Journal of Visualized Experiments

Three-dimensional imaging of bacterial cells for accurate cellular representations and precise protein localization

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60350R2
Full Title:	Three-dimensional imaging of bacterial cells for accurate cellular representations and precise protein localization
Section/Category:	JoVE Immunology and Infection
Keywords:	protein localization, cell shape, mircoscopy, morphogenesis, forward convolution, computational image processing
Corresponding Author:	Randy Morgenstein oklahoma state university stillwater, UNITED STATES
Corresponding Author's Institution:	oklahoma state university
Corresponding Author E-Mail:	randy.morgenstein@okstate.edu
Order of Authors:	Randy M Morgenstein
	Benjamin P Bratton
	Brody Barton
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	stillwater, OK, USA

Cover Letter

OKLAHOMA STATE UNIVERSITY

College of Arts and Sciences
Microbiology and Molecular Genetics
307 Life Science East
Stillwater, Oklahoma 74078-3020
405-744-6243, FAX 405-744-6790

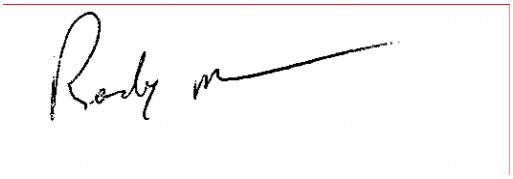
Dear Editor,

We are pleased to resubmit our manuscript titled “Three-dimensional imaging of bacterial cells for accurate cellular representations and precise protein localization” for your consideration for publication in JoVE.

Thank you for your help in editing the protocol. We have tried our best to address all of your concerns, however there were a few places where it was unclear what to do. In section 4.2 we were requested to add how to perform this task, but feel that subsection 4.2.2-4.2.4 already do this. Please advise if these instructions are not sufficient. In section 5.3 we have added a second note to explain what we edited for this experiment, but were unclear the best way to display. Additionally, we have reordered figure 3 and 4 for clarity.

Thank you.

Sincerely,



Randy Morgenstein, Ph.D.
Assistant Professor
Microbiology Department
420 Life Science East
Stillwater Ok, 74074

TITLE:

Three-Dimensional Imaging of Bacterial Cells for Accurate Cellular Representations and Precise Protein Localization

AUTHORS AND AFFILIATIONS:

Benjamin P. Bratton[2], Brody Barton[1], Randy M. Morgenstein[1]

[1]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK, USA

[2]Department of Molecular Biology and Lewis-Sigler Institute of Integrative Genomics, Princeton University, Princeton, NJ, USA

Corresponding Author:

Randy M. Morgenstein (randy.morgenstein@okstate.edu)

Email Addresses of Co-authors:

Brody Barton (brodylb@okstate.edu)

Benjamin P. Bratton (bratton@princeton.edu)

KEYWORDS:

protein localization, cell shape, microscopy, three-dimensional microscopy, morphogenesis, forward convolution, computational image processing

SUMMARY:

This protocol explains how to prepare and mount bacterial samples for live three-dimensional imaging and how to reconstruct the three-dimensional shape of *E. coli* from those images.

ABSTRACT:

The shape of a bacterium is important for its physiology. Many aspects of cell physiology such as cell motility, predation, and biofilm production can be affected by cell shape. Bacterial cells are three-dimensional (3D) objects, although they are rarely treated as such. Most microscopy techniques result in two-dimensional (2D) images leading to the loss of data pertaining to the actual 3D cell shape and localization of proteins. Certain shape parameters, such as Gaussian curvature (the product of the two principal curvatures), can only be measured in 3D because 2D images do not measure both principal curvatures. Additionally, not all cells lie flat when mounting and 2D imaging of curved cells may not accurately represent the shapes of these cells. Accurately measuring protein localization in 3D can help determine the spatial regulation and function of proteins. A forward convolution technique has been developed that uses the blurring function of the microscope to reconstruct 3D cell shapes and to accurately localize proteins. Here, a protocol for preparing and mounting samples for live cell imaging of bacteria in 3D both to reconstruct an accurate cell shape and to localize proteins is described. The method is based on simple sample preparation, fluorescent image acquisition, and MATLAB-based image processing. Many high-quality fluorescent microscopes can be simply modified to take these measurements. These cell reconstructions are computationally intensive and access to high-throughput computational

resources is recommended, although not necessary. This method has been successfully applied to multiple bacterial species and mutants, fluorescent imaging modalities, and microscope manufacturers.

