procedures used. All the needed chemicals and instruments are specified, and every step of the protocol is described with sufficient details. Generally, the figures are expressive and informative. In summary, my opinion is that this high quality manuscript deserves a publication in Jove. I'm looking forward to the produced video based on the manuscript.

We are grateful to the Reviewer for his/her positive assessment of our manuscript.

However, I have a few minor critiques that would be desirable to address before publication.

Minor Concerns:
1. There are two distinct PDMS structures fabricated. The microfluidic design is used for two purposes: either as bacterial chamber in single pulse experiments, or as a washing channel when sandwiched together with the millifluidic piece. This double use is not described very clearly in the text. For example, the caption for Fig. 1A refers to this microfluidic structure as the washing channel only, while step 1.1 of the single pulse device fabrication protocol refers to this figure. I think the figure caption should be changed. Additionally, a new panel on this figure similar to Fig. 1E, but showing the single pulse device would be useful. Also a clear labelling of the microfluidic and millifluidic parts on Fig. 1E would help understanding the protocol.

We thank the Reviewer for pointing this inconsistency out, which could have generated some confusion. As suggested by the Reviewer, we introduced a new panel in Figure 1 (panel A), which shows the single pulse microfluidic device. Following the suggestions of this Reviewer (and of

Reviewer #2), we have decided to expand the schematics of the millifluidic setup by adding clearer labeling and the optical ray diagram that was implemented.

2. All experimental demonstrations of the method are done with single nutrient pulses only, although the multi pulse capabilities are just as important (most of the steps described, as well as the major part of Fig. 1. deals with the multi pulse capabilities). I think it would be desired to show some results in a multi pulse scenario.

We thank the Reviewer for pointing this out. It is, of course, important to demonstrate the working capacity of the device, and this is fully accomplished in a quantitative way by using the single nutrient pulse scenario. We have now significantly expanded panel F in Figure 1, where we added bacterial accumulation (qualitative) results in multi pulse scenario. Given the focus of the manuscript is methodological in nature and that the multi-pulse response of bacteria is driven by the same mechanisms illustrated by the single pulse results, we feel that the quantitative single-pulse results are sufficient to demonstrate that there is a behavioral response to these kinds of chemical manipulations. As such, the video will be primarily focused on the *method* of multi pulse generation, rather than explicit scientific results. Finally, since the method is expected to find broad appeal for investigating a range of microbial behaviors – including quorum sensing, antibiotic resistance, nutrient uptake, chemotaxis – we believe it most impactful to highlight the generality of the method rather than a specific use.

3. Fig. 4 shows practically a control experiment. I don't see much reasons to include this on a figure by itself, without presenting similar data in case of a nutrient pulse. So I'd suggest to either leave this figure out, or extend with nutrient pulse data.

We thank the Reviewer for their suggestion. The Reviewer is right when stating that Figure 4 shows the swimming statistics for a control experiment. The data shown here, namely the distribution of the swimming speed and the reorientation statistics, is required for the determination of bacterial diffusion, which is an important parameter for the chemotactic process and for the calibration of the individual based model used in Brumley, Carrara et al. 2019. We have now further clarified the need for the unbiased swimming statistics in the text. For these reasons, we decided to keep this figure in the text. We refer the Reviewer to the panels in Figure 2D for a visualization of the radial chemotactic drift in case of a nutrient pulse.

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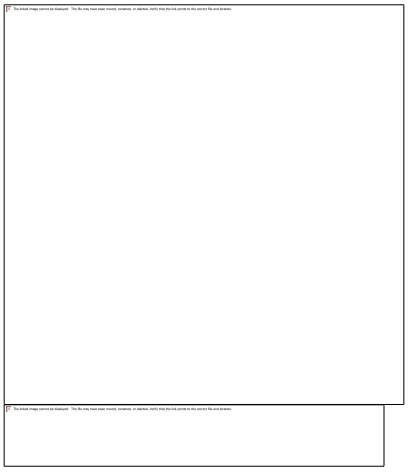

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