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Live-cell fluorescence microscopy to investigate subcellular protein localization and cell morphology changes in bacteria

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Dear JoVE Editor,

Please find attached our manuscript titled “Live-cell fluorescence microscopy to investigate subcellular protein localization and cell morphology changes in bacteria” which we are submitting as requested by Science Editor Jaydev Upponi.

In this manuscript we provide detailed protocols to conduct timelapse microscopy in *Bacillus subtilis* as well as *Staphylococcus aureus*, some of the results were a part of our recent *eLife* paper (Eswara et al., 2018).

We look forward to hearing from you soon.

Sincerely,

Prahathees Eswara, Ph.D.
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TITLE:

Live-Cell Fluorescence Microscopy to Investigate Subcellular Protein Localization and Cell Morphology Changes in Bacteria

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KEYWORDS:

Fluorescence microscopy, timelapse microscopy, deconvolution microscopy, *Bacillus subtilis*, *Staphylococcus aureus*, FtsZ, GpsB, GFP, protein localization, PC190723

SUMMARY:

This article provides a step-by-step guide to investigate protein subcellular localization dynamics and to monitor morphological changes using high-resolution fluorescence microscopy in *Bacillus subtilis* and *Staphylococcus aureus*.

ABSTRACT:

Investigations of factors influencing cell division and cell shape in bacteria are commonly performed in conjunction with high-resolution fluorescence microscopy as observations made at a population level may not truly reflect what occurs at a single cell level. Live-cell timelapse microscopy allows investigators to monitor the changes in cell division or cell morphology which provide valuable insights regarding subcellular localization of proteins and timing of gene expression, as it happens, to potentially aid in answering important biological questions. Here, we describe our protocol to monitor phenotypic changes in *Bacillus subtilis* and *Staphylococcus aureus* using a high-resolution deconvolution microscope. The objective of this report is to provide a simple and clear protocol that can be adopted by other investigators interested in conducting fluorescence microscopy experiments to study different biological processes in bacteria as well as other organisms.

INTRODUCTION:

The field of bacterial cell biology has been significantly enhanced by recent advancements in microscopy techniques[1,2]. Among other instruments, microscopes that are capable of conducting timelapse fluorescence microscopy experiments remain a valuable tool. Investigators can monitor various physiological events in real-time using fluorescent proteins such as, green

fluorescent protein (GFP)-based transcriptional and translational reporter fusions, fluorescent D-amino acids (FDAA)[3], or use other stains for labeling the cell wall, membrane and DNA. It is therefore of no surprise that fluorescence microscopy remains popular among microbial cell biologists. In addition to simply showing the end phenotypes, providing information as to how the observed phenotypes arise using timelapse microscopy could add significant value to the findings and potentially offer clues as to what cellular processes are being targeted by potential drug candidates[4].

The protocols to conduct high-resolution imaging using a fully motorized, inverted, wide-field fluorescence microscope (see the **Table of Materials**) are provided in this article. These protocols could be adapted to suit the needs of other fluorescence microscopes that are capable of conducting timelapse microscopy. Although the software discussed here corresponds to the specific manufacturer-supplied software as indicated in the **Table of Materials**, software commonly supplied by other microscope manufacturers or the freely available ImageJ[5], have equivalent tools for analyzing microscopy data. For conditions where timelapse is not conducive, time-course experiments could be conducted as described in this article. The protocols described here provide a detailed guide to study the phenotypic changes in two different bacterial species: *B. subtilis* and *S. aureus*. See **Table 1** for strains used.

PROTOCOL:

1. General growth conditions

1.1. Inoculate 2 mL of appropriate growth medium supplemented with antibiotics (where required) with a single colony of the strain(s) to be imaged. Incubate these seed cultures overnight at 22 °C in a shaking incubator.

NOTE: The specific bacterial growth conditions used in this article are provided under the representative results section.

1.2. Dilute overnight cultures 1:20 in fresh media in a 125 mL flask, supplement with antibiotics (where required).

1.3. Grow culture(s) at 37 °C in a shaking incubator until mid-logarithmic phase (OD_{600} = 0.5).

NOTE: Inducer or inhibitor could be added directly to growing culture(s) at the appropriate growth phase.

1.4. Harvest cells at desired culture conditions for microscopy sample preparation as described in the following section.

2. Sample preparation

2.1. Prepare 1% agarose by combining 0.25 g of molecular biology grade, low EEO, agarose with

either 25 mL of growth medium supplemented with appropriate antibiotics (where required) for timelapse microscopy or sterile water. Heat the mixture with the help of a microwave for approximately 30 s and pour it into a sterile 100 mm x 15 mm Petri dish and let it solidify.

2.2. Take a 5–50 µL aliquot of the cell culture depending on the need and stain the cells. Stain cells with appropriate dyes at this stage (e.g., add fluorescent dye FM4-64 (membrane stain) for an experiment (**Figure 1**)).

2.2.1. Pipette a 5 µL aliquot of the culture sample or stained sample to be imaged onto the bottom of a 35 mm glass bottom culture dish (with 14 mm microwell diameter and uncoated No. 1.5 coverslip; see the **Table of Materials**).

CAUTION: Do not use poly-L-lysine coated coverslips especially for timelapse microscopy as they could induce different growth pattern (we have observed this with poly-D-lysine as well; data not shown) and/or affect protein dynamics[6].

2.2.2. To minimize the use of dyes, stain 3–4 µL cell suspension (harvested at OD_{600} = 0.5; approximately 2.7 x 10^7 and 3.4 x 10^7 for *B. subtilis* and *S. aureus* respectively per 1 mL culture) directly on the glass bottom dish.

2.2.3. Place a pre-cut agarose slab (11 mm in diameter or of any desired size to overlap the area of the coverslip; cut using the open end of a sterile tube or a razor blade) on top of the sample and gently tap to make sure the agarose slab is lying flat against the coverslip.

2.3. Subsequent to imaging (see following section), if the number of cells in the field of view are not desirable, adjust the cell density of the sample via dilution or concentration by centrifugation and alter the ratio of cells in the sample using growth medium/buffer as necessary.

NOTE: To minimize autofluorescence emanating from the culture medium, cells can be washed in buffer (for example in standard 1x phosphate buffer saline) and resuspended in appropriate amount of buffer prior to imaging. It is possible that in this step smaller cells or vesicles may not be retained after centrifugation and will hence be lost.

2.4. Add water using a pipette (~5 µL drops) to the space inside the culture dish (around the coverslip) to prevent the agarose pad from drying, and to help maintain humidity during the course of image acquisition, especially for timelapse experiments.

2.5. Allow culture dishes to equilibrate to the temperature inside the incubation chamber, a built-in opaque compartment provided by the manufacturer, of the microscope for 15–20 min.

NOTE: Turn on the heating element and set the incubation chamber to a desired temperature several hours prior to imaging. This will ensure that the hardware stabilizes to the new temperature. For prolonged timelapse imaging, place a beaker or flask with water inside the microscope chamber, away from the working area, to maintain humidity.