INTRODUCTION:

Cells of all types regulate their shapes for specific functions. For example, neurons are shaped differently than blood cells and have different functions. Similarly, bacterial cells come in a variety of shapes and sizes, although the purpose of these shapes is not always known[1,2]. Therefore, it is important that the shape of bacterial cells be accurately determined. The method outlined shows an easily implemented way to collect data suitable for the 3D analysis of most live or fixed bacterial cells.

The method described enables one to take 3D images of bacterial cells in order to accurately represent the 3D cell shape of the sample and to precisely localize proteins within these shapes. Traditional microscopy techniques take 2D images, which is problematic when studying cells that have abnormal or nonsymmetrical shapes, such as mutants of *Escherichia coli*, or curved bacteria such as *Vibrio cholerae* and *Helicobacter pylori*. While high-resolution 3D images are the key input to this method, the method does not return a resolution-enhanced image. Rather, this method reconstructs the 3D surface coordinates and shape of the cell using a forward convolution algorithm using active contours and the apparent blurring function of the microscope[3] (**Figure 1**). It has been used to study the bacterial actin homolog MreB in *E. coli*[4-7], the novel periskeletal element CrvA in *V. cholerae*[8], and the putative bactofilin CcmA in *Helicobacter pylori*[9] (**Figure 2**).

The localization of proteins can give insight into their functions. For example, proteins involved in cell division are normally localized to the midcell[10,11]. High-throughput studies have been undertaken to localize all the proteins of a bacterium in hopes of gaining insight into their functions[12]. Unfortunately, these studies were performed with 2D imaging and 1D or 2D analysis, making it impossible to measure specific aspects of protein localization, such as localization to cellular geometric features.

For example, MreB, a dynamic protein required for the rod shape of many bacteria, is hypothesized to work by directing the localization of cell wall synthesis, and its localization mirrors the localization of cell wall synthesis[7,13]. MreB from multiple species shows geometric enrichment[4,6,7,14,15]. Dynamic surface polymers, such as MreB, may couple the geometry of the surface to the time averaged enrichment profile[16] and may be able to orient to specific geometries by minimizing the energy associated with binding to the membrane[17]. While the importance of twisting, bundling, bending, and dynamics has not been entirely resolved for MreB, it is important to note that accurate measurements of both principal curvatures of a surface require a full 3D representation of the cell. Therefore, to most accurately measure the curvatures to which proteins localize, it is preferential to use 3D, rather than 2D imaging. 3D imaging eliminates the need to computationally estimate those curvatures which cannot be measured in 2D, an estimate that might not be accurate in asymmetrical cells[18].

Although 2D imaging of cells is quicker and does not require as much postimaging computational

work, 3D imaging provides a more accurate representation of the cell, as well as the ability to measure surface features, such as curvature, which cannot be measured in 2D. Therefore, as 3D imaging becomes more commonplace, new insights into cell shape and protein localization will become possible.

PROTOCOL:

1. Sample preparation

1.1. Make *E. coli* fluorescent for imaging either by engineering them to express a cytoplasmic fluorescent protein[6] or using a membrane dye[5]. Other bacterial species can be used instead of *E. coli*.

1.1.1 Transform cells via electroporation with a plasmid that encodes the cytoplasmic mCherry fluorescent protein.

NOTE: Different fluorescent proteins of other colors of can be used.

1.1.2 Grow the transformants on an LB plate with the appropriate antibiotic at 37 ˚C. Obtain single colonies and identify positive clones by microscopy. Antibiotic resistant colonies that appear red under the microscope contain the plasmid carrying mCherry.

1.2. Grow 2 mL of the overnight culture in LB liquid media with the appropriate antibiotics at 37 ˚C in a shaking incubator. Use either a colony from a plate or a small amount of a freezer stock as the inoculum.

NOTE: Use the media necessary for the bacterial cells selected for the experiment.

1.3. Subculture the overnight culture 1:1,000 into 5 mL of fresh LB media and let the cells grow to exponential phase at 37 ˚C in a shaking incubator for 3−4 h. Measure the OD_{600} of the cells to confirm they are in the exponential phase (i.e., OD_{600} = 0.2−0.4).

