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Multi-scale analysis of bacterial growth under stress treatments

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Dear Jaydev,

Please find the enclosed manuscript "**Multi-scale analysis of bacterial growth under stress treatments**" for consideration as a *JoVE* article.

This manuscript presents a method to analyze the effect of stress treatments on bacterial growth, at the population and the single-cell levels. The procedure combines four complementary experimental approaches i.e., (i) traditional monitoring of cell viability and cell mass synthesis (ii) flow cytometry to evaluate cell size and DNA content parameters (iii) microscopy snapshot imaging to analyze cell morphology and (iv) time-lapse single-cell imaging in microfluidic chambers for examination of the temporal dynamics of cells fate. Data provided by this multi-scale framework allow interpreting the observed growth deficiencies in the light of the behaviour of individual cells.

We believe this procedure will be of great interest to all readers interested in the description of bacterial growth, in particular those willing to characterize the influence of conditions and stress treatments. Indeed, this procedure can be applied to address bacterial response to virtually any stress of interest, including growth under particular conditions (medium, pH, temperature, salt concentration), or exposure to antibiotics or other antimicrobial compounds for instance.

We hope this first version of the manuscript will conform to *JoVE* editorial scope and format requirement. We are looking forward to receiving feedback from referees and yourself, which should help to improve and to finalize this work for publication.

Thanks again for inviting us to share our expertise by submitting this method.

Sincerely,
Christian Lesterlin and Julien Cayron

TITLE:

Multi-scale Analysis of Bacterial Growth Under Stress Treatments

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KEYWORDS:

bacterial growth and viability, effect of stress-inducing treatment, live-cell imaging, microfluidics, flow cytometry, plating assay, cell cycle.

SUMMARY:

This protocol allows a time-resolved description of bacterial growth under stress conditions at the single-cell and the cell population levels.

ABSTRACT:

Analysis of the bacterial ability to grow and survive under stress conditions is essential for a wide range of microbiology studies. It is relevant to characterize the response of bacterial cells to stress-inducing treatments such as exposure to antibiotics or other antimicrobial compounds, irradiation, non-physiological pH, temperature, or salt concentration. Different stress treatments might disturb different cellular processes, including cell division, DNA replication, protein synthesis, membrane integrity, or cell cycle regulation. These effects are usually associated with specific phenotypes at the cellular scale. Therefore, understanding the extent and causality of stress-induced growth or viability deficiencies requires a careful analysis of several parameters, both at the single-cell and at the population levels. The experimental strategy presented here combines traditional optical density monitoring and plating assays with single-cell analysis techniques such as flow cytometry and real time microscopy imaging in live cells. This multiscale framework allows a time-resolved description of the impact of stress conditions on the fate of a bacterial population.

INTRODUCTION:

The overall purpose of this protocol is to analyze the behavior of bacterial cells exposed to stress treatments at the population and at the single-cell levels. Bacterial growth and viability are traditionally addressed at the population level using optical density monitoring (OD_{600nm}), which is a proxy of bacterial cell mass synthesis, or by plating assays to determine the concentration of viable cells in the culture (colony forming unit per milliliter, CFU/mL). Under normal (unstressed)

growing conditions, OD_{600nm} and CFU/mL measurements are strictly correlated because bacterial doubling time depends directly on cell mass increase[1,2]. However, this correlation is often disrupted under conditions that affect cell mass synthesis[3], cell division[4], or that trigger cell lysis. A simple example is provided by stress treatments that inhibit cell division, which result in the formation of filamentous bacterial cells[5,6]. Filamentous cells elongate normally because cell mass synthesis is unaffected, but they are unable to divide into viable cells. The culture optical density will consequently increase over time at a normal rate but not the concentration of viable cells determined by plating assays (CFU/mL). In this case, as in many others, optical density and plating measurements are informative but fail to provide a comprehensive understanding of the observed stress-induced effect. These ensemble assays need to be combined with single-cell analysis techniques to allow an in-depth characterization of stress-induced growth deficiencies.

Here, a procedure that combines four complementary experimental approaches is described: (1) traditional plating assays and basic optical density monitoring to monitor cell viability and cell mass synthesis, respectively; (2) flow cytometry to evaluate cell size and DNA content parameters on a large numbers of cells; (3) microscopy snapshot imaging to analyze cell morphology; and (4) time-lapse single-cell imaging in microfluidic chambers for examination of the temporal dynamics of cell fate. This multi-scale framework allows interpreting the global effects on cell growth and viability in the light of the behavior of individual cells. This procedure can be applied to decipher the response of diverse bacterial species to virtually any stress of interest, including growth under particular conditions (i.e., growth medium, pH, temperature, salt concentration), or exposure to antibiotics or other antimicrobial compounds.

PROTOCOL:

1. Cell culture, stress-induction, and sampling procedure

NOTE: Use sterile culture glassware, pipette tips, and growth medium filtered at 0.22 µm to avoid background particles. Here, cell cultures are grown in low autofluorescence rich defined medium (see **Table of Materials**)[7,8].

1.1. Streak the bacterial strain of interest from a frozen glycerol stock on a Luria-Broth (LB) agarose plate (with selective antibiotic if required) and incubate at 37 °C overnight (17 h).

NOTE: The example experiment presented here uses *Escherichia coli* MG1655 *hupA-mCherry.* This strain produces the fluorescently tagged subunit α of the HU nucleoid associated protein, thus allowing light microscopy visualization of the chromosome in live cells[9].

1.2. Inoculate 5 mL of medium with a single colony and grow at 37 °C with shaking at 140 rotation per minute (rpm) overnight (17 h). Flasks (≥50 mL) or large diameter (≥2 cm) test tubes must be used to ensure satisfactory aeration of the agitated culture.

1.3. The following morning measure the optical density at 600 nm (OD_{600nm}) and dilute the culture into a test tube containing fresh medium to an OD_{600nm} of 0.01. The total volume of the

culture needs to be adjusted depending on the number of time points to be analyzed during the experiment.

1.4. Load a 200 µL sample of the culture into a microplate (0.2 mL per well of working volume with a clear transparent bottom) and place it in an automated plate reader (see **Table of materials**) for OD_{600nm} monitoring during incubation at 37 °C.

1.5. Incubate the inoculated test tube at 37 °C with shaking (140 rpm) to OD_{600nm} = 0.2, corresponding to full exponential phase in rich medium.

NOTE: It is critical to grow the cells for at least 4−5 generations before achieving proper exponential growth. The initial inoculum used in step 1.3 (OD_{600nm} = 0.01) needs to be adapted in the case of growth in poorer medium (i.e., where the exponential phase is reached below OD 0.2), or if more generations are wanted (e.g., for specific physiological studies or to extended stress treatments).

1.6. At OD_{600nm} = 0.2, take the following culture samples corresponding to the t_0 time point (exponentially growing cells before stress induction): (1) A 150 µL sample to be immediately loaded in the microfluidic apparatus for time-lapse microscopy imaging (see section 2); (2) A 200 µL sample for the dilution and plating assay (see section 3); (3) A 250 µL sample to be put on ice for flow cytometry analysis (section 4); (4) A 10 µL sample to be immediately deposited on an agarose-mounted slide for microscopy snapshot imaging (see section 5).

1.7. Expose the cell culture remaining in the test tube to the specific stress treatment you want to investigate and incubate at 37 °C with shaking (140 rpm).

NOTE: The culture growing in the automated plate reader for OD_{600nm} monitoring should also be subjected to the stress treatment.

1.8. At relevant time points after the stress treatment (t_1, t_2, t_3, etc.), take the following cell samples from the stressed culture: (1) A 200 µL sample for the dilution and plating assay (see section 2); (2) A 250 µL sample to put on ice for flow cytometry analysis (section 3); (4) A 10 µL sample to be immediately deposited on an agarose-mounted slide for microscopy snapshot imaging (see section 4).