3. Imaging

3.1. On the day of the experiment, turn on the microscope system. Start the imaging software (see **Table of Materials**) by clicking the appropriate icon on the desktop. Initialize the microscope by clicking the **Initialize Microscope** option (button depicted with a microscope on it) on the software's start-up dialog box. Ensure that the objective is fully lowered using the microscope coarse adjustment prior to initialization.

NOTE: Following initialization three additional dialog boxes should appear in addition to the start menu: resolve3D, data collection, and filter monitor.

3.2. Place a drop of 1.517 (refractive index) oil on the 100x oil immersion objective supplied by the manufacturer (Numerical Aperture = 1.4, Working Distance = 0.12 mm; see **Table of Materials**).

NOTE: It is important to choose the appropriate immersion oil for the temperature at which imaging is conducted.

3.3. Load the glass bottom dish containing sample into the metal housing (coffin) and gently slide into the stage clamp.

3.4. Use the coarse adjustment knob to raise the objective until the oil makes contact with the glass bottom of the dish. Use the eye piece and fine adjustment knob to bring the sample into focus. Once the cells are in focus turn the knob from the eye piece to camera mode by moving the selector switch located on the front of the microscope body to the left.

3.5. Begin experiment using the imaging software. On the **resolve3D** window, select the **Design/run experiment** icon depicted by a flask. A new dialog box should appear entitled **design/run experiment**.

3.5.1. Set the number of Z-stacks and sample thickness using the **design** and then **sectioning** tab on the **design/run experiment** dialog box.

NOTE: For the experiments in representative results section, 17 Z-stacks at 200 nm interval for still images and four Z-stacks at 200 nm interval for timelapse microscopy were used.

3.5.2. To measure the thickness of the cells in the sample, manually adjust the Z-plane incrementally by using the up and down arrows on the **resolve3D** dialog box. Mark where the cells go out-of-focus as the upper and lower limit for image acquisition. Import this information prior to running the experiment.

3.5.3. To help minimize phototoxicity and photobleaching during timelapse imaging, reduce the number of Z-stacks and choose the mid-plane of the cells for image acquisition.

3.6. Select the appropriate filter set for the experiment using the **design** and then **channels** tab on the **design/run experiment** dialog box (TRITC: EX 542/27; EM 597/45; FITC/GFP: EX 475/28; EM 525/48; mCherry: EX 575/25; EM 632/60; Cy5: EX 632/22; EM 676/34).

3.6.1) Also, select a reference for the collection of POL/DIC information. Adjust percentage transmission (light intensity) and duration of exposure for individual channels selected prior to imaging by selecting the appropriate options on the **resolve3D** dialog box.

NOTE: In a test field of view that is not considered for the experiment test these settings to identify if the selected parameters obtain meaningful fluorescence data without missing weak signal or oversaturating it. Then import these parameters for the experimental set up.

3.7. Open the points list by selecting the **Points list** button on the **resolve3D** dialog box. A new dialog box should appear entitled **points list**. Mark several fields of view to be used in the experiment by finding appropriate fields of view using the microscope stage controls and selecting the mark point option on the points list dialog box.

NOTE: A replace point will have to be selected each time the microscope is refocused on a point in the points list. This can be done by focusing the microscope on the appropriate point in the list and then selecting the **replace point** button. It is important to not touch the analog coarse/fine adjustment knob when refocusing; use only the software to adjust the focus.

3.8. Set timelapse parameters by first selecting the design and then **timelapse** tab on the design/run experiment dialog box. Select the timelapse check box. Enter the appropriate timelapse parameters in terms of timelapse images/total time.

3.9. Set points to be imaged from the points list by first selecting the **design** and then **points** tab on the design/run experiment dialog box. Select the visit points list option and enter points to be imaged in the text box separated by commas or hyphens if it is a complete sequence.

3.10. Prior to beginning an experiment, edit file names and file locations using the **run** tab on the design/run dialog box. File location can be changed by selecting the **settings** button, selecting the data folder, and by then selecting the appropriate folder or creating a new one. Change file names by entering in the new file name in the image file name text box.

3.11. Begin the experiment by selecting the **play** button on the **begin experiment dialog** box.

NOTE: For timelapse, continually check focus at each field of view throughout the experiment using DIC setting (to avoid unnecessary photobleaching) and refocus and update the information in points list as cells tend to go out of focus over time.

4. Image processing

4.1. Open the desired raw (R3D) image files for generation of figures.

NOTE: Recently saved files can be located using the data folder button on the imaging software's start-up dialog box.

4.2. Run the deconvolution program, to remove out-of-focus fluorescence light[7,8], in order to produce a deconvolved D3D image file. Select the **process** tab on the start-up dialog box, and then select **deconvolve**. A new dialog box will appear entitled **deconvolve**.

4.2.1. Set the deconvolution parameters by dragging the appropriate image number (located in the upper left-hand corner of each image file) to the input on the deconvolution dialog box. On the deconvolution dialog box, select the **more options** button, and deselect the **crop border rolloff** after **processing** check box (otherwise the images cannot be superimposed over the corresponding DIC file).

4.2.2. Click the **do it** button on the deconvolution dialog box to deconvolve raw image file.

NOTE: The deconvolution parameters could be adjusted as indicated by the software manufacturer's guidance manual for desired results.

4.3. If necessary, perform manual background noise subtraction and brightness/contrast adjustment in any or all wavelength (color) channels. Do this by selecting the **contrast adjustment** button on the deconvolved image file.

4.4. Save the image as a TIFF file by first selecting the **file** option at the top of the **D3D** dialog box. Then select save as TIFF, identify and select appropriate Z-stacks (that are within focus), select or unselect desired filter sets.

4.5. Perform data quantification using any manufacturer-supplied software or freely available programs such as ImageJ[5].

4.5.1. For cell size quantification click on the **tool** tab on the appropriate D3D image file, and then select the **measure distances** option. Measure distances by selecting start and end points on the desired image file using the mouse through left clicks.

NOTE: It is best to zoom in on the image during cell size quantification.

4.5.2. For fluorescence signal quantification use the **data inspector** option under the **tool** tab on the appropriate D3D image file.

NOTE: It is best to zoom in on the image and to have only relevant filters selected when completing fluorescence signal quantification.

4.5.3. To quantify the fluorescence signal, draw a box with column/row option set to specific

dimension. Select an area with signal to be quantified and record the value. Measure the background signal by using the same size box to select an area immediately outside of the cells. Subtract the background value from the value recorded from the fluorescence signal obtained within the cell.

