

Video Article

Live Cell Imaging of *Bacillus subtilis* and *Streptococcus pneumoniae* using Automated Time-lapse Microscopy

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Abstract

During the last few years scientists became increasingly aware that average data obtained from microbial population based experiments are not representative of the behavior, status or phenotype of single cells. Due to this new insight the number of single cell studies rises continuously (for recent reviews see ^{1,2,3}). However, many of the single cell techniques applied do not allow monitoring the development and behavior of one specific single cell in time (e.g. flow cytometry or standard microscopy).

Here, we provide a detailed description of a microscopy method used in several recent studies ^{4, 5, 6, 7}, which allows following and recording (fluorescence of) individual bacterial cells of *Bacillus subtilis* and *Streptococcus pneumoniae* through growth and division for many generations. The resulting movies can be used to construct phylogenetic lineage trees by tracing back the history of a single cell within a population that originated from one common ancestor. This time-lapse fluorescence microscopy method cannot only be used to investigate growth, division and differentiation of individual cells, but also to analyze the effect of cell history and ancestry on specific cellular behavior. Furthermore, time-lapse microscopy is ideally suited to examine gene expression dynamics and protein localization during the bacterial cell cycle. The method explains how to prepare the bacterial cells and construct the microscope slide to enable the outgrowth of single cells into a microcolony. In short, single cells are spotted on a semi-solid surface consisting of growth medium supplemented with agarose on which they grow and divide under a fluorescence microscope within a temperature controlled environmental chamber. Images are captured at specific intervals and are later analyzed using the open source software ImageJ.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3145/>

Protocol

1. Preparation of *B. subtilis* cultures

1. Inoculate cells from -80°C stocks in 10 ml time-lapse microscopy (TLM) medium (62 mM K₂HPO₄, 44mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 6.5 mM sodium citrate, 0.8 mM MgSO₄, 0.02 % casamino acids, 27.8 mM glucose, 0.1 mM L-tryptophan, the pH was set to 7 using a KOH solution) supplemented with antibiotics, if necessary.
2. Grow the cells overnight in a shake flask (30°C, 225 rpm).
3. The following morning, dilute the cells 1:10 in pre-warmed chemically defined medium (CDM) (62 mM K₂HPO₄, 44mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 6.5 mM sodium citrate, 0.8 mM MgSO₄, 2.2mM glucose, 2.1 mM L-glutamic acid, 6 μM L-tryptophan, 7.5 μM MnCl₂, 0.15 x metal mix (prepare 50x MT mix stock (ref ⁸) which contains: 0.2 M MgCl₂, 70 mM CaCl₂, 5 mM MnCl₂, 0.1 mM ZnCl₂, 0.2 mM thiamin-hydrochloride, 2 mM HCl, 0.5 mM FeCl₃ (add last)) without antibiotics.
4. Grow the *B. subtilis* cells to mid-exponential phase (30°C, 225 rpm). Usually, this takes approximately four hours. Importantly, prepare the agarose slide one hour before the cells reach mid-exponential phase (see section 2).
5. Measure the absorbance of the culture at 600nm (A₆₀₀) and dilute the cells to an approximate A₆₀₀ of 0.035 using CDM. This OD ensures that single cells with appropriate spacing are spotted on the microscope slide for time-lapse microscopy.

2. Preparation of the microscope sample (also see Figure 2)

One hour before cells reach mid-exponential growth, prepare the microscope slide as follows:

1. Clean two microscope glass slides (e.g. Knittel Glass, 7.6 x 2.6 cm,) with 70% ethanol and water.
2. Take a gene frame (ABgene; 1.7 x 2.8 cm) and carefully remove one of the plastic foils from the gene frame without causing disassembly of the plastic cover on the other side of the gene frame.