NOTE: Each stress-inducing treatment has an efficiency that is dose- and time-dependent. Thus, it might be necessary to run preliminary tests to determine the dose and duration of treatment to be used for optimal results. This can be done by performing OD monitoring using an automated plate reader (potentially associated with plating assays) of a cell culture treated with a range of doses and exposure times. In the experiment presented here, the cell culture was treated with the cell division-inhibiting antibiotic cephalexin (Ceph.) at 5 µg/mL final concentration for 60 min. Cephalexin was then washed away by pelleting the cells in a 15 mL tube using centrifugation (475 g, 5 min), removing the supernatant, resuspending the cell pellet in an equal volume of fresh medium by gentle pipetting, and transferring into a clean tube. The washed cells were incubated

at 37 °C with shaking (140 rpm) to allow recovery. The sample was taken at t_{60} (60 min after cephalexin addition corresponding to the 'cephalexin-60min-treated' sample), t_{120} (60 min after washing), and t_{180} (120 min after washing).

2. Plating assay

NOTE: The plating assay allows for measuring the concentration of cells able to generate a CFU in the culture samples. This procedure reveals the rate at which one cell divides into two viable cells and allows to detect cell division arrests (e.g., increase of the bacterial generation time of cell lysis).

2.1. Prepare 10-fold serial dilutions up to 10^{-7} of the 200 µL of culture sample in fresh medium. Plate 100 µL of the appropriate dilution on non-selective LB agarose plates in order to obtain between 3−300 colonies after overnight incubation at 37 °C.

NOTE: Serial dilution in fresh medium must be performed rapidly to limit bacterial divisions. Alternatively, researchers might consider using a saline solution without a carbon source to prevent cell divisions during the dilution process.

2.2. The next day, count the number of colonies to determine the concentration of viable cells (CFU/mL) in each culture sample. Plot the CFU/mL as a function of time for untreated and treated cell cultures.

3. Flow cytometry

NOTE: The following section describes the preparation of cell samples for flow cytometry analysis. This analysis technique reveals the distribution of cell size and DNA content for a large number of cells. When possible, it is recommended to process the flow cytometry samples immediately. Alternatively, samples can be kept on ice (for up to 6 h) and analyzed simultaneously at the end of the day, once plating and microscopy imaging have been performed.

3.1. Dilute the 250 µL of culture sample to obtain 250 µL at a concentration of ~15,000 cells/µL (corresponding to an OD_{600nm} ~0.06) in fresh medium at 4 °C.

NOTE: Incubation on ice will limit the growth and morphological modification of the cells. Alternatively, the user might consider performing fixation of the cells in 75% ethanol, as usually recommended for flow cytometry[10].

3.2. For DNA staining, mix the bacterial sample with a 10 µg/mL solution of DNA fluorescent dye (ratio 1:1) and incubate in the dark for 15 min before analyzing the sample.

3.3. Pass the sample into the flow cytometer with a ~120,000 cells/min flow rate. Acquire forward-scattered (FSC) and side-scattered (SSC) light as well as DNA fluorescent dye fluorescence signal (FL-1) with the appropriate settings.

3.4. Plot the FSC and FL-1 cell density histograms to represent the distribution of cell size and DNA content in the cell population.

4. Snapshot microscopy imaging

NOTE: The following part describes the preparation of microscopy slides and image acquisition for population snapshot analysis. This procedure will provide information regarding the morphology of the cells (cell length, width, shape) and the intracellular organization of the nucleoid DNA.

4.1. Preheat the thermostated microscope chamber at 37 °C to stabilize the temperature before starting the observations. This chamber allows for temperature modulation of the microscope optics and sample stage during time-lapse experiments.

4.2. Prepare the agarose-mounted slides as described in Lesterlin and Dubarry [7].

4.2.1. Remove the plastic film from the bottom of the blue frame (see **Table of Materials**), leaving the hollowed plastic film on the other side. Stick the blue frame on a microscope glass slide.

4.2.2. Pipette ~150 µL of melted 1% agarose medium solution and pour within the blue frame compartment. Rapidly cover with a clean coverslip to remove the excess liquid and wait a few min for the agarose pad to solidify at room temperature.

4.2.3. When the cell sample is ready, remove the coverslip and the plastic film from the blue frame. Pour 10 µL of cell sample on the agarose pad and tilt the glass slide gently to spread the droplet. When all the liquid has been adsorbed, stick a clean coverslip on the blue frame to seal the sample. The microscopy slide is now ready for microscopy.

4.3. Place the slide on the microscope stage and perform image acquisition using transmitted light (with a phase contrast objective) and with light source excitation at the appropriate wavelengths (560 nm for mCherry).

4.4. Select fields of view that contain isolated cells in order to facilitate automated cell detection during image analysis. Make sure that at least 300 cells are imaged to allow robust statistical analysis of the cell population.

5. Microfluidics time-lapse microscopy imaging

NOTE: The following part explains the preparation of the microfluidic plates (see **Table of Materials**), cell loading, microfluidics program, and time-lapse image acquisition. This imaging procedure reveals the behavior of individual cells in real-time.

5.1. Remove the conservation solution from the microfluidic plate and replace it with fresh medium preheated to 37 °C, as described in the microfluidic software user guide.

5.2. Seal the microfluidic plate to the manifold system and click on the **Priming** button.

5.3. Place the microfluidic plate with the manifold system on the microscope stage and preheat at 37 °C for ~2 h before starting the microscopy acquisition.

NOTE: This preheating step is critical to avoid the dilation of the microfluidic chamber, which would alter the focusing of the microscope during the time-lapse experiment and compromise image acquisition.

5.4. Seal off the microfluidic plate. Replace the medium from well 8 with 150 µL of culture sample and replace the medium from well 1 to 5 by the desired medium with or without the stress-inducing reagent.

5.5. Seal the microfluidic plate and place it on the microscope stage.

5.6. Open the microfluidic software (see **Table of Materials**) and run the cell loading procedure. Check that the loading of the cells is satisfactory by looking under the microscope in transmitted light. Run the cell loading procedure a second time if the cell density in the chamber is insufficient.

5.7. Perform carefully focus in transmitted light mode and select several fields of view that show isolated bacteria. It is important to select fields that are not overcrowded to be able to monitor the growth of isolated cells over time (~10−20 cells per 100 μm^2 is recommended). This will also facilitate cell detection during image analysis.

5.8. On the microfluidic software, click on the **Create a Protocol** button. Program the injection of growth medium for 1−2 generation time equivalents to allow for the cells to adapt (optional). Then program the injection of the stress-inducing medium during 10 min at 2 psi, followed by injection at 1 psi for the wanted duration of the stress treatment. If you intend to analyze the recovery of the cells after stress, program the injection of fresh growth medium for the wanted duration.

NOTE: In the experiment presented here, cephalexin was injected for 10 min at 2 psi, followed by 50 min at 1 psi. Then, fresh growth medium was injected at 2 psi for 10 min, followed by 3 h at 1 psi.

5.9. Perform microscopy imaging in time-lapse mode with 1 frame every 10 min using phase contrast in transmitted light and a 560 nm excitation light source for the mCherry signal if required.

NOTE: It is important to start microscopic image acquisition at the same time as the start of the microfluidic injection protocol.

6. Image analysis

NOTE: This section briefly describes the key steps of processing and analyzing snapshot and time-lapse microscopy images. Opening and visualization of microscopy images is done with the open source ImageJ/Fiji (https://fiji.sc/)[11]. Quantitative image analysis is performed using the open source ImageJ/Fiji software together with the free MicrobeJ plugin[12] (http://microbej.com). This protocol uses the MicrobeJ 5.13I version.

6.1. Open the Fiji software and the MicrobeJ plugin.

6.2. For snapshot analysis, drop all images corresponding to one microscope slide (one sample) into the MicrobeJ loading bar to concatenate images and save the obtained image stacks file. For time-lapse data, just drop the image stack into the loading bar of MicrobeJ.

6.3. Run the automated detection of the cells' outlines based on the segmentation of phase contrast image and, if relevant, of the nucleoids based on the segmentation of the stained DNA fluorescence signal. Check the accuracy of the cell detection visually and use the MicrobeJ editing tool for correction if needed. Save the result file obtained.