NOTE: Imaging can be performed at any growth stage depending on the experiment being performed.

2. Slide preparation

NOTE: It is important to use media that has low autofluorescence. Any medium which has low autofluorescence can be used. In this experiment, 1% agarose M63 minimal media pads and sealing with VaLaP[19] is required to image cells in 3D.

2.1. Prepare a 1% solution of agarose in 20 mL of minimal media.

2.2.1. Microwave the solution until the agarose is fully dissolved and the solution appears clear.

2.2.2. Keep the solution in a 60 ˚C water bath until ready to use.

NOTE: This solution can be solidified and melted as needed or can be aliquoted into smaller amounts that are melted as needed. After multiple uses the media may become discolored and should be replaced.

2.2. Melt VaLaP sealant on a hot plate at 80−100 ˚C.

NOTE: Prepare VaLaP, to be used as a sealant, by melting 50 g each of petroleum jelly, lanolin, and paraffin together in a beaker on a hot plate at 80−100 ˚C. This large amount of VaLaP can be stored at room temperature indefinitely and will be enough for >500 slides. Heating at >100 ˚C will cause it to degrade and its color will darken.

2.3. Place two stacks each of three 20 mm x 20 mm cover slips on the opposite ends of a slide (six total coverslips).

2.4. Pipette 200 µL of agarose pad solution onto the slide.

NOTE: If not using an engineered fluorescent stain, a membrane dye may be added to the agarose pad solution before this step. Resuspend the dye in water and add dye to 1 mL of the agarose pad solution to a final concentration of 5 µg/mL.

2.5. Immediately and firmly place a second slide down on the stack of cover slips to flatten the agar and let it solidify for 1 min at room temperature.

2.6. Carefully slide off the top slide.

2.7. Use the large end of a 200 µL pipette tip to cut out individual pads from the gel (~5 mm in diameter) on the slide and discard malformed or unneeded gel.

NOTE: If imaging multiple strains or conditions, a separate pad will be needed for each one. If only one strain is to be imaged, make 3−4 pads to help balance the coverslip.

2.8. Pipette cells up and down multiple times to disrupt cell clumps and ensure the culture is well mixed. Pipette 1 µL of subculture from step 1.3 onto a pad.

NOTE: Cell shape reconstructions require individual cells that are not touching other cells. If using stationary phase or high cell density cultures, it may be necessary to dilute the sample 1:10 before placing it on the pad.

2.9. Let the sample air dry for 5−10 min. Ensure that the droplet is completely absorbed into the pad. If any liquid remains, the cells will move around in the liquid and cannot be imaged.

2.10. Place a cover slip on the top of the pads.

2.11. Seal the cover slip with melted VaLaP by gently brushing around the edge of the cover slide with a cotton swab. Make sure to keep it away from the top of the cover slip where the objective will touch. The VaLaP will harden in a matter of seconds, sealing the sample.

2.12. Immediately image the sample.

NOTE: The sample should be imaged as soon as the slide is prepared. The cells can grow on the pads, and if they divide the reconstructions will be more difficult.

3. Imaging requirements

3.1. Ensure that the z or focusing axis of the microscope can make precise movements of less than 50 nm. Use z piezo stages (see **Table of Materials**), available on research grade microscopes, because typical motorized focus devices are unable to provide this precision.

NOTE: Assuming the Nyquist-Shannon sampling criterion[20], one needs to have the ability to move 0.5x the smallest desired step size, or 2x the desired spatial frequency. For the 100 nm steps needed in this protocol, a stage with a precision of 50 nm or less is required.

3.2. Ensure that the microscope contains a 100x objective with a minimum numerical aperture (NA) of 1.45.

3.2.1. Collect z-stacks of between 200−400 different cells to ensure enough cells are obtained for downstream applications.

NOTE: The number of cells needed depends on the underlying variability of the sample of interest. Some of the cells collected at this point will not make it through the reconstruction steps.

3.3. Ensure that the z-stack matches the settings used for the shape channel if a secondary fluorescent channel (protein, metabolic label, etc.) is measured.

4. Imaging

4.1. Insert the sealed slide onto the microscope and allow it sit for 5 min to equilibrate the temperature with the surroundings, because the microscope room may be at a different temperature than the sample preparation room.