REPRESENTATIVE RESULTS:

GpsB phenotypes

Previously we have shown that Sa-GpsB is an essential protein as depletion of GpsB using an antisense RNA results in cell lysis[9]. Here we describe how the emergence of various cell division phenotypes and changes in protein localization could be captured using the timelapse microscopy protocol described in this article. For this purpose, *S. aureus* strains RB143 [SH1000 harboring pEPSA5 (empty vector)] and GGS8 [SH1000 harboring pGG59 (P_{xyl}-$gpsB^{antisense}$ *bla cat*)] reported previously[9], were grown as follows. Strains RB143 and GGS8 were inoculated in 2 mL of tryptic soy broth (TSB) supplemented with 5 µg/mL chloramphenicol (chlor) in a 15 mL test tube and were incubated overnight at 22 °C while shaking. The overnight cultures were diluted 1:20 in 10 mL of fresh TSB + chlor in a 125 mL flask and grown at 37 °C with shaking until mid-logarithmic phase (OD_{600} = 0.5). The inducer, 1% xylose, was added to the culture medium to trigger the expression of antisense RNA of *gpsB* and the culture was grown for another 3 h. Cells were then stained with fluorescent dye FM4-64 (membrane stain), where required, by the addition of 0.5 µL of a 10 µg/mL stock of FM4-64 directly onto the 5 µL aliquot of culture on the microscope dish as described in the protocol section. As shown in **Figure 1** and **Video 1**, addition of xylose to induce GGS8 strain resulted in a "sick" cell phenotype, as described previously[9], while empty vector control (RB143) appeared similar to our control—cells grown in the absence of inducer.

Our group also reported that overproduction of *S. aureus* GpsB (Sa-GpsB) disrupts cell division in *B. subtilis*[9]. We use this overexpression phenotype as an example to demonstrate the protocol described here. To this end, a *B. subtilis* strain GG9 (*amyE::*$P_{hyperspank}$-$gpsB^{Sa}$ *spc; ftsAZ::ftsAZ-gfpΩerm*) was used[9]. Subcellular localization of fluorescently-labeled FtsZ, a key cell division protein which marks the cell division sites[10,11], was used to monitor the status of cell division. The sample for microscopy was prepared as follows. A single colony of GG9 was inoculated in 2 mL Luria-Bertani (LB) medium and incubated overnight at 22 °C in an incubator shaker. The overnight cultures were 1:20 in 10 mL of fresh LB, and grown at 37 °C with shaking until mid-logarithmic phase (OD_{600} = 0.5). GG9 cells (5 µL aliquot) to be imaged were placed on the bottom of a glass bottom culture dish and covered with a 1% agarose pad made with LB supplemented with 250 µM (final concentration) of isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the expression of *Sa-gpsB* (**Figure 2** and **Video 2**). Timelapse microscopy and cell length quantification were performed as described in the protocol section.

Inhibition of FtsZ

FtsZ, being a protein essential for cell division, is considered an attractive drug target and multiple groups are developing FtsZ inhibitors as a way to develop new antibiotics[12]. Localization patterns of FtsZ or one of the proteins associated with it, such as ZapA, can be used as a reporter to study

and/or identify novel antimicrobial compounds. We use the protocol provided here to demonstrate this approach using *S. aureus* RB197 [SH1000 harboring pRB42 (P_{Cd}-*zapA*^{Sa}-*gfp bla erm*)][9] and *B. subtilis* PE92 (*ftsAZ::ftsAZ-gfpΩerm*)[13] strains. RB197 and PE92 strains were grown as described above in TSB (containing 5 µg/mL erythromycin; and 1.25 µM $CdCl_2$ to induce the expression of *zapA-gfp*) and LB respectively. At mid-logarithmic phase, a well-characterized FtsZ inhibitor, PC190723 [14,15], was added at 2 µg/mL final concentration and its effect on the *S. aureus* and *B. subtilis* cells were monitored using microscopy at different time intervals (**Figure 3** and **Figure 4**). Quantification of cell diameter of *S. aureus* and cell length of *B. subtilis* was performed as described in the protocol section.

FIGURE AND TABLE LEGENDS:

Figure 1: High-resolution micrograph of *S. aureus* cells displaying sick phenotype. Fluorescence micrographs of *S. aureus* strains harbouring either empty vector (left; RB143) or an inducible copy of antisense RNA of *gpsB*^{Sa} (right; GGS8) in the presence and absence of 1% xylose (inducer). Cells awere stained with FM4-64 membrane stain (stocks dissolved in sterile water) and imaged using TRITC filter set. Scale bar: 1 µm.

Figure 2: Representative data showing cell division inhibition in *B. subtilis*. (**A**) Timelapse micrographs of *B. subtilis* strain GG9 with images acquired at 20-min intervals for 120 min using the DIC/FITC channels. Fluorescence data of FtsZ-GFP (green) are shown. Arrows follow one cell throughout the experiment. Scale bar: 1 µm. (**B**) Quantification of cell lengths at all time points. Average cell length with error bars indicating standard deviation (n = 50) are shown.

Figure 3: Time course investigation of cell division inhibition in *S. aureus*. (**A**) Mid-logarithmic phase cells of strain RB197 untreated (top) or treated (bottom) with FtsZ inhibitor (PC190723), subsequent to 30 min growth, aliquots of growing cultures were taken every 10 min for 90 min and imaged using DIC and FITC filter sets. Fluorescence from ZapA-GFP is shown. Scale bar: 1 µm. (**B**) Quantification of microscopy data. Average cell width with error bars indicating standard deviation (n = 50) and percentage of cells (n = 50) displaying proper ZapA-GFP localization (mid-cell and periphery) are shown. Data points of cells treated with inhibitor are shown in red. Shapes with green outline corresponds to the right Y-axis.

Figure 4: Investigation of cell division inhibition by a synthetic inhibitor in *B. subtilis*. *B. subtilis* strain PE92 was either untreated or treated with an FtsZ inhibitor (PC190723) at mid-logarithmic phase and were monitored for the subsequent 90 min. Aliquots of growing cultures were taken every 10 min for microscopy and images were acquired using DIC/FITC channels. Fluorescence from FtsZ-GFP is shown. Scale bar: 1 µm. (**B**) Quantification of microscopy data. Average cell length with error bars indicating standard deviation (n = 50) and percentage of cells (n = 50) displaying proper mid-cell FtsZ-GFP localization are shown. Data points of cells treated with inhibitor are shown in red. Shapes with green outline corresponds to the right Y-axis.

Video 1: Timelapse microscopy of *S. aureus* cells developing sick phenotype. Strain GGS8 (*gpsB* antisense) treated with 1% xylose. Cells were stained with FM4-64 membrane stain and imaged

at 10 min intervals for 60 min using the TRITC channel as described in the protocol.

Video 2: Overexpression of *Sa-gpsB* leads to inhibition of cell division in *B. subtilis*. Timelapse video showing filamentation and change in FtsZ-GFP localization in GG9. Images were taken at 20 min intervals for 120 min using DIC and FITC channels.

Table 1: Strains used.

DISCUSSION:

Microscopy has remained a mainstay in studies pertaining to microbial organisms. Given their micron-scale cell size, single-cell level studies have traditionally relied on electron microscopy (EM). Although EM has become quite a powerful technique in recent years, it has its own intrinsic limitations in addition to limited user access[16]. Improvements in fluorescence microscopy techniques and development of different fluorescent probes, such as FDAA[3], have provided microbial cell biologists with a vast array of tools to study various cellular processes in live cells. Researchers are also actively building fluorescent tools to monitor changes, for example in the level of signaling molecules such as c-di-GMP among others, in living cells[17,18]. In addition, high-resolution timelapse fluorescence microscopy allows investigators to monitor changes as they happen and to study relevant phenotypes.