NOTE: The settings used for detection of *E. coli* cells are indicated in the **Table of Materials** (see Comments/Description column of MicrobeJ). For other bacterial species (especially for non-rod-shape bacteria), the user must refine the settings before detection (see MicrobeJ tutorial). For time-lapse images, running a semi-automated detection of the cells using the MicrobeJ editing tool might be preferred to allow focusing on the fate of individual cells (see MicrobeJ tutorial).

6.4. Click on the icon **ResultJ** to complete the analysis and obtain the **ResultJ** window. Many different types of output graphs can be generated from that point. Plot the normalized histograms of cell shape/length and mean nucleoid number per cell.

REPRESENTATIVE RESULTS:

The procedure described was used to analyze the behavior of *Escherichia coli* K12 cells during transient exposure to cephalexin, an antibiotic that specifically inhibits cell division (**Figure 1A**)[13]. The *hupA-mCherry E. coli* strain that produces the fluorescently labeled HU protein associated with the chromosomal DNA was used to investigate the dynamics of the chromosome throughout this treatment[8,9]. The exponentially growing *hupA-mCherry E. coli* cells were analyzed before (t_0) and 60 min after incubation with cephalexin (t_{60}). Then, the antibiotic was washed away and the recovery of the cell population after 1 h (t_{120}) and 2 h (t_{180}) was analyzed (**Figure 1B**).

[Place Figure 1 here]

The parallel evolution of OD and CFU/mL is a first indicator that helps to understand the effect of the stress treatments. These two parameters are strictly correlated during unperturbed

growth but are often uncoupled and evolve independently under stress. Cell cultures growing in the presence of cephalexin exhibited similar OD_{600nm} increases as the unstressed cultures (**Figure 2A**), showing that the drug did not affect cell mass synthesis. However, the concentration of viable cells did not increase when cephalexin was present due to strict inhibition of cell division (**Figure 2B**). Cells started dividing again when cephalexin was removed and eventually reached a concentration equivalent to the unstressed culture at (t_{180}). These results reflect the bacteriostatic effect of cephalexin, which induces a fully reversible inhibition of cell division. Different stresses will result in different uncoupling of the OD and CFU/mL curves, depending of the effect induced (e.g., modification of the cell morphology such as filamentation or bulging, cell death with or without lysis). A non-exhaustive list of possible outcome results indicative of different stress-induced effects is presented in **Figure 2C**.

[Place Figure 2 here]

Single-cell analysis is essential to accurately interpret the stress response observed at the population level. Flow cytometry allows the examination of cell size and DNA content of several thousands of cells[14,15] (**Figure 3**). Exposure to cephalexin provoked the parallel increase of cell size and DNA content (t_{60}). When cephalexin was removed, the population cell size and DNA content gradually decreased to become similar to the unstressed population at t_{180}. These results show that cephalexin did not inhibit DNA replication and provoked the formation of filamentous cells that contained several chromosome equivalents. These filaments divided into cells with normal cell size and DNA content when the drug was washed away. Flow cytometry results would be very different for stresses that inhibit DNA synthesis, which lead to the formation of filamentous cells containing only one non-replicating chromosome. In that case, cell size would increase similarly but would not be associated with increase in DNA content.

[Place Figure 3 here]

Snapshot microscopy imaging was used to examine the cell morphology and the intracellular organization of the DNA shown by HU-mCherry localization (**Figure 4A**). Cephalexin provoked the formation of long cells with normal cell width and no division septa. These smooth filaments contained regularly spaced DNA structures called nucleoids, confirming that cephalexin did not affect chromosome replication and segregation. Quantitative image analysis largely confirmed the cell size and DNA content increase previously observed with flow cytometry (**Figure 4B,C**). Results would be very different for stresses that induce DNA-damage, which lead to the formation of filamentous cells in which replication continues but segregation is impaired. In that case, cell size and DNA content would increase similarly, but cells would harbor a single unstructured mass of DNA. Snapshot images could also reveal other kind of aberrant cell shapes or the presence of mini, anucleate, or lysed cells (ghost cells).

[Place Figure 4 here]

Time-lapse microscopy associated with the microfluidic apparatus[16] helped to determine the phenotypes previously observed and provides additional insights regarding the development and causality of the growth deficiency. Time-lapse images (**Figure 5A** and **Movie 1**) confirmed that cell elongation (cell mass synthesis), and chromosome replication and segregation were not inhibited by exposure to cephalexin. In addition, it revealed the process of recovery when

cephalexin was removed. Analysis of the filamentous cell lineage showed that cell division restarts ~20 min after washing away the drug (**Figure 5B**). The resulting divided cells were viable, because they in turn divided, eventually leading to the formation of 33 cells exhibiting normal size and DNA content. This allowed calculation of an overall generation time of ~31 min over the 180 min of the experiment, which is similar to the generation time calculated for the unstressed population from CFU/mL measurements (~33 min).

[Place Figure 5 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Procedure for the analysis of bacterial response to stress treatments. (**A**) Schematic of the method. (**B**) Cartoon illustrating the cell morphology during normal growth in rich medium and during transient exposure to cephalexin (Ceph.), from addition at (t_0) and after cephalexin washing from (t_{60}) to (t_{180}).

Figure 2: Bacterial growth monitoring of untreated and cephalexin-treated cells at the population level. (**A**) Optical density monitoring (OD_{600nm}/mL). (**B**) Concentration of viable cell (CFU/mL) within untreated and cephalexin-60min-treated cultures. Error bars indicate the standard deviation for an experimental triplicate. (**C**) Schematics of possible results and associated stress effects.

Figure 3: Representative flow cytometry analysis of untreated and cephalexin-60min-treated cells. (**A**) Cell size distribution histograms (FSC-H). (**B**) DNA content histograms (FL1-SYTO9). n= 120,000 cells analyzed.

Figure 4: Microscopy snapshot analysis of untreated and cephalexin-60min-treated cells. (**A**) Representative microscopy images showing phase contrast (grey) and HU-mCherry signal (red). (**B**) Cell length distribution histograms. Scale Bar = 5 µm. (**C**) Histograms of the number of nucleoid per cell. Between 800 and 2,000 cells were analyzed for each sample.

Figure 5: Microscopy time-lapse analysis of cephalexin-60min-treated cells. (**A**) Representative microscopy images showing phase contrast (grey) and HU-mCherry signal (red). The monitored filamentous cell is indicated by the white outline, and divided cells by different colors. Scale Bar = 5 µm. (**B**) Schematic representation of the filamentous cell lineage corresponding to panel (**A**) and to **Movie 1**.

Movie 1: Microfluidic movie of *E. coli* HU-mCherry treated with cephalexin. Cephalexin was injected after 60 min, followed by injection of fresh RDM medium for 3 h. Time indicated in yellow (1 frame every 10 min). Scale Bar = 5 µm.

DISCUSSION:

It is essential to pay attention to the growth state of the cells during the procedure. Grow the cells over several generations before reaching a full exponential phase. For the success of this method, it is important that all cells samples are collected simultaneously, and it is best to analyze only one treated and one untreated culture at the same time. Cell samples for microscopy

imaging must be maintained at the experimental temperature throughout the procedure. It is then essential to preheat the microscope chamber and microfluidic chamber before the beginning of the experiment. If cell samples for flow cytometry cannot be analyzed readily, they can be kept on ice for up to 6 h. Incubation on ice will limit the growth and morphological modification of the cells. Alternatively, the user might consider using fixation of the cells in ethanol 75%, which is usually recommended for flow cytometry[10]. If the protocol requires washing the stress inductor from the medium, centrifuge and pipette cells very carefully to avoid damaging the potential aberrant cells.

