Video Article

Multi-scale Analysis of Bacterial Growth Under Stress Treatments

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/60576/

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. 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].

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.
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.
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.
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).
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).
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.
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).

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. 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.

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].
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. 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.
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.

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

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.
2. Seal the microfluidic plate to the manifold system and click on the **Priming** button.
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.
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. Seal the microfluidic plate and place it on the microscope stage.
6. On the microfluidic software (see **Table of Materials**) 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.
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.
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.
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] (https://microbej.com). This protocol uses the MicrobeJ 5.13I version.

1. Open the Fiji software and the MicrobeJ plugin.
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.
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).
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**).

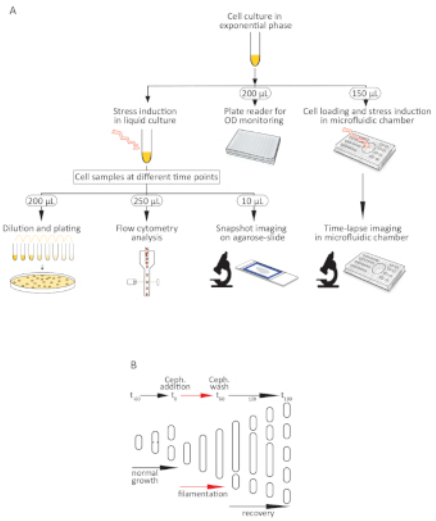


Figure 1: Procedure for the analysis of bacterial response to stress treatments. (**A**) Schematic of the method. (**B**) Cartoon illustrating the cell morphology during normal growth in rich medium and during transient exposure to cephalexin (Ceph.), from addition at (t_0) and after cephalexin washing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

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**.

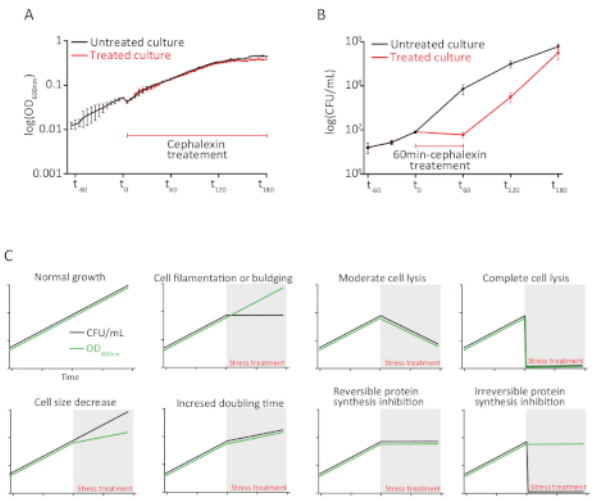


Figure 2: Bacterial growth monitoring of untreated and cephalexin-treated cells at the population level. (**A**) Optical density monitoring (OD_{600nm}/mL). (**B**) Concentration of viable cell (CFU/mL) within untreated and cephalexin-60min-treated cultures. Error bars indicate the standard deviation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

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.

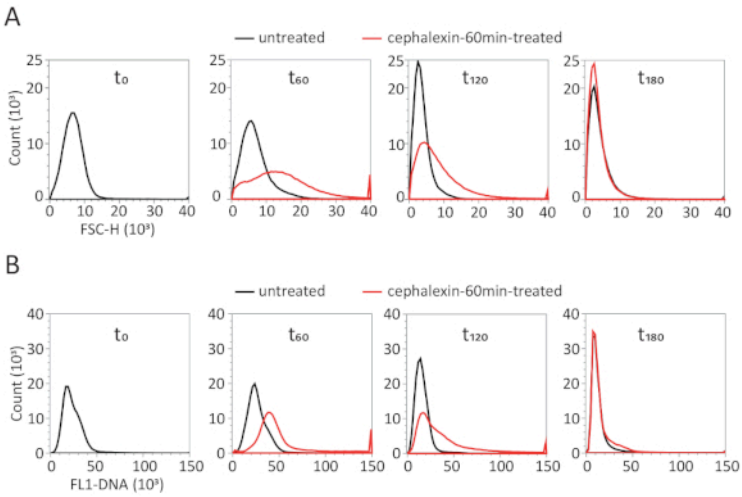


Figure 3: Representative flow cytometry analysis of untreated and cephalexin-60min-treated cells. (**A**) Cell size distribution histograms (FSC-H). (**B**) DNA content histograms (FL1-SYTO9). n = 120,000 cells analyzed. Please click here to view a larger version of this figure.