We have provided detailed protocols to conduct microscopy experiments with a high-resolution fluorescence microscope (see **Table of Materials**). However, the steps in the protocols could be altered to fit the needs of the user and the microscope used. We use *S. aureus* and *B. subtilis* as our model organisms to show how to monitor various cell division phenotypes, track the changes in protein localization, and quantify the data. In addition, for cases where timelapse is not conducive, we show with the help of an FtsZ inhibitor, how to set up a time course experiment.

The inherent limitation with fluorescence microscopy is the resolution set by the diffraction limit, which could be overcome to some extent with the aid of advanced super-resolution microscopy techniques[19,20]. Other issues such as phototoxicity and photobleaching could be circumvented by collecting fewer Z-stacks or minimizing the duration and/or frequency of exposure to laser. Other guidance materials specific to live-cell microscopy are available[21]. Apart from Gram-positive organisms *B. subtilis* and *S. aureus*, using this set up, we have successfully imaged Gram-negative bacterium *Escherichia coli*, yeast *Saccharomyces cerevisiae*, and nematode *Caenorhabditis elegans*.

In addition to the experiments described here, similar methodologies could be used to identify compounds that target specific cellular processes in a high-throughput fashion. Algorithms that automate the quantification process can also be incorporated for large datasets[22,23]. There is an immense need to study different bacterial species to address the antibiotic-resistance crisis and more studies are warranted to understand the mechanisms of basic biological processes and to identify novel therapeutic compounds. Various fluorescence microscopy techniques have gained the power and momentum to aid researchers in addressing these challenges among others.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Coltharp, C., Xiao, J. Superresolution microscopy for microbiology. *Cellular Microbiology.* **14** (12), 1808-1818, (2012).

2 Holden, S. Probing the mechanistic principles of bacterial cell division with super-resolution microscopy. *Current Opinion in Microbiology.* **43** 84-91, (2018).

3 Hsu, Y. P. et al. Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chemical Science.* **8** (9), 6313-6321, (2017).

4 Nonejuie, P., Burkart, M., Pogliano, K., Pogliano, J. Bacterial cytological profiling rapidly identifies the cellular pathways targeted by antibacterial molecules. *Proceedings of the National Academy of Sciences of the USA.* **110** (40), 16169-16174, (2013).

5 Rueden, C. T. et al. ImageJ2: ImageJ for the next generation of scientific image data. *BioMed Central Bioinformatics.* **18** (1), 529, (2017).

6 Colville, K., Tompkins, N., Rutenberg, A. D., Jericho, M. H. Effects of poly(L-lysine) substrates on attached Escherichia coli bacteria. *Langmuir.* **26** (4), 2639-2644, (2010).

7 McNally, J. G., Karpova, T., Cooper, J., Conchello, J. A. Three-dimensional imaging by deconvolution microscopy. *Methods.* **19** (3), 373-385, (1999).

8 Wallace, W., Schaefer, L. H., Swedlow, J. R. A workingperson's guide to deconvolution in light microscopy. *Biotechniques.* **31** (5), 1076-1078, 1080, 1082 passim, (2001).

9 Eswara, P. J. et al. An essential Staphylococcus aureus cell division protein directly regulates FtsZ dynamics. *Elife.* **7**, (2018).

10 Haeusser, D. P., Margolin, W. Splitsville: structural and functional insights into the dynamic bacterial Z ring. *Nature Reviews Microbiology.* **14** (5), 305-319, (2016).

11 Du, S., Lutkenhaus, J. At the Heart of Bacterial Cytokinesis: The Z Ring. *Trends in microbiology.* 10.1016/j.tim.2019.04.011, (2019).

12 Haranahalli, K., Tong, S., Ojima, I. Recent advances in the discovery and development of antibacterial agents targeting the cell-division protein FtsZ. *Bioorganic and Medicinal Chemistry.* **24** (24), 6354-6369, (2016).

13 Brzozowski, R. S. et al. Deciphering the Role of a SLOG Superfamily Protein YpsA in Gram-Positive Bacteria. *Frontiers in Microbiology.* **10** 623, (2019).

14 Elsen, N. L. et al. Mechanism of action of the cell-division inhibitor PC190723: modulation of FtsZ assembly cooperativity. *Journal of the American Chemical Society.* **134** (30), 12342-12345, (2012).

15 Haydon, D. J. et al. An inhibitor of FtsZ with potent and selective anti-staphylococcal activity. *Science.* **321** (5896), 1673-1675, (2008).

16 Pilhofer, M., Ladinsky, M. S., McDowall, A. W., Jensen, G. J. Bacterial TEM: new insights from cryo-microscopy. *Methods in Cell Biology.* **96** 21-45, (2010).

17 Ni, Q., Mehta, S., Zhang, J. Live-cell imaging of cell signaling using genetically encoded fluorescent reporters. *Federation of European Biochemical Societies Journal.* **285** (2), 203-219, (2018).
18 Weiss, C. A., Hoberg, J. A., Liu, K., Tu, B. P., Winkler, W. C. Single-Cell Microscopy Reveals That Levels of Cyclic di-GMP Vary among Bacillus subtilis Subpopulations. *Journal of Bacteriology.* **201** (16), (2019).
19 Schneider, J. P., Basler, M. Shedding light on biology of bacterial cells. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences.* **371** (1707), (2016).
20 Yao, Z., Carballido-Lopez, R. Fluorescence imaging for bacterial cell biology: from localization to dynamics, from ensembles to single molecules. *Annual review of microbiology.* **68** 459-476, (2014).
21 Ettinger, A., Wittmann, T. Fluorescence live cell imaging. *Methods in Cell Biology.* **123** 77-94, (2014).
22 Fredborg, M. et al. Automated image analysis for quantification of filamentous bacteria. *BioMed Central Microbiology.* **15** 255, (2015).
23 Ursell, T. et al. Rapid, precise quantification of bacterial cellular dimensions across a genomic-scale knockout library. *BioMed Central Biology.* **15** (1), 17, (2017).