Both flow cytometry and snapshot analysis give access to cell size and DNA content parameters, with snapshots providing additional observation of the cell morphology. DNA staining with DAPI[10] (4',6-diamidino-2-phenylindole) or other stable DNA dyes can be performed if no fluorescent fusion is available to observe the nucleoids in the organism of interest. If flow cytometry analysis cannot be performed, it is important to image and analyze a large number of cells by microscopy. Microscopy imaging can also be performed using strains carrying fluorescent fusions to proteins involved in specific pathways of interest. This would help reveal the effect of stress on a variety of cellular processes such as replication, transcription, cell wall synthesis, or cell division. The method can be applied to a range of bacterial species, the only requirement being that the microfluidic apparatus must be compatible with the morphology of the cells. Standard microfluidic plates are convenient for rod-shape bacteria with a cell width between 0.7 µm and 4.5 µm. However, cocci, ovococci, or other bacterial strains with peculiar shapes need to be tested. Alternatively, if microfluidic experiments cannot be performed due to the unavailability of the equipment or incompatible bacterial strains, time-lapse imaging can be performed on agarose-mounted slides for a maximum duration of 2h.

The overall advantage of this multi-scale analysis is to provide a global vision of the effect of stress induction on several aspects of bacterial growth ability (i.e., mass synthesis, cell viability, cell morphology, membrane integrity, DNA content) and the way these evolve with time in a bacterial population growing under stress conditions. It also allows analyzing the restoration of normal growth at the single-cell level and population level. The approach is applicable to a wide range of bacterial species and to virtually any kind of stress treatment, such as exposure to antibiotic or other antimicrobial compounds, analysis of the influence of interaction with other organisms in multispecies populations, or the effect of genetic mutation.

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DISCLOSURES:

The authors declared no competing interests.

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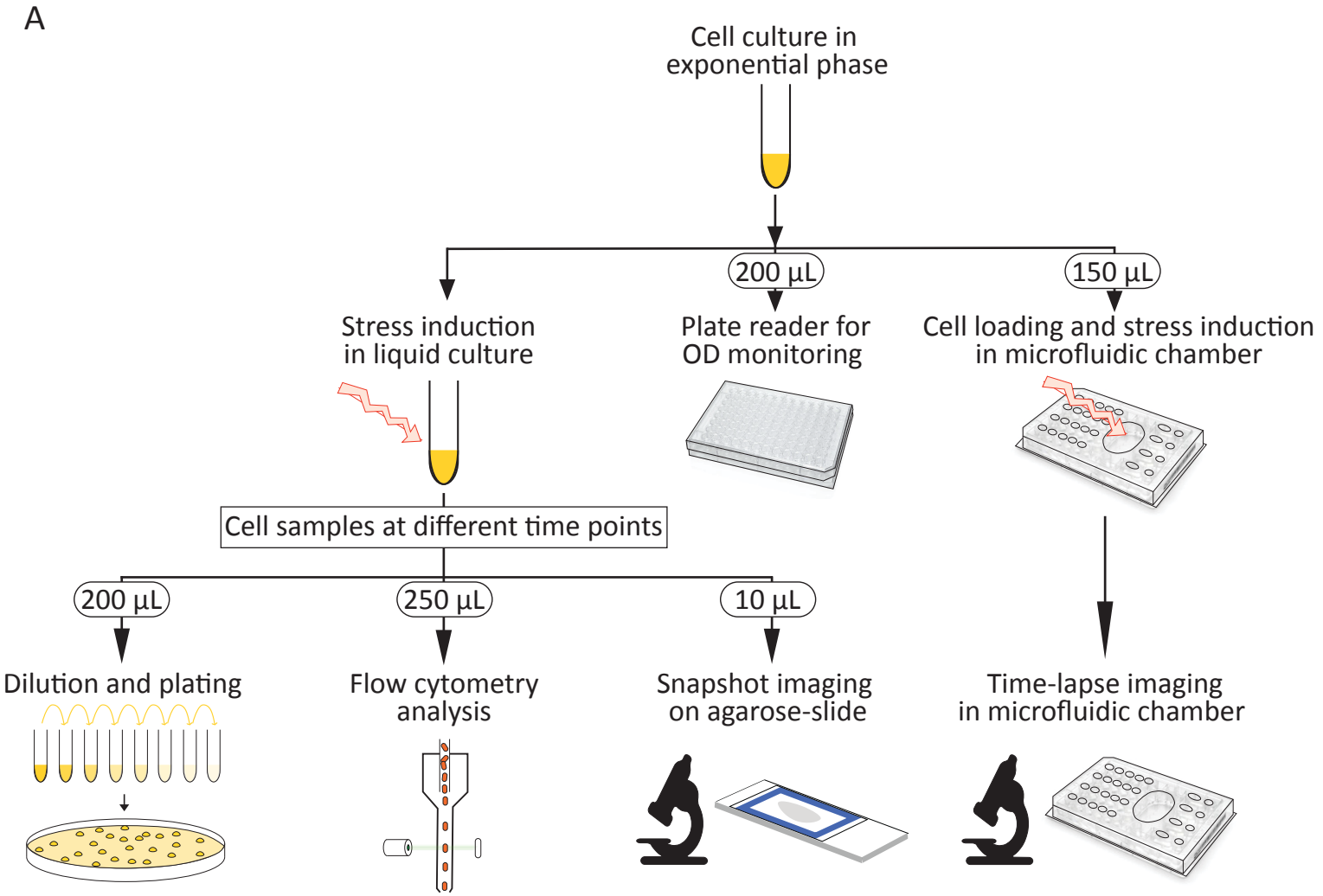