3. Attach the gene frame in the middle of one of the glass slides by first facilitating contact on just one side, followed by guided attachment of the remaining gene frame with a fingernail. Prevent air bubbles while attaching the gene frame to the glass slide.
4. Use a microwave to dissolve 150 mg (1.5%) high-resolution low-melting agarose (Sigma) in 10 ml CDM. The agarose needs to be fully dissolved to obtain minimal background required for the time-lapse microscopy experiments. If required, supplement the agarose-CDM with inducer or other compounds at this time
5. Transfer 500 μ l of the warm agarose-CDM in the middle of the gene frame. Make sure the whole area including (the borders) is fully covered. The following steps (2.6 - 2.10) have to be carried out quickly to prevent excessive drying of the agarose-CDM.
6. Place the second glass slide on the agarose-CDM filled gene frame. Try to avoid air bubbles. Place the sandwiched slides in a horizontal position for 45 min at 4°C in the refrigerator to allow the agarose-CDM to solidify sufficiently.
7. Carefully slide off the upper glass slide. Use a razor blade to cut out agar strips of ~5 mm width within the gene frame, on which the cells will be grown. A maximum of three strips can be used per slide, separated by ~4 mm space to either side. These spaces will provide air which is essential for *B. subtilis* growth. If four different strains need to be followed in time, two strips can be made and be cut in half to result in four small squares. Remove any residual solid medium.
8. Carefully remove the second and final plastic cover from the gene frame to expose the sticky side of the gene frame
9. Load single cells (from step 1.5) on the solid medium without touching it with the pipet tip. Use 2.5 μ l for a whole strip, or 1 μ l for a small square. Always start on top of the agarose pad and allow the liquid to disperse equally on its assigned growth area by turning the slide up and down. The slide is ready, as soon as the edges of the liquid become corrugated and movement of the liquid is no longer visible when turning the slide.
10. Place a clean microscope slide cover slip (24 x 50 mm) on the gene frame from one side to the other (avoid air bubbles). Assure complete attachment by applying pressure on the cover slip along the gene frame with your fingernail. If the cover slip is placed on the cells without allowing them to dry long enough, cells tend to grow on top of each other during the experiment. Also be careful not to wait too long before applying the cover slip, since the agarose will then be too dry.
11. Pre-warm the slide for 1 h at 30°C. If the slide would directly be placed in the pre-warmed environmental chamber (see step 3.1) of the microscope, the temperature fluctuations might cause autofocus problems in the first hours of the experiment.

3. Time-lapse fluorescence microscopy (also see Figure 3 and Movie 1)

1. Pre-warm the environmental chamber on time (in our hands at least 2h before the start of the experiment) in order to prevent autofocus problems after starting the experiment. The time required depends on the environmental chamber used as well as the heating system and the microscope.
2. Select the appropriate objective, filters and dichroic mirror according to your experimental set-up. For long experiments make sure that a UV-filter is placed between the light source and the sample. Also, if possible, block some of the excitation light using neutral density filters to minimize exposure.

The following equipment (provided by DeltaVision, UK) was used for the time-lapse microscopy experiments published in de Jong *et al.* 2010⁵: IX71 Microscope (Olympus), CoolSNAP HQ2 camera (Princeton Instruments), 300W Xenon Light Source, 60x bright field objective (1.25 NA), GFP filterset (Chroma, excitation at 470/40 nm, emission 525/50 nm), mCherry filterset (Chroma, excitation at 572/35 nm, emission 632/60 nm). Autofocus was performed using diascopic light and using the autofocus routine present in Deltavision's Softworx software. It should be noted that there are now a number of other autofocus systems that are also suitable such as the Zeiss Definite Focus, the Nikon Perfect Focus System and the Leica Adaptive Focus Control.

3. Program your experiment according to your experimental set-up. It is wise to determine the amount of light required for specific constructs, as well as the autofocus settings for other time-lapse microscopes or bacteria prior to the actual experiment. Shorter exposure times and less amount of excitation light will minimize bleaching and phototoxicity. Use diascopic light for the autofocus routine.

