PNAS

Supporting Information for

Simple optical nanomotion method for single-bacterium viability and antibiotic response testing

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Extended Methods

Bacteria and culture conditions.

The *E. coli* (DH5α) and *S. aureus* strains were cultivated in Luria-Bertani (LB) agar plates for 24 h at 37°C. The colonies were sub-cultured overnight in LB broth at 37°C with shaking at 160 revolutions per minute (rpm). *L. rhamnosus* was cultivated with the same temperature and shaking conditions in MRS (Oxoid™) broth and agar. The overnight cultures were centrifuged at 6500 rpm for 5 min and washed with sterile phosphate-buffered saline (PBS). After that, the culture was diluted to obtain an OD650 of 0.75 in fresh LB, MRS, or PBS liquid medium, depending on the experiment.

M. smegmatis (mcc 155) cells were growing in LB agar plate for 3 days at 37°C. The cultures were grown for 20 h in 3 ml of LB broth with 0.1% v/v Tween 20 (Sigma-Aldrich, P1379) at 37°C and 160 rpm. The culture was centrifuged at 6500 rpm for 5 min and washed with PBS supplemented with 0.1% v/v Tween 20. Finally, the cell pellet was diluted in fresh LB supplemented with 0.1% v/v Tween 20 (OD650 of 0.75).

Nutritional conditions, viability, and antibiotic susceptibility test

We tested the viability by exposing bacterial cells to different physicochemical killing agents. *L. rhamnosus* from an overnight culture was killed by incubation in a 115°C bath for 30 min (1). *S. aureus* was exposed to a germicidal UV lamp (1800 µW/cm2, 254 nm, 2x8 W, power of 32 W) for 30 min (2, 3), where there is a significant reduction in the displacement of UV light-exposed bacteria when compared with untreated cells. *M. smegmatis* was incubated for 40 min in a 5% v/v glutaraldehyde (AppliChem, A0589) solution. We assessed the efficiency of the treatments by re-growing the bacteria on an agar plate.

The nutritional conditions tests on *E. coli* and *L. rhamnosus* nanomotion consisted in suspending the cells in PBS supplemented with different glucose concentrations. The washed and diluted overnight cell cultures were incubated for 3 h in a shaker at 160 rpm and 37°C. The cells were then centrifugated at 6500 rpm for 5 min and resuspended in PBS with 0, 2, 4, and 8% m/v D-glucose (Sigma-Aldrich, G7528).

We finally explored the effect of antibiotics on bacterial nanomotion. Washed and diluted overnight cell suspensions were incubated with antibiotics in 1.5 ml tubes (Eppendorf) for 2, 3, or 5 h. Ampicillin (Sigma A0166) and kanamycin sulfate (Gibco, 11815024) were dissolved in water, vancomycin hydrochloride (CELLPURE, 0242.3) and doxycycline hyclate (BioChemical, A2951.0010) in dimethyl sulfoxide (DMSO; Sigma-Aldrich, D5879) and streptomycin (Sigma S6501) in pure ethanol.

Data acquisition

The analysis chamber was eventually placed under the microscope and the recording started after a delay of 3 min, to achieve stabilization of the cell suspension. The recording consisted of 10 s long avi or mp4 movies acquired at 30 fps using a XIMEA camera (MU9PC-MH, XIMEA, Germany). The camera was mounted on a Zeiss Axio Observer 7 microscope with a 63x objective. Simpler microscopes (AmScope B120, USA; and Tasco LM400 400x) were also used to demonstrate the flexibility of the technique. In these cases, an objective of 100x was used and the movies were recorded with a cell phone (model 2020 Xiaomi Redmi Note 9, Xiaomi, USA) with a camera of 48 MP. We tested several variants of this protocol and noticed that even a very basic and low-cost (< 100 EUR) microscope and a mobile phone equipped with a camera, can fulfill the task, as demonstrated by the viability results.

Microscopy chamber design

The analysis chamber was prepared by punching a 5-8 mm diameter hole through a 0.01 mm thick double-coated adhesive tape (Nitto, 5601). The tape was stuck on a microscope glass slide and its hole was filled with 0.6-1 µl of bacterial solution (mixed with oil or not). Finally, a microscope cover glass was deposited on top of the tape to close the chamber.

In some experiments, to avoid the interaction of the bacteria with the glass surfaces, we suspended the cell suspension with a pipette (i.e., cells and medium with or without the drug) in oil. We used InmersolTM 518F oil (Zeiss) or HFE 7500 fluorinated oil (3MTM Novec™) with the surfactant Pico-Surf™ (2% m/m in Novec™ 7500). In this mixture, the cells were embedded in 50-500 µm diameter medium droplets that were suspended in oil. To assess the effect of the oil on bacteria, we incubated *E. coli* and *S. aureus* in oil for 5 h. This control experiment confirmed that the "oil droplet" methodology does not affect bacteria nanomotion nor cell viability.

Nanomotion analysis software

We developed homemade software in Matlab to analyze cellular nanomotion. Its user interface permits to indicate, among other parameters, the number of frames (deltaF) between the two images used to calculate cellular displacements. The cells can be recognized "manually" or automatically. In the manual selection mode, the user labels the cells he wants to process whereas in the automatic mode a cell recognition algorithm (based on https://www.mathworks.com/help/images/detecting-a-cell-using-image-segmentation.html) automatically detects individual cells. The user is left with the freedom to adjust the identification parameters (size of opening /closing masks, thresholds, etc) and to suppress regions of interest that he considers as irrelevant for further analysis. An algorithm based on Guizar-Sicairos *et al.* (4) calculates the cellular displacement between two frames (separated from each other by deltaF) and stores it in an Excel (Microsoft) table. A "lighter" version of the software calculates the absolute differences between two frames (i.e., pixel intensities) and highlights the changes in false colors. The pixels that change the most appear in red, while the ones that change the least appear in blue. Full population analysis was carried out on *S. aureus* exposed to vancomycin (Fig. 2*C*) whereas all the other results were obtained using single-cell analyses.

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