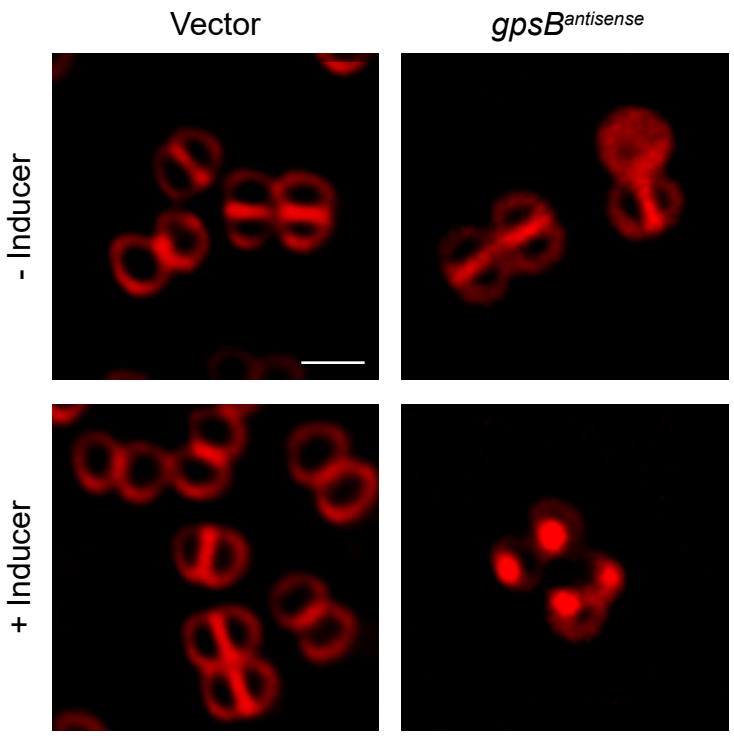
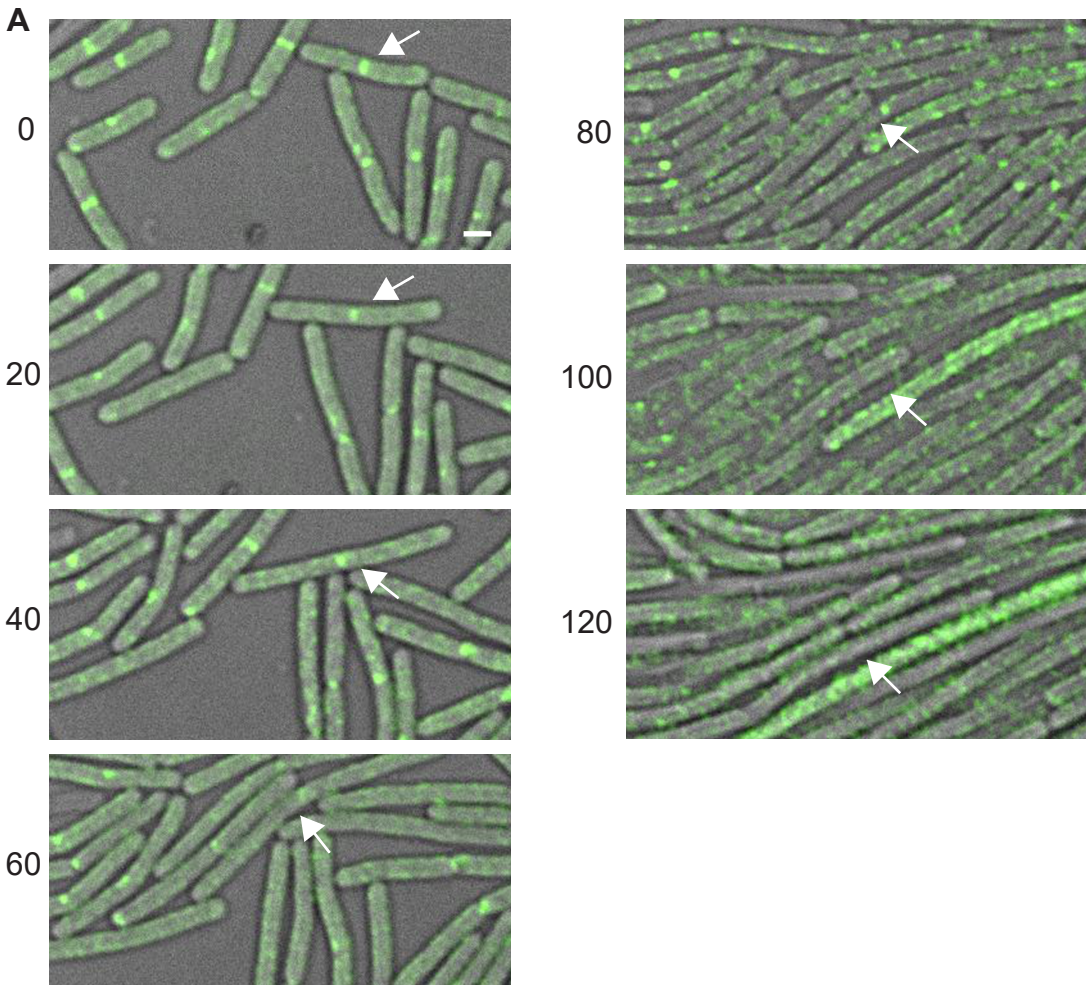