The following settings were used for the time-lapse microscopy experiments published in de Jong *et al.* 2010⁵: snapshots for movies were taken at intervals of 8 or 12 minutes using 10 % APLLC White LED light and 0.05 s exposure for bright field pictures, 10% Xenon light and 0.5 s exposure for GFP detection, and 32% Xenon light and 0.8 s exposure for mCherry detection, respectively. Raw data were stored using softWoRx 3.6.0 (Applied Precision). The autofocus was programmed for 0.06 μ m steps and a total range of 1.2 μ m.

4. Place the prepared slide (section 2) in the pre-warmed environmental chamber of the microscope and monitor the outgrowth of single cells into a microcolony monolayer at 30°C.

Specific tips:

- Select single cells that are located in the middle of the agar pad. The edges of the agar pad dry out more easily. Store the X,Y,Z position using the microscope's software.
- Large movements of the stage in the X,Y and Z direction might perturb the agarose and consequently hamper identification of cells by the autofocus routine. In general, to minimize X,Y,Z movement, we do not select more than 10 positions per experiment even if a slide contains multiple strains.
- After having selected the first cell, only adjust the Z-focus with the software. From this point, do not alter the focus manually on the microscope body using the "Z-knob" unless this is digitally encoded. Ensure that after each autofocus routine the new X,Y,Z position is stored by the software.
- Check whether the autofocus settings are appropriate for the experiment, before starting the run. The use of phase-contrast microscopy might improve the autofocus routine compared to using brightfield or DIC microscopy, due to enhanced contrast. However, the phase ring in phase-contrast objectives makes them less sensitive (approximately 10%) in collecting fluorescence light. So for weak fluorescent samples, an objective without phase ring is better suited.

- Check whether the selected cells are still in focus every half hour, until the experiment is running stably. When cells at this point are out of focus, adjust manually. Due to temperature variations, as well as poorly dried samples, this may be necessary during the first hours. Furthermore, due to enhanced contrast, the autofocus works better when more cells are in the field of view.
- 5. After the experiment has finished, separate the different channels of the movie and save them as separate files (i.e. phase contrast, GFP, mCherry) if required (certain acquisition packages will place all channels in a single stacked file). For publication, pictures can be enhanced by 2D deconvolution, which is especially useful for protein localization studies. Deconvolve the images using your microscope's software or with a commercial package such as Huygens (www.svi.nl).
- 6. Analyze the data using ImageJ (<http://rsbweb.nih.gov/ij/>) (use the raw, unprocessed image files for this) and Microsoft Excel or Sigma Plot. Stacks can for example be saved as an ".avi" movie file in ImageJ. A detailed description of how fluorescence of single cells can be measured in time is given below.

4. Data analysis of promoter activity dynamics using ImageJ

We note that other good software packages are available which are specialized in analyzing time-lapse microscopy images such as BHV software^{9,4}, Schnitzcell¹⁰, PSICIC¹¹, and Microbe-Tracker¹², but here we focus on the freely available ImageJ package.