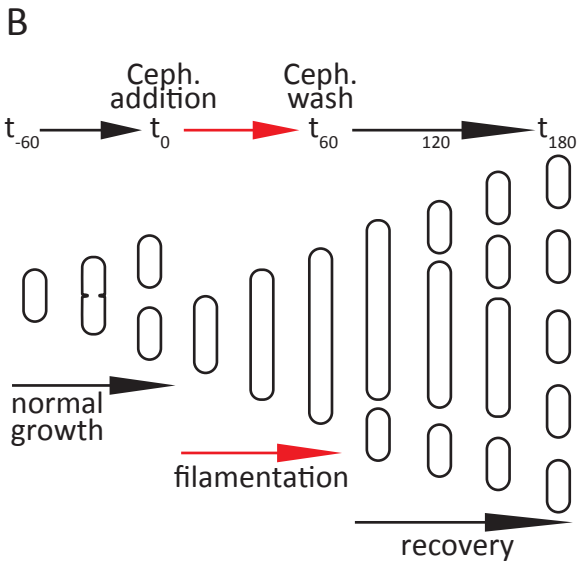


FIGURE 1

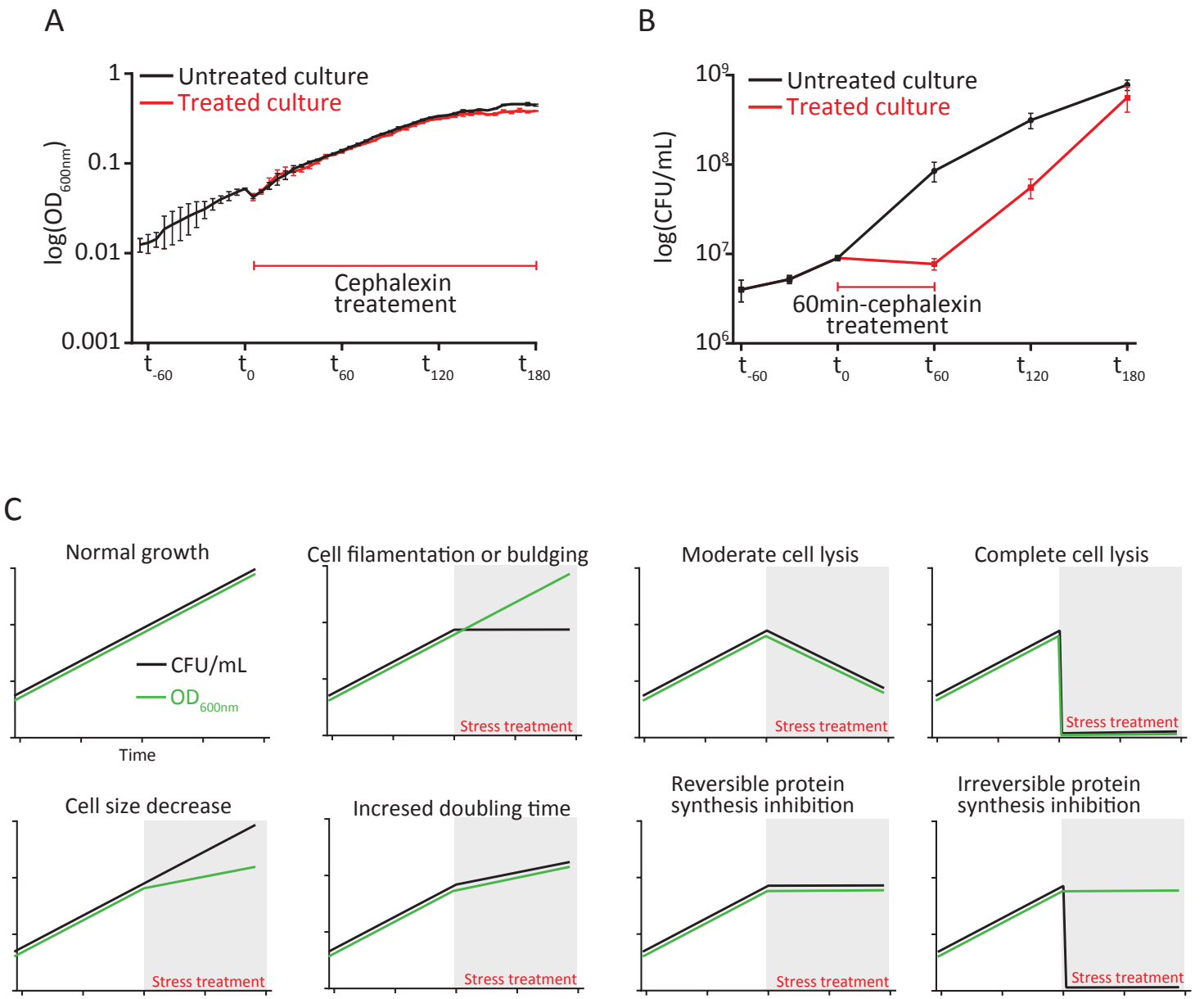


FIGURE 2

Figure 3

Click here to access/download;Figure;Figure 3.ai

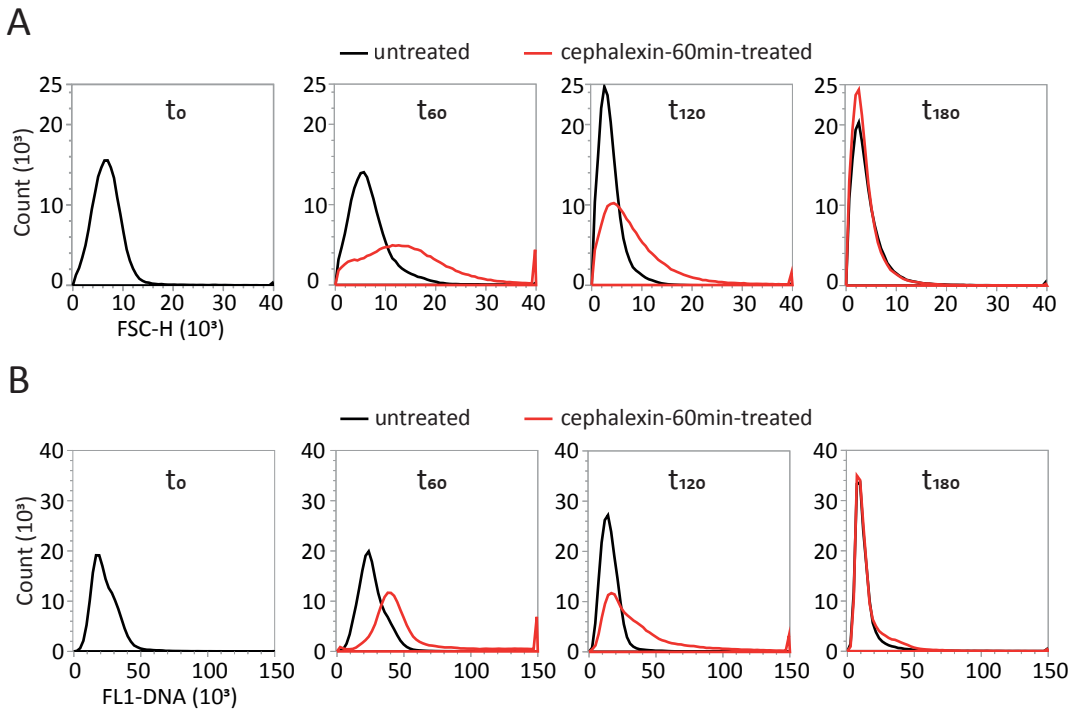


FIGURE 3

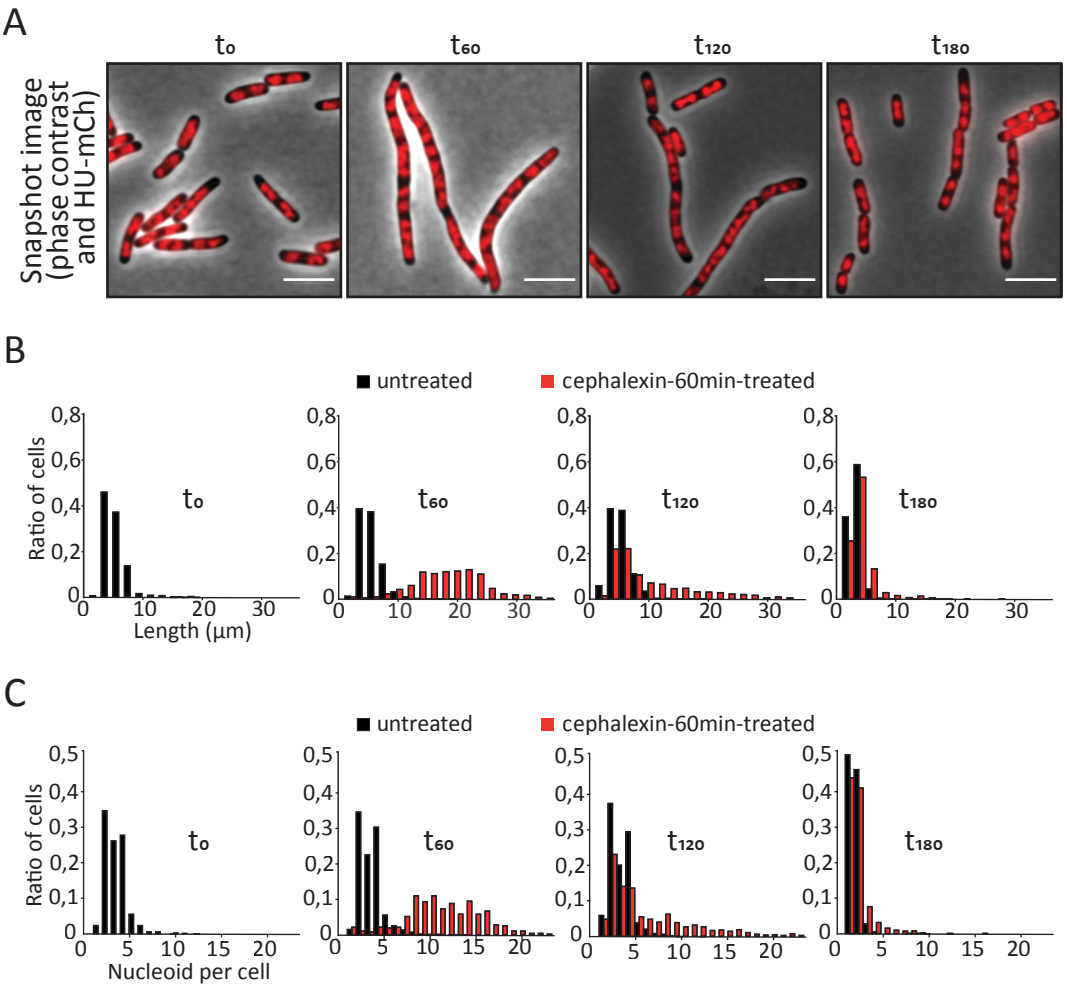


FIGURE 4

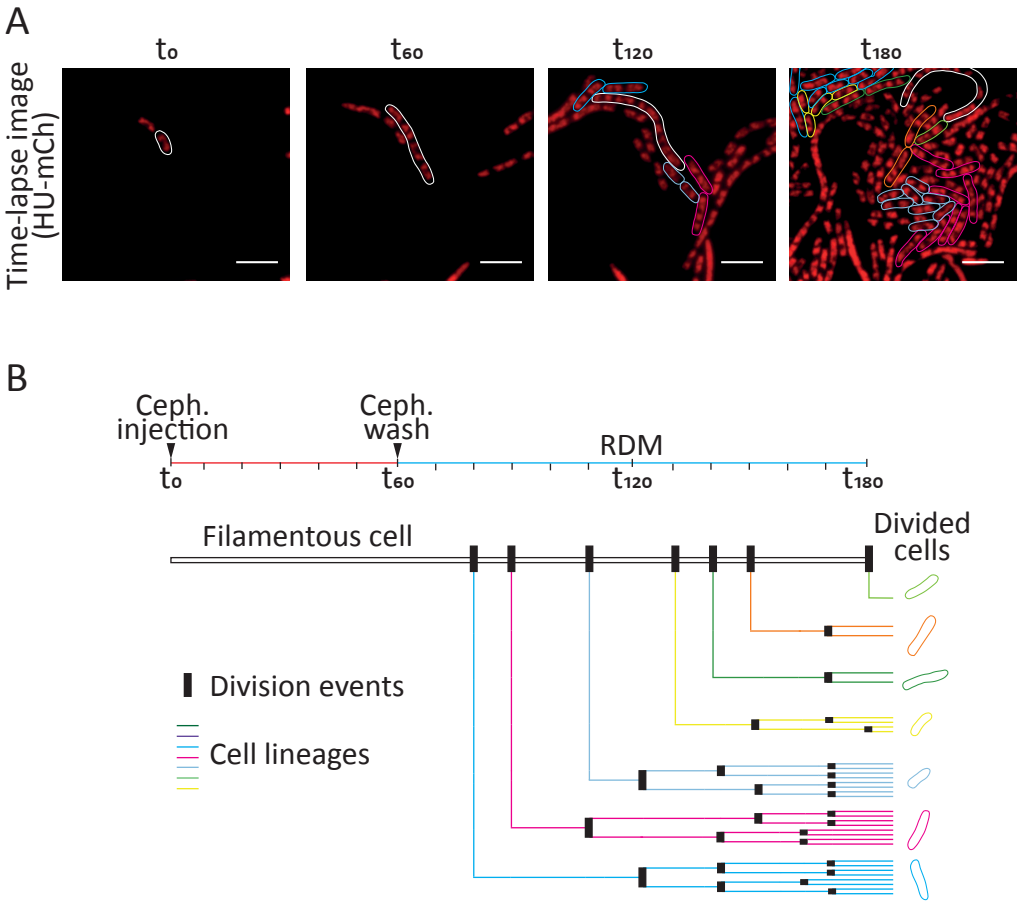
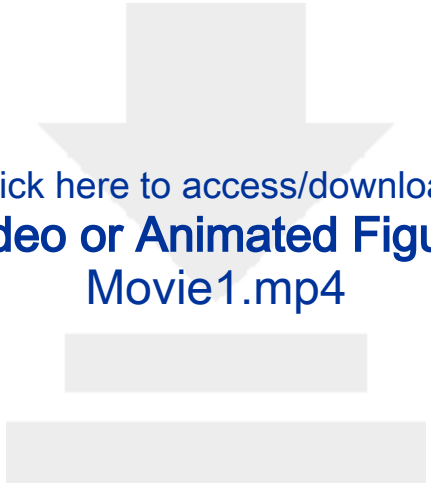


FIGURE 5

Video or Animated Figure



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Video or Animated Figure

Movie1.mp4

Name of Material/Equipment	Company	Catalog Number
Agarose	BioRad	1613100
Attune NxT Acoustic Focusing Cytometer	ThermoFisher scientific	A24858
CellASIC ONIX Microfluidic System	Merck Millipore	CAX2-S0000
CellASIC ONIX2 FG	Merck Millipore	ONIX2 1.0.1
CellASIC ONIX2 Manifold Basic	Merck Millipore	CAX2-MBC20
CytoOne 96-well plate with lid	Starlab	CC7672-7596
E. coli strain carrying a chromosomal insertion for a *hupA-mCherry* fusion		
Fiji	ImageJ	https://fiji.sc/
Gene Frame	Thermo Scientific	AB-0578
Luria-Broth agarose medium	MP Biomedicals	3002232
MicrobeJ	Imagej/Fiji plugin	https://www.microbej.com/
Microfluidic Plates CellASIC ONIX	Merck Millipore	B04A-03-5PK
Microscope Nikon eclipse Ti	Nikon	
MOPS EZ Rich Defined Medium (RDM)	Teknova	M2105
SYTO9 Green Fluorescent Nucleic Acid Stain	ThermoFisher scientific	S34854
TECAN Infinite M1000	TECAN	30034301

Comments/Description
Certified molecular biology agarose
Cytometer
Microfluidic system
Microfluidic software Manifold system Microplate with 0,2 mL well working volume and clear flat bottom, for automated plate reader
Created by P1 transduction of *hupA-mCherry* in *E. coli* MG1655
Image software. Cite Schindelin *et al.* if used in publication Blue frame (125 µL, 1,7 x 2,8 cm) Growth medium for plating assay Microscopy image analysis plugin. Cite Ducret *et al.* If used in publication;Detection settings: For bacteria : Area (µm2): 0,1-max; Length (µm): 0,5-max; Width (µm): 0,6-max; Range (µm): 0,5-max; Angularity (rad): 0-0,3; 0-max for all other parameters. For nucleoid: Tolerance: 500; Threshold: Local; 0-max for all other parameters
Plate for Microfluidic system