Figure 1



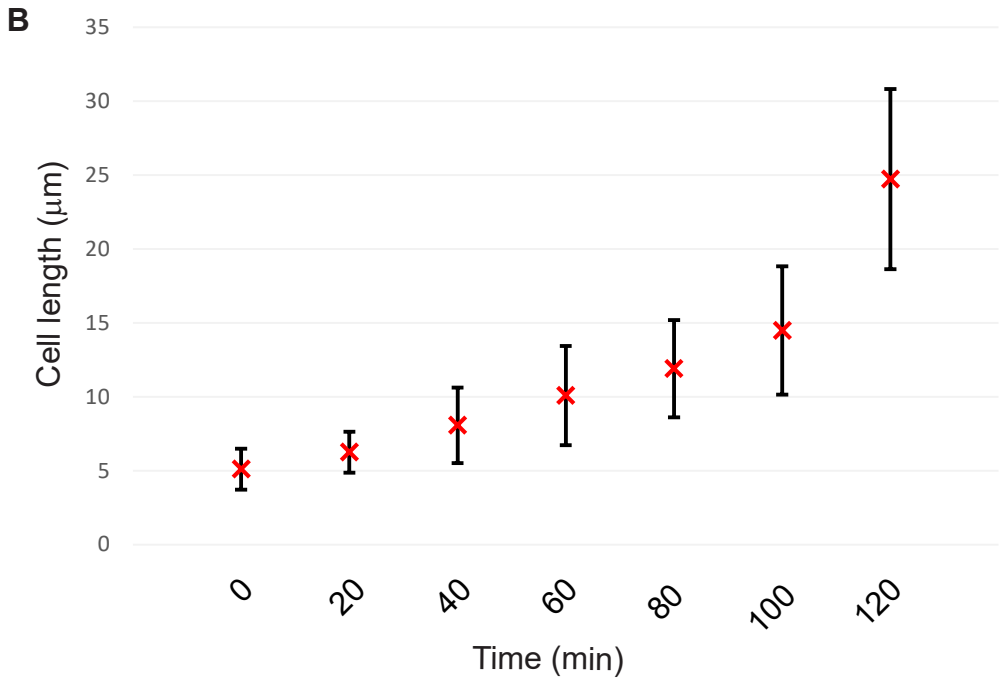
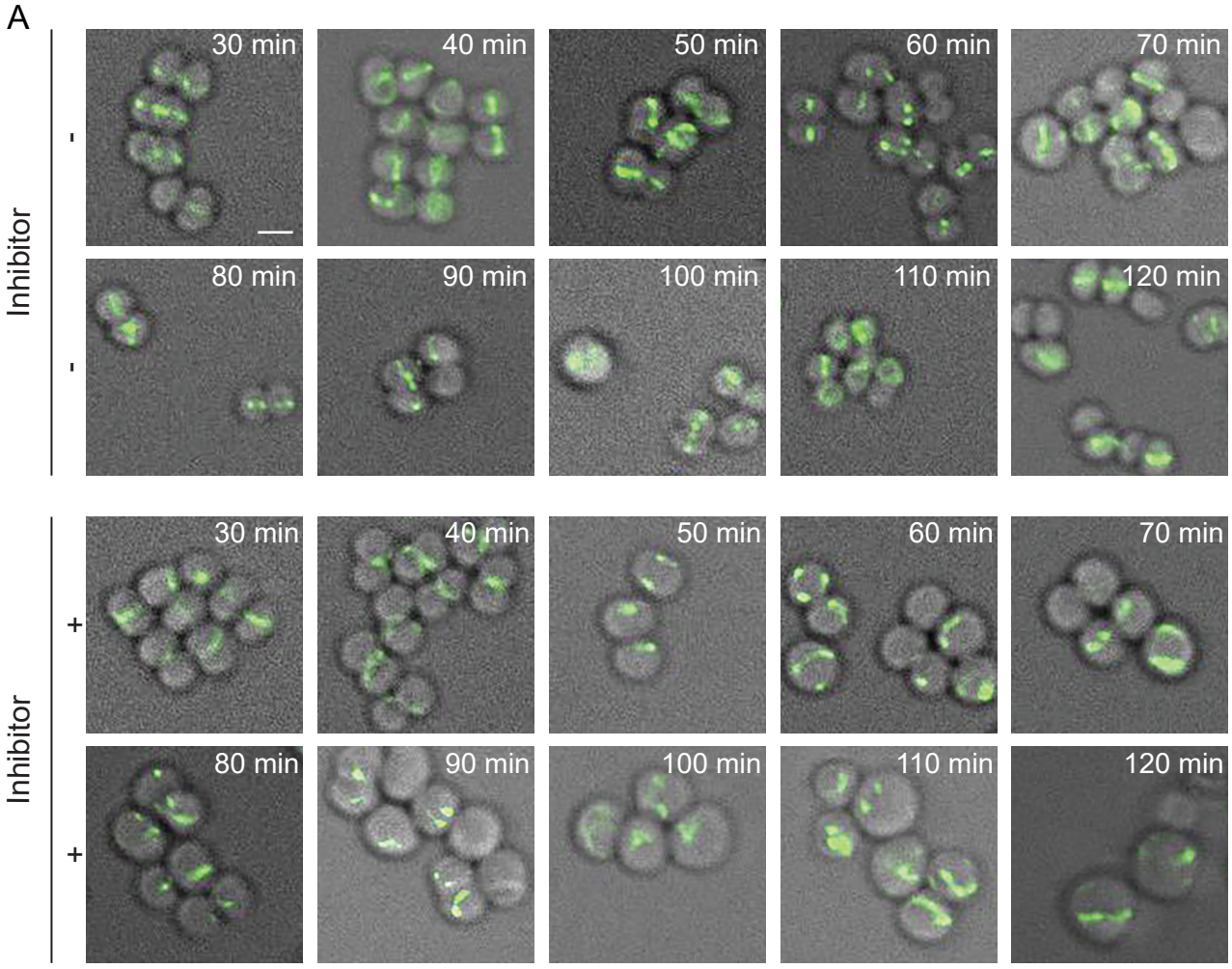


Figure 2



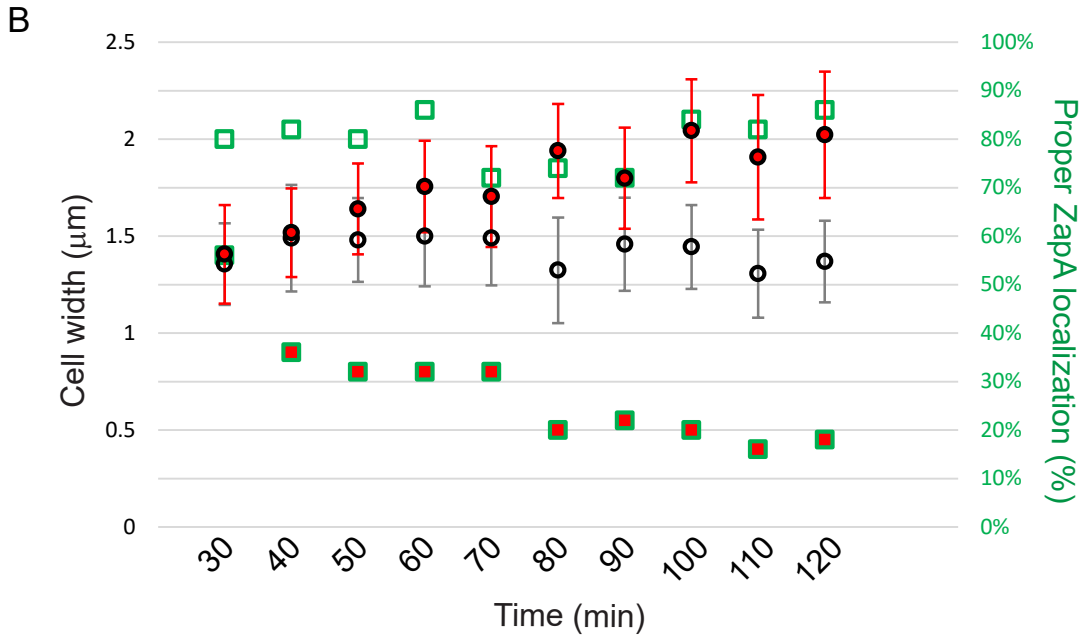
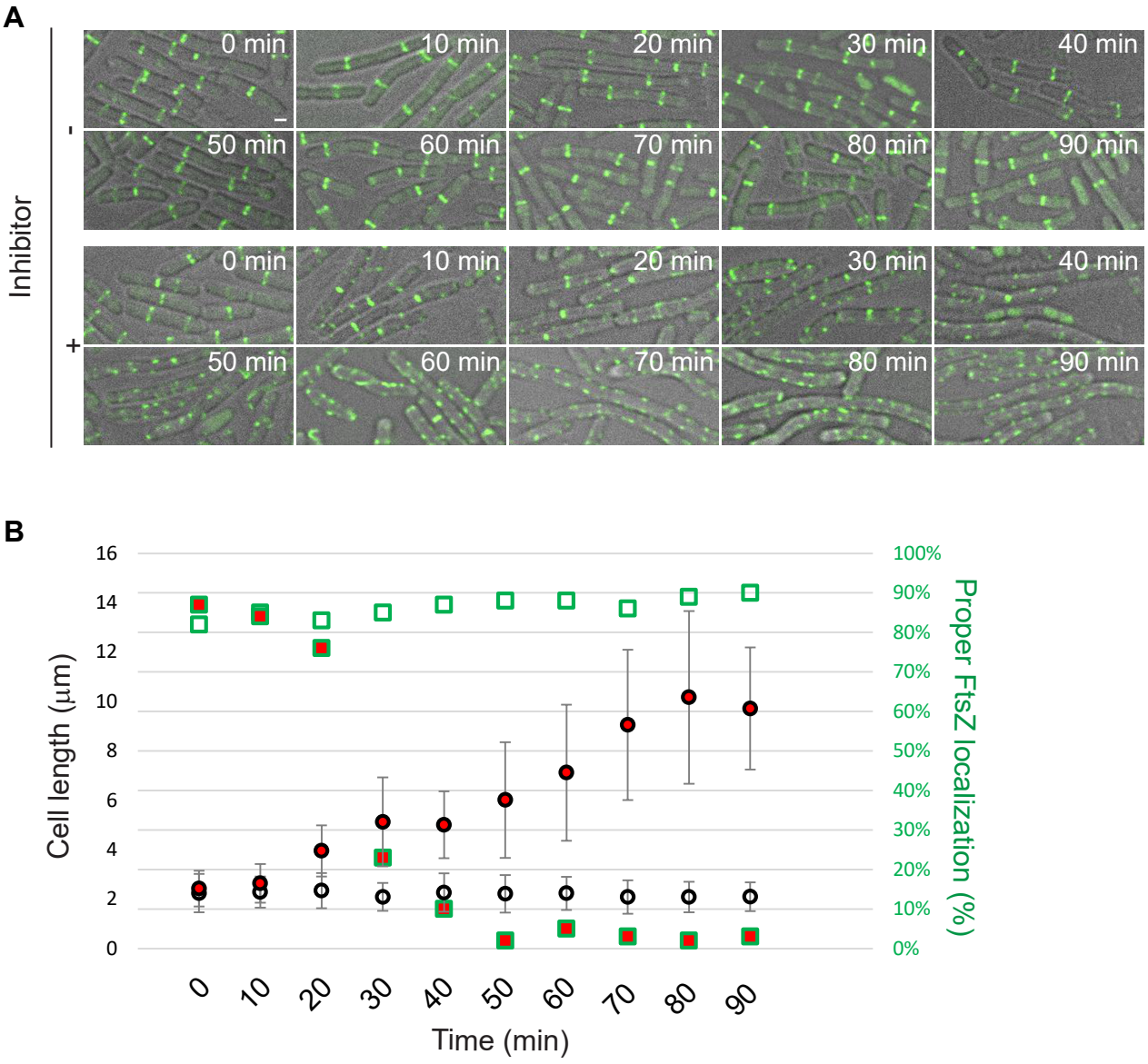
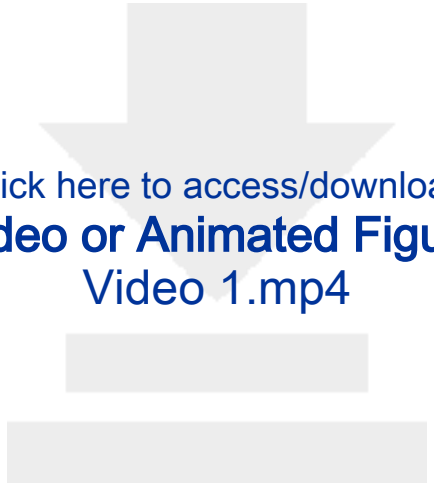