1. Download ImageJ (<http://rsbweb.nih.gov/ij/>) and (if necessary) the correct plugin to open your (stacked) file. For instance, movies recorded using a delatavision microscope can only be opened in ImageJ with the delatavision opener plugin. Copy the DV-plugin in the ImageJ plugin folder and start the program. Change the memory capacity in edit/option/memory&threads to 1250. This allows working with bigger files such as those obtained from time-lapse movies.
2. To assess the cell history of one single cell, open the original phase contrast movie of one microcolony and scroll to the last frame of interest in the movie. Select the "segmented lines" selection button in the menu.
3. Draw a line in the background and press "CTRL" + "T". This will open the regions of interest (ROI) manager. (The corresponding background fluorescence value can be used to manually subtract the background - see below. Alternatively, use the background subtraction routine present within ImageJ.) Also draw a line in the cell of interest and add the ROI to the ROI manager. Since we are investigating promoter-GFP fusions in this case study and the GFP is diffused throughout the cytoplasm, the whole cell should have similar fluorescence values for each single pixel across the length of the cell. Scroll one frame back in time and select a new ROI in the same cell of interest. Save this third ROI and continue with the procedure until the corresponding ROI in the first frame of the movie has been saved. Be aware that the fluorescence of daughter cells can vary greatly after cell division. Either use a membrane dye which can be applied together with fluorescence proteins produced by the cells (such as the combination of the red membrane dye FM[®] 5-95 (Invitrogen) and GFP) to visualize septum formation. In this case the corresponding fluorescence channel and not the phase contrast movie should be used to follow cells in time. Alternatively, one can stay on the safe side by selecting ROI's in only one half of a cell.
4. Within the ROI manager, click "save". If the ending of the file is ".roi", then one roi is selected in the list, and only this one will be saved. If the ending of the file is ".zip", then the whole set will be saved (required).
5. Close the phase contrast movie and open the original fluorescence movie (e.g. GFP). Click "show all" and "measure" in the ROI manager. A new window will open (Results). Copy the results into an Excel sheet and subtract the mean fluorescence for each single cell from the background fluorescence of the medium. The resulting net fluorescence can be plotted against the time to reveal the promoter activity of the cell of interest in time.
6. Alternatively, the fluorescence of all cells of a microcolony can be analyzed at a specific time-point. In order to do this select and save ROI's for the background and every single cell in one frame as described above, copy the fluorescence values to Excel and produce histograms using the "histogram" function in the "tool" menu.
7. To obtain pictures from single frames for publication, select the frame of interest, select "image" - "duplicate image" and change the type of image to "RGB" or "8-bit" by selecting "image" - "type" - "RGB color" or "8-bit". Save the duplicated frame as ".tiff". The RGB/8-bit pictures can be opened by conventional drawing programs such as CorelDraw or Adobe Illustrator. If required, the pictures may be adapted using "image" - "adjust" - "brightness/contrast" or "image" - "adjust" - "window/level" in ImageJ.

5. Producing movies for publication with ImageJ

1. Open the original phase contrast and the corresponding fluorescence movie(s) in ImageJ as described above. Select the rectangular selections button (1st on the left) and draw a rectangle ROI in the first frame in such a way, that the developing microcolony is enclosed by the ROI throughout the whole movie. Select "image" - "crop" and save this smaller version of the movie under a new name. Select the same ROI via the ROI manager in the fluorescence movie and proceed as before.
2. To combine the movies either horizontally or vertically, select "plugin" - "stack combiner". If desired, a time stamper can be added via "plugin" - "time stamper". Save the combined stack once as ".dv" or ".tiff" and once as ".avi" or quicktime movie.

Alternative Protocol Adaptations for *Streptococcus pneumoniae* (Figure 4 and Movie 2):

6. Preparation of *S. pneumoniae* cultures

1. Grow *S. pneumoniae* cells (capsulated strain D39¹³, or unencapsulated strain R6¹⁴ as standing cultures in C+Y medium¹⁵ at 37°C until an OD at A_{600nm} of approximately 0.4 is reached. Centrifuge the cells for 2 min at 14000 rpm and resuspend the cell pellet in a volume of fresh C+Y medium containing 14.5% glycerol (v/v) that would result in an A_{600nm} of exactly 0.4. Aliquot the cells and store them at -80°C for future use.
2. For time-lapse microscopy, take an aliquot of previously cultured *S. pneumoniae* cells. Inoculate 4 ml of fresh C+Y medium 1:100 with cells from the -80°C aliquot. Grow the cells until mid-exponential phase to an OD of A_{600nm} of 0.1 - 0.2. Usually, this takes about 2 hours when using cells from -80°C aliquots.

7. Preparation of the microscope sample

1. Prepare a microscope slide as described above for *B. subtilis* but make sure that the agarose contains complex C+Y medium. Since *S. pneumoniae* is a microaerophile, the air pockets between agarose strips should be smaller than for *B. subtilis* (~1 mm space on both sides).
2. Measure the absorbance of the culture at 600 nm (A_{600}), dilute the exponentially growing *S. pneumoniae* cells to an approximate A_{600} of 0.05 using C+Y medium and use this dilution to load the agarose slide.