Fluorescence microscope Growth rich medium, 10x MOPS Mixture, 0,132 M K2HPO4, 10x AGCU, 5x Supplement EZ, 20% Glucose. Filtered at 0,22 µm
DNA fluorescent dye
Automated plate reader

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Author(s):	Julien Cayron and Christian Lesterlin

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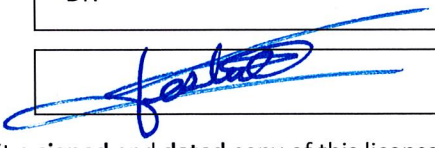
Signature:	
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Dear Alisha,

With this letter we would like to submit a revised version of our manuscript entitled " Multi-scale analysis of bacterial growth under stress treatments". We would like to thank the reviewers for their positive evaluations, helpful comments and valuable suggestions. The manuscript has been revised to answer to all their concerns and recommendations.

The text has been edited using words' track changes system and is now more precise and comprehensive. Additions to the Table of Materials have been left in red font. As recommended, we provided further details and additional notes to several sections, to make the procedure clearer. In particular, step 4 has been expended so it can be included in the film. The image analysis section 6 has also been reshaped to be more straightforward and useful for the user. We finally made sure that the highlighted text precisely corresponds to 3 pages and constitutes a logical flow of steps for the film to cover all key steps of the procedure.

These revisions have helped us to improve the clarity of our method. We hope that this new manuscript provides a satisfactory answer to the peer-review and editorial concerns and convince you to accept our work for publication.

Below, we respond to editorials and reviewers' comments in turn.
Sincerely,
Christian Lesterlin and Julien Cayron

Line-by-line response to editorial and reviewers' comments

Editorial comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The text has been proofread, corrected and rephrased in several places.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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.

1) 4.2: If you wish to film this step more details are required.