Figure 3



Video or Animated Figure



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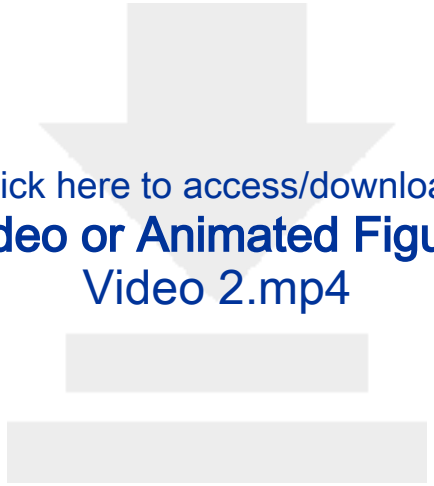
Video or Animated Figure

Video 1.mp4

Video or Animated Figure



Click here to access/download

Video or Animated Figure

Video 2.mp4

Table

Species	Strain	Genotype	Reference
S. aureus	RB143	SH1000 pEPSA5, *bla, cat*	Eswara et a
S. aureus	GGS8	SH1000 pGG59 (pEPSA5 backbone) P_{xyl}-*gpsB* ^{an}	Eswara et a
S. aureus	RB197	SH1000 pRB42 (pJB67 backbone) P_{Cd}-*zapA* ^{SA} -g	Eswara et a
B. subtilis	GG9	*amyE::Phyperspank-gpsB* ^{SA} *spc; ftsAZ Ωfts AZ-g*	Eswara et a
B. subtilis	PE92	*ftsAZ::ftsAZ-gfp Ωerm*	Brzozowski

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et al, 2019

Name of Material/Equipment	Company	Catalog Number
Agarose	Fisher BioReagents	BP160-100
DAPI	Invitrogen	D3571
FM4-64	Invitrogen	T3166
Glass bottom dish	MatTek	P35G-1.5-14-C
IPTG	Fisher BioReagents	BP1755-10
Microscope	GE	DeltaVision Elite
PC190723	MilliporeSigma	3445805MG
SoftWorx	GE	

Comments/Description

Molecular Biology Grade - Low EEO

Microscopy
Microscopy
Microscopy
Dioxane-free

Customized Olympus IX-71 Inverted Microscope Stand; Custom Illumination Tower and Transmitted Light Illuminator Module. Objectives: PLAPON 60X (N.A. 1.42, WD 0.15 mm); OLY 100X OIL (N.A. 1.4, WD 0.12 mm); DIC Prism Nomarski for 100X Objective; CoolSnap HQ2 camera; SSI Assembly 7-color; Environmental control chamber - opaque.

FtsZ inhibitor
Manufacturer-supplied imaging software

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Title of Article:	Seeing is believing: high resolution fluorescence timelapse microscopy to investigate protein localization and cell division in bacteria
Author(s):	Robert S. Brzozowski, Maria L. White, and Prahathees J. Eswara

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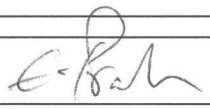
Signature:	
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August 1, 2019

Dear JoVE Editor,

Please find below our point-by-point response to the comments of the Editor and peer reviewers for our manuscript titled “Live-cell fluorescence microscopy to investigate subcellular protein localization and cell morphology changes in bacteria.”

Sincerely,

Prahathees Eswara, Ph.D.
Assistant Professor

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

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

We have fixed all errors that we were able to find.

• Please avoid the split title (i.e., remove the colon), and make the it more concise.

We have now fixed the title as per your recommendation.

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• Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) For example lines 78-80 should be a note.

We believe the protocol language has been updated as suggested.