8. Time-lapse phase-contrast microscopy

Adjust the microscope settings for *S. pneumoniae*: use phase-contrast microscopy since *S. pneumoniae* is difficult to identify using bright-field microscopy. Continue the protocol as described for *B. subtilis* (follow steps 2.9 - 3.7). *S. pneumoniae* cells can be grown at either 30°C or 37°C (they grow faster at 37°C).

9. Representative results:

The time-lapse fluorescence experiment has been carried out successfully, if the bacteria grew into a microcolony monolayer, which is completely located within the field of view at the end of the experiment (see Figure 5A-C). If cells grew on top of each other, it is not only impossible to trace back their history accurately, but also the fluorescence levels of overlapping cells can not be measured correctly. Cells tend to grow on top of each other, if the spotted cells were not dried sufficiently (step 2.9) or if the medium composition needs to be adjusted to obtain slower growth. If a microcolony grew out of view, then the distribution of fluorescence signals within one colony can not be determined. Causes for "microcolony movement" can be insufficient drying of spotted cells (step 2.9), or if the software was not programmed to track the microcolony during development. Furthermore, it is important that local patches of increased fluorescence are not detectable in the medium as this obscures the fluorescence signals coming from the cells (see Figure 5D-F). Background related problems can arise from media compounds, airbubbles or undissolved agarose clumps. To visualize this, we show in Fig. 5F background signals of this specific slide when the image was taken using excitation/emission filters for red fluorescent dyes. As seen, bright autofluorescent spots are present which could hamper imaging. To prevent such spots, make sure the agarose is completely dissolved and there are no airbubbles when placing the coverslip on the microscope slide.

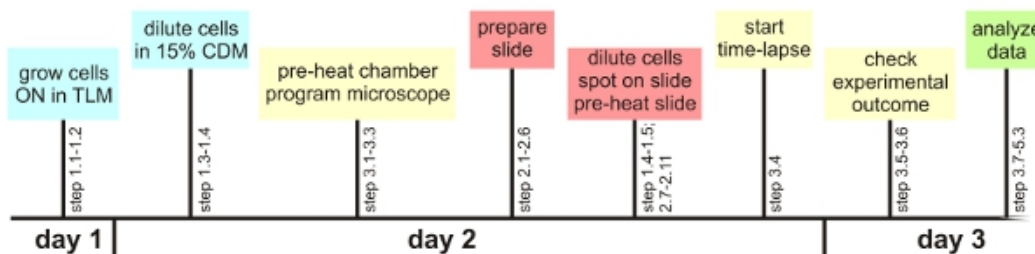


Figure 1: Experimental overview

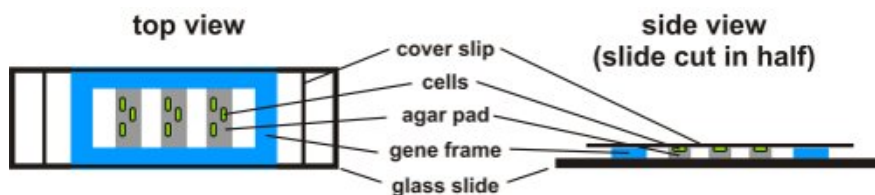


Figure 2: Preparation of the microscope sample

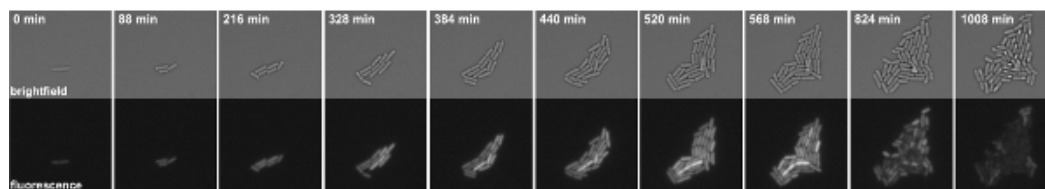


Figure 3: Time-lapse fluorescence microscopy of *B. subtilis* cells harboring a P_{kinB} -*gfp* fusion. Snapshots are taken from Movie 1. Top panels: bright field, bottom panels: GFP channel.