4.2. Take a fluorescent z-stack of the sample.

NOTE: The z-stack should entirely cover the sample with a z-spacing less than the depth of field. For a 1.45 NA 100x objective and ~1 μm thick *E. coli* cells, 40 steps at 100 nm per step work

well. For larger cells or cells that do not lie perfectly flat on the surface, 50 or more steps may be necessary. Include enough steps and ensure that the sample is fully blurred above and below.

4.2.1. Use the software associated with the microscope (see **Table of Materials**) to control the microscope.

4.2.2. Focus on the middle of the cell using the microscope focus wheels. Under **ND acquisition** check the **Z box** to take a z-stack. Click the **Home** button to set the middle of the cell as the starting point. Set the **Step size** to 0.1 µm and set the **Range** to 4 µm. Make sure that the Z device is set to the piezo stage.

4.2.3. Set the fluorescent channels under the **Lambda Window** to the settings for the fluorescent molecules being imaged. In this experiment GFP and mCherry were used.

NOTE: Take an additional z-stack with the same **Step Size** and **Range** in the second color channel if the 3D distribution of an additional fluorescent channel is desired. In this experiment, cytoplasmic mCherry was used to determine cell shape and MreB-GFP was used as a second color channel[21].

4.2.4 Ensure that the **Order of Experiment** is set to lambda (z series) so that it will take a complete z-stack in each color channel before switching.

5. Cell reconstruction

5.1 Crop individual cells and save the images as a stacked tiff file so that there is only one cell per file. Ensure that this cell is well isolated from any other cells (i.e., roughly 5x the full-width half-maximum of the blurring function in xy.

NOTE: This can be done using freely available image analysis software (see **Table of Materials**).

5.1.1 Draw a box around an individual cell and **Duplicate** that cell 2x, once for each channel. Make sure the duplicate hyperstack box is checked and change the channel to either 1 or 2, making sure that the slices include the entire z-stack.

NOTE: If imaging only the shape of the cells and not an additional fluorescent protein, only one channel will be present.

5.1.2 Once both stacks are available go to **Images**|**Stacks**|**Tools**|**Concatenate** to combine the images with the protein channel first and the shape channel second.

5.2 Measure the blurring function of the microscope using subdiffraction limited fluorescent beads[22]. This needs to be done for each microscope and microscope objective but can be performed before or after imaging the samples of interest.

5.2.1 Average together multiple independent beads with some manual intervention using available software (see **Table of Materials**).

NOTE: The final product should be a 3D image of the blurring function with the same xyz spacing as the samples of interest.

5.3 Run the forward convolution cell shape reconstruction scripts using available software. The latest version of these scripts can be freely downloaded from https://github.com/PrincetonUniversity/shae-cellshape-public.

5.3.1 Make a folder inside a folder on the desktop that contains the cropped images and the *cell_shape_settings_tri.txt* file from shae-cellshape-public/exampleData_tri.

5.3.2 Edit *cell_shape_setting_tri.txt* to have the correct settings for the experiment of interest. For this experiment, the settings file includes the following lines:

```
nm_per_pixel 70
Z_scale 0.65
stack_z_size 41
stack_t_size 1
Fstack_z_size 41
Fstack_t_size 1
stack_seperation_nm 100
Fstack_seperation_nm 100
psfScript -999 osuPSF20180726
gradient 1
```

NOTE: This text file is organized into sections and each section is parsed into its own variable. While many of the settings do not need to be changed from experiment to experiment, one should make sure that the size of the z-stack (*stack_z_size* for shape, *Fstack_z_size* for protein of interest), spacing of the z-stack (*stack_seperation_nm*, *Fstack_seperation_nm*), relative focal shift of the microscope (*Z_scale*)[23], the pixel size in xy (*nm_per_pixel*), and the name of the script that loads the blurring function of the microscope (*psfScript*) match the experiment. The *gradient* field should be set to 1 if the shape channel is cytoplasmically filled and 0 if it is a membrane-stained object.

5.3.3 Run **Cell_shape_detector3dConvTriFolder** function (shae-cellshape-public/CellShapeDetectorTri/) with the string to the folder location followed by the number of cells you want to run, and the number of the cell to start on.