We have added several steps to the section 4.2 (4.2.1, 4.2.2 and 4.2.3) explaining the preparation of agarose-mounted slides for microscopy observation. These steps have been highlighted in yellow as we think it is important to show them in the film.

2) 6.1.3: What exactly are the settings?

The settings used for detection of *E. coli* cells on microscopy images have been added to the table of materials, in MicrobeJ « Comments/Description » cell. These settings are as follow:

- For bacteria detection: Area (µm^2): 0,1-max; Length (µm): 0,5-max; Width (µm): 0,6-max; Range (µm): 0,5-max; Angularity (rad): 0-0,3; 0-max for all other parameters.
- For nucleoid detection: Tolerance: 500; Threshold: Local; 0-max for all other parameters

• Protocol Numbering: Please add a one-line space after each protocol step.

This has been done.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol

text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
Following these recommendations, we have highlighted 3 pages of text in total, resulting in a logical and consistent flow of steps.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
The revised discussion covers these different points. Some parts have been added to answer to the reviewers' concerns.

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• Please define all abbreviations at first use.
This has been done throughout the text.

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Comments from Peer-Reviewers:
Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript entitle "Multi-scale analysis of bacterial growth under stress treatments" by Cayron J. and Lesterlin C. gives a perfect "boost" start for scientist that would initiate that kind of research. Indeed, it is frequent that only one or two technics are used to describe a "cell cycle phenotype" leading to potential misinterpretation. The manuscript is very concise, giving straightforward and precious advices to perform experiments in efficient and easy ways.
We thank the reviewer for her/his comments. Below, we provide point-by-point answers that address

all concerns.

Minor Concerns:
1°) On the Fig.1, it seems that the samples for OD measurement were not indicated.
The sample for OD monitoring is now indicated in Fig. 1.

2°) I would propose to modify "cephalexin-treated" by "cephalexin-60min-treated". The readers might be confused to see that the red curves, red histograms at t120 or t180 (cpx treated) are closer to untreated than t60; specially in fig.3 and fig.4 where cpx washing is not indicated (even if Fig. 1 indicates precisely the protocol).
“Cephalexin-treated” has been replaced by “cephalexin-60min-treated” throughout the text when appropriate and in Figures 3 and 4. Also, for the sake of clarity, we now indicate when cephalexin is added and washed out in Fig. 1A and Fig. 5B.

3°) I would suggest that the authors could a give short comment in the manuscript about the duration of the stress- treatment that will be studied. It might be needed to perform some preliminary test to adjust the protocol: some stresses might only be efficient in a certain duration window; hence the interpretation of the data could be confusing if a "wt" phenotype is finally observed after x.hours of treatment; it is important to know that the stress is still or not efficient at this moment. For instance, I think that cephalexin (depending on the concentration used) is not preventing cell division after a time correspond to 5-6 cell cycles?
This reviewer is right. We have added a NOTE stating: “Each stress-inducing treatment has an efficiency that is dose- and time-dependent. Then, it might be needed to run preliminary tests to determine the dose and duration of treatment to be used for optimal results. This can be done by performing OD monitoring (using automated plate reader) potentially associated with plating assays when required, of a cell culture treated with a range of doses and exposure times. “

4°) The authors importantly mention that cells should be cycling well before the stress (several generations before starting stress-treatment). It might be important to also mention that the ability to perform a sufficient number of generations is also an important parameter. If a stress will produce a phenotype only after 5 generations, the early stationary phase might be reach before and mask the stress effect.
This comment is consistent with reviewer #2 remark on point 1.5. To answer to these concerns, we have added a NOTE to the current section 1.5, stating: “It is critical to grow the cells for at least 4-5 generation times before achieving proper exponential growth. The initial inoculum used in step 1.3 (OD 0.01) needs to be adapted in the case of growth in poorer medium (where the exponential growth will be reached at OD<0.2), or if more generations are wanted before stress treatment (for thorough physiological study or to accommodate to extended stress treatments for instance).”

Reviewer #2:
Manuscript Summary:
In this manuscript the authors describe in detail a combination approach to record cell growth and morphology parameters, both via single cell and population approaches. The methodology is particularly useful for any type of stress induction, but can be easily adapted for other experimental approaches as well.
Overall I find the work very insightful and very suitable for publication in JoVE. The approaches are not new, of course, and have been used in various combinations in a wide variety of publications. However, this particular manuscript highlights the specific complementarity of the methods described and how a more systematic joint approach can be useful and highly informative. I am not aware that this has been done systematically by any study, which makes this work overdue.
However, to make this really useful to a variety of users, particular attention to all details should be paid by the authors, and I found a number of issues the addressing of which I would consider to be mandatory before publication can go ahead.
We thank the reviewer for the valuable assessment of the method. As requested, we provide additional details that contribute to make the procedure description more precise and useful for the user.

Major Concerns:
Protocol 1, point 1.1. The authors state that cells should be grown in the presence of the relevant antibiotic. This is a common misconception and a very bad practice and should not be promoted. To

be perfectly clear: if there is any chance of instability of the genetic marker in question then maintaining antibiotic selection is extremely important. This is almost always the case for plasmids, but for some other constructs as well. However, for markers stably integrated into the chromosome the presence of antibiotics in the growth medium is not only superfluous but rather dangerous. Inoculation of fresh medium with cells is in itself a stress for cells that results in the observed early lag of growth, but the presence of antibiotics greatly exacerbates the situation. Ironically the authors could use their experimental approach to demonstrate that cells get stressed in the presence of antibiotics, even if they carry the appropriate resistance markers. Cells do respond to different antibiotics in different ways - some cause stronger effects than other. But antibiotics such as kanamycin are a serious problem for cells, for example if streaked from stock cultures, despite the presence of the resistance gene. This stress can have serious unwanted side effects and should be avoided. Growth in medium without antibiotic is by far the best way to grow strains with stable deletions or point mutations. There is no excuse for adding antibiotics. It is nothing but bad strain handling.
We agree with this comment. As a consequence, we no longer recommend the addition of antibiotics in liquid culture (but only in the LB agar plates used for the initial isolation of the bacterial strain form the glycerol stock, step 1.1).

Protocol 1, point 1.5. This section needs more explanation. The authors note in their Discussion that care must be taken to ensure cells are indeed in exponential growth and I could not agree more. For growth curves with wild type-like cells roughly 4 cell divisions are needed before a clean linear correlation on a semi-logarithmic plot is observed. However, this is deceptive: for physiological studies 7-10 divisions are required - only then will protein or RNA levels be at a steady state. As such the description here is misleading. If the cells have grown overnight to stationary phase growing them from an OD.600 of 0.01 to 0.02 is completely inadequate. They will be just about in the transition from stationary to exponential growth. However, even if the overnight culture is in exponential growth cells will be stressed by the dilution into fresh medium and more than 2 divisions are required for the culture to return to exponential growth. The authors either need to adapt their protocol or they need to explain the precise conditions in their cultures very clearly so that other researchers can adapt the protocol. In addition, the authors also should note that for passing OD.600 ~0.3 in LB there is a growth shift and therefore the cells in their experiment are probably not physiologically homogeneous (J Bact. 2007 Dec; 189(23): 8746-8749). Does the same apply to the medium they are using? It will be important to add some comments to make other readers aware, as some might want to use LB or similar rich media, but a slight adaptation of the procedure to lower ODs might eliminate the problem altogether.
The initial text recommended to grow the cells from an initial inoculum of OD 0.01 to OD 0.2 (and not 0.02), thus allowing for 4-5 generation times before sampling and stress treatment. This reviewer is right that more generation times might be required for specific studies (or for longer stress exposures, as also mentioned by reviewer #1 in the comment number 4°). We have then added a NOTE to the current section 1.5, stating: “It is critical to grow the cells for at least 4-5 generation times before achieving proper exponential growth. The initial inoculum used in step 1.3 (OD 0.01) needs to be adapted in the case of growth in poorer medium (where the exponential growth will be reached at OD<0.2), or if more generations are wanted before stress treatment (for thorough physiological study or to accommodate to extended stress treatments for instance).”