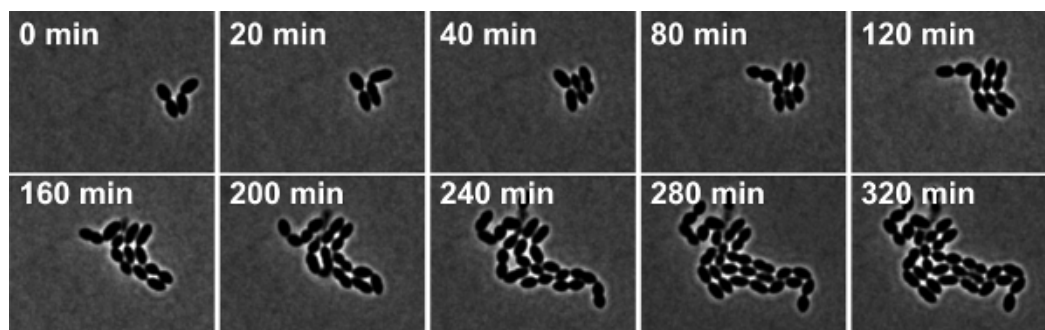


Figure 4: Time-lapse phase-contrast microscopy of *S. pneumoniae* wild type strain R6. Snapshots are taken from Movie 2.