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).

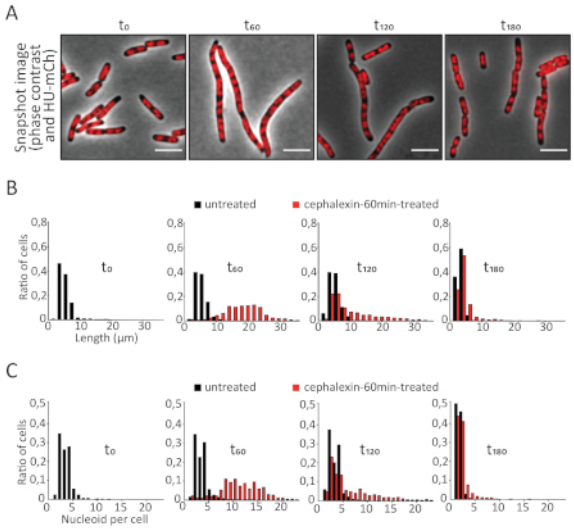


Figure 4: Microscopy snapshot analysis of untreated and cephalexin-60min-treated cells. (**A**) Representative microscopy images showing phase contrast (grey) and HU-mCherry signal (red). (**B**) Cell length distribution histograms. Scale Bar = 5 µm. (**C**) Histograms of the number of nucleoid per cell.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

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).

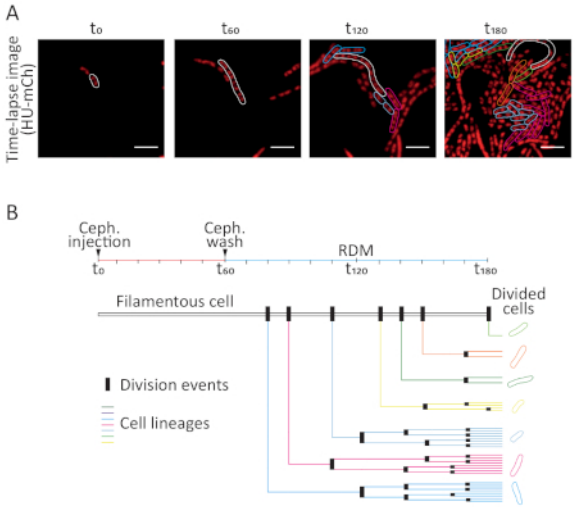
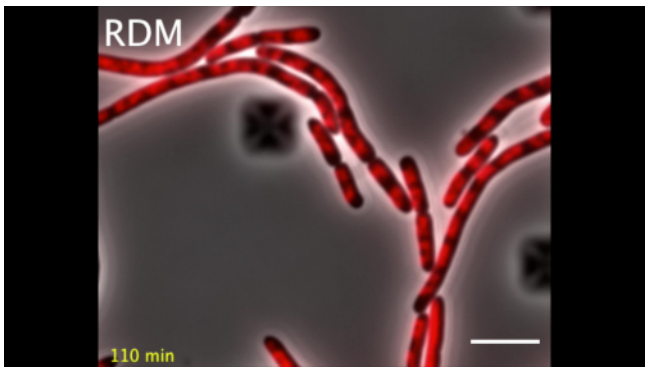


Figure 5: Microscopy time-lapse analysis of cephalexin-60min-treated cells. (**A**) Representative microscopy images showing phase contrast (grey) and HU-mCherry signal (red). The monitored filamentous cell is indicated by the white outline, and divided cells by different colors. Scale Bar = 5 µm. (**B**)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



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. Please click here to view this video (Right click to download).

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.

Disclosures

The authors declared no competing interests.

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