Protocol 2, point 2.1. The authors dilute their samples in medium that is proficient for growth. This is not ideal, as time can impact the results quite significantly. A variation of saline solution without a carbon source (M9 minimal medium without glucose or similar) eliminates this problem while keeping cells reasonably happy.
This reviewer is right, though serial dilutions are performed in a couple of minutes, thus limiting the impact of ongoing cell divisions. Nonetheless, for the user to be aware of this phenomenon, we have added a NOTE to section 2.1 stating: “Serial dilution in fresh medium must be performed rapidly to limit bacterial divisions. Alternatively, authors might consider using a saline solution without a carbon source to prevent cell divisions during the dilution process.”

Protocol 3, point 3.1. The authors ought to measure the viable titer of a culture from the moment when it is put on ice to the actual processing by flow cytometry. They might be surprised by the result. Cells grow significantly in the cooling period, but they also grow while kept on ice, slowly but measurably. This needs to be stated. For some applications it will be important to fix the cells to avoid this problem. The same point needs to be addressed in the Discussion (line 330).
For the user to be aware of this important point, we have added a NOTE to section 3.1: “Incubation on ice will limit the growth and morphological modification of the cells. Alternatively, the user might

consider using fixation of the cells in ethanol 75%, as usually recommended for flow cytometry". We have also added a comment in the discussion "Note that incubation on ice efficiently limits, but does not strictly stop cell division. As a consequent, the user might consider fixing the cells in ethanol 75% as usually recommended for flow cytometry analysis (see reference 10) and for a fixation faster than with formaldehyde or glutaraldehyde. However, cell fixation requires additional washing of the cells and is known to affect the cell morphology (shrinking of the cells and partial lysis) and the brightness of fluorescent dye". An additional reference Nº17 was added to support that statement.

Minor Concerns:
There are minor grammar and spelling issues throughout ("cultures tubes", line 73, "Platting assay", line 112, "if older or recently version", line 212, to name but a few).
The manuscript has been corrected for grammar and spelling errors, including those indicated by this reviewer.

Protocol 4, point 4.1. The "microscope chamber" might, depending on the experience of the audience member, be slightly cryptic. Slightly more information of what it does will make this part more highly accessible to a wide audience.
Section 1.1 now reads: "Preheat the thermostated microscope chamber at 37°C to stabilize the temperature before starting the observation. This chamber allows for temperature modulation of the microscope optics and sample stage during time-lapse experiments."

Some confusion about the precise order of steps in 6.1.4 (line 217).
Indeed, this has been corrected.

Reviewer #3:
The ms by Cayron and Lesterlin describes an integrated protocol allowing to analyze the effects of any treatment on E. coli cells (or other bacterial species) at both the population and single-cell levels. I found the protocol very clear, concise and complete. I only have minor comments.
We thank the reviewer for her/his positive evaluation of the manuscript. Below, we provide point-by-point answers to her/his concerns.

Minor concerns:
This protocol can be used I guess with other bacterial species. this should be stated in the ms.
The applicability of this procedure to other bacterial species is now stated in the introduction "This procedure can be applied to decipher the response of diverse bacterial species to virtually any stress of interest…" and in the discussion "The method can be applied to any bacterial species, the only requirement being that the microfluidic apparatus has to be compatible with the morphology of the cells." and "The approach is applicable to a wide range of bacterial species…".

L73. Remove hupA-mCherry, it is a particular case, the protocol can be used with any strain
The *E. coli* strain used as an illustrative example is now indicated in section 1.1 as a NOTE: "We applied the procedure to the *Escherichia coli* MG1655 *hupA-mCherry*, which carries a fusion of the *mCherry* gene to the subunit α of HU nucleoid associated protein[7]. The *hupA-mCherry* fusion was inserted at the native chromosomal locus by replacing wild-type gene using P1 transduction."

L76. List the complete composition of RDM medium.
RDM composition has been detailed in the Table of Materials.

L81. For certain types of experiments such as persistence to antibiotics, duration of the overnight cultures strongly influence the persister levels. Overnight is too vague. Set a precise timing e.g. 16h
It is now clearly stated that overnight incubation corresponds to 17 hours (see section 1.2)

L84. Log phase culture are really performed in a test-tube? Not in flasks? For good aeration, a small volume of culture in a large flask (like 5 ml medium in 50 or 100 ml flask) is the best.
In section 1.2, we now state "Flasks (≥50 ml) or large diameter (≥2 cm) test-tubes must be used to ensure satisfactory aeration of the agitated culture".

L87. What type of microplate? Volume of the wells? There is the possibility to measure Fluorescence and OD in the same exp. This should be stated as well as the type of plates to use if fluorescence is measured.

Microplate are now described properly in the text and in Table of Materials (0.2 mL well working volume with clear transparent bottom). However. we do not recommend to measure fluorescence in the same experiment as HU-mCherry signal reflects the intracellular amount of HU protein rather than strictly the cell DNA content. Also, we do not recommend growing the cells after DNA staining with fluorescent dye, as these DNA intercalants molecules disturb cell growth.

L94. Samples for flow cytometry are kept on ice. Could that influence? Isn't it better to measure directly?

Indeed, it would be best to analyze all the samples directly if possible. We then state: “When possible, we recommend processing the flow cytometry samples directly. Alternatively, samples can be kept on ice (for up to 6 hours) and analyzed simultaneously at the end of the day, once plating and microscopy imaging have been performed. »

This reviewer’s comment is consistent with reviewer #2 major point 3.1, and we agree that incubation on ice might have an effect on flow cytometry results. For the user to be aware of this important point, we have added a NOTE to the section 3.1: “Incubation on ice will limit the growth and morphological modification of the cells. Alternatively, the user might consider using fixation of the cells in ethanol 75%, as usually recommended for flow cytometry10.” We have also added a comment in the discussion “Incubation on ice will limit the growth and morphological modification of the cells. Alternatively, the user might consider using fixation of the cells in ethanol 75%, as usually recommended for flow cytometry”. An additional reference Nº17 was added to support that statement

L123. The plot of CFU/ml as a function of time should be performed also for the untreated cells and before treatment.

This is now stated clearly in section 2.3.

L125. For the flow cytometry exp., in the case of the strain carrying the HU-GFP, is the SYTO9 treatment necessary?

HU-mCherry fluorescence reports the intracellular concentration of HU protein rather than the DNA content *per se*. Quantification of the cells DNA content by flow cytometry then required the use of DNA stains, which binding reflects to the amount of DNA (ethidium bromide, DAPI, SYTO9 or similar).

L182. How many cells/field? Cells should be allowed to adapt and grow in the microfluidics. It is a different mode of growth as compared to tubes or flasks.

For snapshot imaging (section 4.4, we added “Select for fields of view that contain isolated cells in order to facilitate automated cell detection during image analysis. Make sure that at least 300 cells are imaged to allow robust statistical analysis of the cell population”. For time-lapse imaging (section 5.7), we wrote: ”we recommend about 10 to 20 cells per field of view of 100 µm²”. In the following section 5.8, we now indicate that “Program the injection of growth medium for 1 to 2 generation time equivalents to allow for the cells to adapt (optional)”.

L227. Do the authors check manually the cell detection by MicrobeJ?

Indeed, we systematically check visually the quality of cell detection and we perform manual correction when required. This is now indicated “Check the accuracy of the cell detection and use the MicrobeJ editing tool for correction if needed”

Figure 2C should be better detailed in the text.

We have added explanations in the part of the text that refers to the Figure 2C. “Different stresses will results in different uncoupling of the OD and CFU/mL curves, depending of the effect induced (modification of the cell morphology such as filamentation or bulging, cell death with or without lysis...). A non-exhaustive list of possible outcome results indicative of different stress-induced effects is presented in **Figure 2C**.”

Again, we are grateful to all three reviewers for their remarks, which allowed us to improve the quality of our method description. We hope that these additional data will provide satisfactory answers to their concerns and questions.

Sincerely,

Christian Lesterlin and Julien Cayron