NOTE: An example for the input will look as follows: Cell_shape_detector3dConvTriFolder ('path to folder with cropped images',# of cells, starting index in folder). A typical cell may take between 5−20 minutes for the reconstruction to converge and finish.

5.4 Screen the cell reconstructions to ensure that they are correct before using the cells for any statistical analysis.

5.4.1 Run **ScreenFits** (shae-cellshape-public/shae-fitViewerGui/) to visually screen individual cell reconstructions.

5.4.2 Click the select folder button when the graphical user interface (GUI) opens, then select the folder with the reconstruction data files (TRI.mat) created in step 5.3.

5.4.3 Select the box next to the cell reconstruction if a cell appears misshapen or did not fully converge (**Figure 3**). This could look like a cell with a hole, a flat side, or a branch coming out of it. This will append '**FLAG**' to the file name so that it can be excluded from any downstream analysis.

NOTE: The reconstructed cell can be compared to the original images. This can be especially important if your cells come from a various heterogenous population of shapes.

5.5 Run **enrichmentSmoothingSpline** (shae-cellshape-public/) to create an enrichment profile of the relative concentration of the protein of interest as a function of the Gaussian curvature at the surface.

NOTE: In addition to the precise geometric localization of a fluorescent protein, there are many other ways to analyze the data from the secondary channel, including counting the number, size, and orientation of objects[4].

REPRESENTATIVE RESULTS:

Bacteria come in a wide variety of shapes and sizes that may determine their functions in nature[1]. The outcome of this procedure is an accurate 3D representation of cells from the forward convolution of a z-stack of images (**Figure 1**). This method is especially important when dealing with curved cells (**Figure 2**), or with abnormally shaped cells (**Figure 4A**), as a 2D representation does not reflect the curvature of the cells accurately. In order to use the forward convolution method (**Figure 1A**), cells need to be either peripherally stained or have a cytoplasmic stain (**Figure 2B** left vs. right).

Figure 4 shows MreB localization in the cell. A GFP fusion was made to the bacterial actin protein MreB[21] in order to study its precise localization in both wild type and mutant *E. coli* cells[4]. Because MreB is associated with the membrane, 3D imaging is required to faithfully reproduce its position in the cell. By making these measurements in 3D, we were able to reconstruct the shapes of both wild type and *rodZ* mutant cells (**Figure 4A**). The localization of MreB was shown to be enriched at small Gaussian curvatures, a geometric feature that can only be measured in 3D, in a RodZ dependent manner (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Forward convolution method reconstructs cells with no prior knowledge of cell shape.

(**A**) The final output of the method is a 3D cell reconstruction derived by comparing a test shape with the calibrated blurring function of the microscope. This is compared with the observed z-stack until the observed 3D image matches the hypothetical one. The image shown here is a rendering of the reconstruction of one *C. crescentus* cell. (**B**) The reconstruction pipeline iteratively updates the estimated positions of the surface elements based on the observed image stack. For full algorithmic details of the method, see Nguyen[3]. (**C**) The reconstruction pipeline starts with no *a priori* knowledge of the shape of the cell and updates the position and number of surface vertices to match the observed z-stack. Representative images from every 30 steps during the 3D reconstruction are shown from three different angles of a *V. cholerae* cell. The surface positions of this shape are updated to minimize the difference between the simulated and observed stacks.

Figure 2: Representative 3D reconstructions of three different shaped bacterial species. Three-dimensional reconstructions can be made from cells with their membrane stained (left) or filled with a cytoplasmic fluorophore (right). The shape and curvature of the starting cell is not important, as a bent rod, twisted rod, or straight rod are all able to be accurately reconstructed. The reconstructed surfaces are colored based on the local Gaussian curvature of the surface. Scale bar is 1 µm.

Figure 3: Reconstructions can fail for multiple reasons. Cells must be screened to ensure that the reconstruction algorithm converged to a reasonable result. Left = representative wild type (top) and *rodZ* mutant (bottom) *E. coli* cells that have passed quality control. Right = cells that failed to reconstruct properly and did not pass quality control. Five classes of failed convergence are shown: (i) cells that are too close to the edge of the cropping region leading to a sharp demarcation, (ii) cells that are too close to another cell, (iii) cells that produced a divot, (iv) cells that did not proceed past the initial starting point, and (v) other unknown errors.