• 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) 1.1: Please list growth conditions in the protocol. We have added a new section.
2) 2.5: What should sample thickness and number of z-stacks be set to? It depends on the sample. We elaborate on that point in our note.
3) 2.8: What should the timelapse parameters be set to? Again, it depends on the experiment. We elaborate on that point in our note.
4) Section 3: Mention add menu selection and button clicks to explicitly describe all software actions. We have now updated this as per your suggestion.

• 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.
We believe the discussion section has been updated as suggested.

• References: Please spell out journal names.
We have now manually spelled out the journal names in EndNote.

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We have now made the suggested changes.

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• Table of Materials:Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as microscope, cell strains, culture reagents, agarose, software etc.

We have now updated the table of materials.

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Our figures, videos, and tables are original.

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Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors propose a simple and effective methodology to investigate bacteria cell division utilizing live cell microscopy. Utilizing membrane dyes and the expression of recombinant proteins, the authors followed the bacteria cell division and filametous growth caused by the inhibition of bacteria cytokinesis. The reported methodology allowed the authors to capture and measure morphological changes of bacteria growing between the coverslip and agarose pads. The proposed methodology may help researchers interested in studying bacteria cell biology and antimicrobials.

Major Concerns:
The authors must provide more information on the microscope set up they utilized in this report. It is possible to infer that the authors utilized a fully motorized, inverted, wild field fluorescence microscope. However, this must be explicitly indicated and fully described in the text, including camera, illumination system, stage and optics.

We now explicitly state the information requested in lines 54-55 and in the table of materials.

Minor Concerns:

Paragraph 1.2 line 73: Please indicate if any particular quality grade for agarose is required to minimize autofluorescence background

We have updated this information in line 87 and in table of materials.

Paragraph 1.3 line 80: Indicate size and supplier and model for the glass bottom culture dish.

This information can be found in lines 95-97 and in table of materials.

Paragraph 1.3 line 81: Indicate a range for bacteria cell numbers in the suspension

We have provided this information in lines 100-102.

Paragraph 1.3 line 83: Indicate appropriate dimensions for the pre-cut agarose slab

We have provided this information in lines 102-105.

Paragraph 1.6 line 101: A comma is missing after "away.

We updated this information in paragraph 2.5 line 124.

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Paragraph 2.1 line 104: Provide more information on the microscope utilized: optics, illumination system, camera (sensor and pixel size), motorized stage etc.

This information is now provided in table of materials.

Paragraph 2.2 line 106: Provide more information on the objective lens utilized.

This information is now provided in table of materials.

Paragraph 2.2 line 108: What is the meaning of "imaging temperature"?

We have clarified this in lines 135-136.

Paragraph 2.5 line 19: Please indicate appropriate z intervals for deconvolution and to avoid oversampling and photo damage of the sample

We have provided the Z-stack information used in the experiments conducted in lines 146-148.

Paragraph 2.6 line 122: Does percentage of transmission refers to light intensity ?

Yes, we have now clarified that in line 157.

Paragraph 2.7 line 127: The terminology used is probably specific to software and microscope brand used by the authors. The authors should consider utilizing more generic terms applicable to other microscope and software brands.

As per the Editor's guidelines, we have to be very specific in how the experiments were conducted (down to even specific button click commands), therefore unfortunately we are unable to explain the details in generic terms.

Paragraph 3.1 line 134: Please replace "fluorescence signals" for "fluorescence light".

Fixed this in line 188.

Paragraph 3.1 line 135: Please indicate optimal parameters, confidence limit and number of iterations utilized for imaging deconvolution

This was conducted with software manufacturer's default settings, we have added that the parameters can be altered to obtain desired results. See lines 195-197.

Paragraph 3.2 line 136: Please consider replacing "brightness adjustment" for "contrast adjustment".

Fixed this in line 200.

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FIGURE 1:
Please, indicate solvent utilized for FM 4-64 stock. Provide additional information about experimental conditions: Bacteria numbers, dye concentration, incubation time, temperature. Was the dye kept or washed after incubation?

We have now provided this information in the protocol section (also line 234) and line 274.

Discussion:
The authors may consider to remove "high resolution" from the title, since the microscope utilized is a conventional wide field system and no additional improvement of its limit of resolution has been applied. On this regard the authors should considering to discuss the use of the proposed methodology in combination with spinning disk or any other type of fast confocal technology, to increase imaging contrast an apparent resolution. Can the proposed methodology be applied for Gram negative bacteria?
Although the manufacturer advertises this DeltaVision Elite as a high-resolution microscope (as the image quality is enhanced due to deconvolution and better optics). We have now removed “high resolution” from the title. We have, in lines 336-338, indicated that this methodology can be used to image other organisms.

Reviewer #2:

Manuscript Summary:
In this article, Brzozowski et al describe a single cell fluorescence microscopy protocol to monitor changes in cell morphology during different stages of cell cycle and in response to different environmental cues. The protocol can be adapted to investigate different cellular processes for various bacteria.

Major Concerns:
While the protocol claims to employ a high resolution fluorescence microscopy method, it lacks clear description of how they achieve sub-diffraction limit resolution. Describing the "high resolution deconvolution" microscopy method in the "Introduction" section will be of use to readers, in determining the scope of the method. The protocol also does not mention the resolution that is achievable by the method and the resolution obtained in the included figures. None of the included figures appear to be superresolution images.

The manufacturer advertises this DeltaVision Elite (now Ultra; https://www.gelifesciences.com/en/us/solutions/cellular-analysis/products-and-technology/microscopy/high-resolution) as a high-resolution microscope as the image quality is enhanced due to deconvolution and better optics. We apologize for the confusion, we never claimed that our method is a super-resolution technique. We have briefly discussed the details of deconvolution in lines 187-189. We have now also removed “high resolution” from the title. We hope this alleviates your concerns.

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The protocol described is extremely specific to the SoftWoRx software. The manuscript will be of use to a greater audience, if at least equivalent ImageJ (a more commonly used software) plugins are mentioned in the article.

As per the Editor's guidelines, we have to be very specific in how the experiments were conducted (down to even specific button click commands), therefore unfortunately we are unable to explain the details for ImageJ. However we have explicitly stated that other software such as ImageJ can be used in lines 204-205.

In line 119, how is the mid plane of the cells determined?

It is section where the cells are mostly in focus, where the cell width is the largest.

In Fig.1 (-Inducer, gpsB anti sense), presence of FM464 in non-membranous region of the cell is shown. Is this a common occurence or only occurs in rare instance?
It is not a common occurrence. Few cells in which there is a leaky expression (of gpsB antisense) that are en route to lysis sometimes appear like that.

Minor Concerns:

In step 1.6, more details on the incubation chamber would be helpful.

We have given more details now in lines 119-120.

In step 2.5 (Line 114 -118), it might be better to include the maximum number of z-stacks possible.

We have imaged C. elegans (mentioned in line 338), and for that we taken more than 50 z-stacks (200 nm interval), but we are unsure of the exact maximum limit. We will contact the manufacturer to find this information if you think it is essential to include.

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