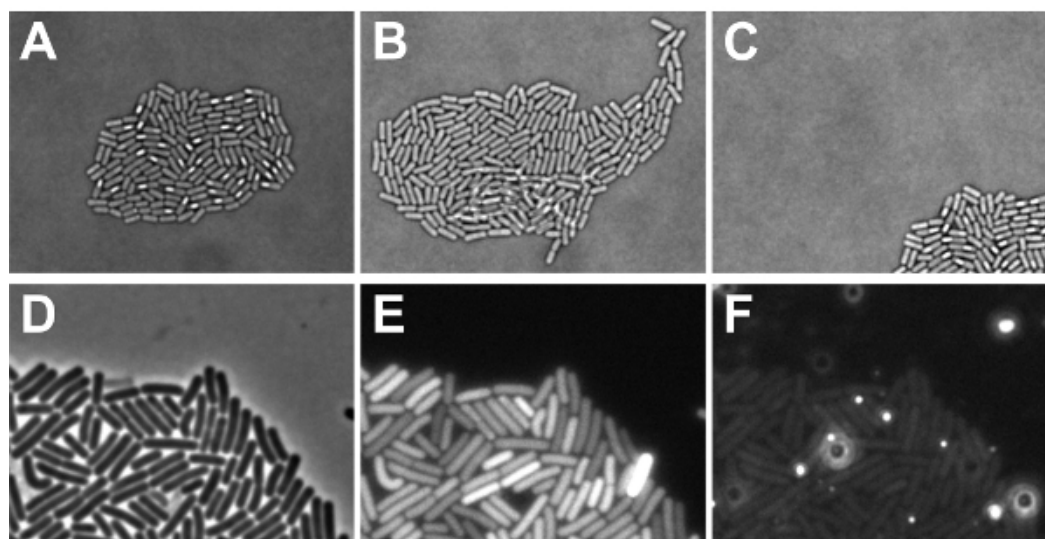


Figure 5: Illustration of possible (time-lapse) microscopy outcomes. A-C shows factors that need to be considered for data obtained with diascopic light settings. (A) Brightfield micrograph of a microcolony monolayer (positive outcome) of sporulating *B. subtilis* cells (B) Brightfield image of a *B. subtilis* microcolony in which some cells grew on top of each other (negative outcome) (C) Brightfield image of a sporulating *B. subtilis* microcolony that grew out of the field of focus (negative outcome). D-F show factors that need to be considered for data obtained with episcopic light settings (D) Phase contrast picture of *B. subtilis* cells in exponential phase depicted to visualize where the fluorescence signals in E and F originate from (E) GFP signals of the cells shown in D. Note that the background signals are similar in each pixel (positive outcome). Also note that the exposure time might be too much since one cell shows a saturated signal (negative outcome) (F) Signals obtained through the red channel of the cells shown in D. Note that the background contains areas with increased red fluorescence levels (negative outcome).

Movie 1. Time-lapse fluorescence microscopy of *B. subtilis* cells harboring a P_{kinB} -*gfp* fusion. Snapshots were taken in 8 min intervals. Left: bright field, Right: GFP channel. [Click here to watch the movie.](#)

Movie 2. Time-lapse phase-contrast microscopy of *S. pneumoniae* wild type strain R6. Snapshots were taken in 10 min intervals. [Click here to watch the movie.](#)

Discussion

In contrast to many other single cell techniques, the time-lapse fluorescence microscopy method described here can be used to follow the history of a specific cell with regard to its ancestors, its behaviour, and division events. In combination with fluorescently labelled target promoters or proteins, specific developmental pathway activation can be followed in time and protein localization as well as protein dynamics can be monitored during bacterial development.

As indicated above, studies concentrating on different bacterial species can be performed by adapting the growth conditions according to the requirements for a specific bacterium. The only limitations we encountered are related to the growth conditions and sample size. Due to a sealed environment, medium conditions cannot be changed during the experiment. Furthermore, a maximum of four strains per experiment can be monitored efficiently.

Considering a few critical steps, the single cell analysis method described here can easily be applied using any automated microscope. In the following, an overview of these critical steps will be given. Detailed information can be found in the main text. **General preparation:** It is wise to check the autofocus settings required for a specific bacterium prior to the experiment. Likewise, approximate optimal settings for the visualization of fluorescence should be determined in advance, if possible. Furthermore, following a prepared time-line helps to have all material ready to use in time (pre-warming the microscope chamber, programming the microscope settings, preparing the slide one hour before the cells are in the desired growth phase, see Figure 1). **Growth of *B. subtilis* in TLM and CDM:** TLM and CDM are chemically defined starvation media in

which *B. subtilis* only grows slowly. The time-period in which the cells are grown in the media might have to be prolonged depending on the specific strain. The slow growth prevents cells from piling up on each other. **Preparation of the microscope sample:** Air bubbles between the gene frame, the glass slide and the cover slip have to be prevented to prevent extensive drying of the agarose-based medium. The same holds for the medium/cover slip interface. It is crucial to let the cells dry enough, to prevent swimming and/or multiple layer growth. **Time-lapse fluorescence microscopy:** Pre-warming of the slide as well as the environmental chamber is crucial to prevent major autofocus problems. Cells should be selected in the middle of an agar pad, since these have the highest chance to stay in field and focus during the experiment (provided the sample was dried well enough). A maximum of 10 locations per experiments still works properly. After having selected the first cell of interest only use the software to adjust the focus (see text for details). Check whether the cells are still in focus during the first hours of the experiment in 30 min intervals. **Analysis:** It is important to check prior to extended analysis procedures whether the background of the medium has similar values in the fluorescence channels. Small dust particles, medium components, dirty lenses or tiny agarose clumps can contribute to locally increased fluorescence, making the movie difficult or impossible to analyse. **Trouble shooting:** If the cells grow on top of each other, this might either indicate that the coverslip was attached too soon or that the medium is not suited for growth of microcolony monolayers. If cells of interest continuously die prematurely, whereas other cells on the slide divide happily, you might want to check whether you put the UV-filter in position. It might also help to decrease the exposure time or light intensity during long experiments.

Disclosures

No conflicts of interest declared.

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