Figure 4: Representative data showing protein localization to specific cellular geometries. (**A**) Three-dimensional reconstructed cells of wild type and *rodZ E. coli* cells with Gaussian curvature and MreB fluorescence intensity displayed. Scale bar is 1 µm. (**B**) Enrichment plots of MreB from wild type and *rodZ E. coli* cells. Values >1 show enrichment and values <1 show depletion of MreB from these cellular regions relative to uniform coverage of the cell. Shaded areas indicate mean ±90% bootstrap confidence interval of the mean. The curve for each strain is a cubic smoothing spline and is truncated using a probability threshold for extreme curvatures of $p > 5 \times 10^{-3}$. This figure has been modified from (Bratton et al.)[4].

DISCUSSION:

A critical step in this protocol is the acquisition of high-quality images. To properly reconstruct the cells, there must be enough blurring above and below the cell. Therefore, it is imperative that the z-stack taken covers a large enough distance. The number of steps taken during the image acquisition can be adjusted for each strain. For example, *E. coli* cells deleted for *rodZ* are wider and require more steps, and therefore, a greater distance, than wild type cells. If the sample drifts during image acquisition, the reconstruction can have major errors. Therefore, it is important to let the slide come to thermal equilibrium with the microscope before imaging to

avoid drift during the z-stack acquisition. Cells should be imaged on pads with low autofluorescence. Media components, such as those found in the common LB medium, have autofluorescence that can cause problems when trying to reconstruct the cells. The density of cells on the imaging pad is important because the reconstruction process is performed independently on each cell. Too few cells will increase the time needed to obtain images of a sufficient number of cells, while too many cells will result in imaging fields that are too dense to easily crop individual cells. Because not all cells reconstruct properly, extra cells should be imaged during the acquisition step and all outputs should be screened before moving forward with statistical analysis (**Figure 3**).

Many of the limitations for this method are technical. On the microscope to be used, one must have an objective that has a high numerical aperture (typically >1.4), because this enables optical sectioning on the size scale of bacteria. Additionally, the microscope needs to be equipped with a piezo stage that can take small, precise steps in the z-direction. Furthermore, while it is not necessary, access to high-throughput computational resources to run the image analysis software is highly recommended because it will reduce the processing time to reconstruct cells.

One conceptual limitation to the method is that the correct energy scales for weighting the smoothness of the reconstruction relative to the signal-to-noise ratio of the images must be chosen. To validate a choice of parameters, the sizes and shapes of cells should be measured using independent methods such as transmission electron microscopy (TEM) or atomic force microscopy (AFM). As a proof of principle, 3D reconstructions of cells were performed either on an AFM to test for z-accuracy (<50 nm) or on a TEM grid to test for xy-accuracy (<30 nm)[3]. Such a correlated approach is time consuming and costly. A simpler approach may be to image standard samples such as wild type cells or 1 µm spherical beads. The diameter and sphericity of the reconstructions can be used to ensure that the size and energy scales used in the reconstruction are correct.

This is not the only method that seeks to extract high resolution spatial information from fluorescence microscopy images. Many review articles describe recent advances in the field of super-resolution microscopy[24,25]. Resolution-enhancing techniques such as deconvolution microscopy[26], spinning disk confocal microscopy[27], pixel reassignment[28], and structured illumination microscopy (SIM)[29] seek to improve the resolution of the images acquired by the microscope. These methods are not incompatible with the approach presented. Recently this method was adapted to allow for SIM-based images as inputs[9]. While the forward convolution method shares some of its underpinnings with deconvolution microscopy, it has a completely different output. Whereas approaches such as deconvolution microscopy seek to improve the resolution of the image, this approach does not generate an image but rather a cell shape reconstruction with roughly 50 nm precision. Single-molecule active-control microscopy techniques based on sparsely labeled samples can provide even higher levels of spatial precision than this method. In many cases, these single molecule approaches require optimization of the fluorescent constructs and can require long acquisition times, making them difficult to use with live or dynamic samples. Each of these methods comes with one or more caveats that this method does not. For instance, the benefits advertised by spinning disk

confocal microscopy are not as applicable to monolayers of bacterial cells, where there is not much out of plane light. Furthermore, this method provides a pipeline to acquire accurate 3D cell shapes and protein localization without the need of any specialized fluorophores. This method has minimal hardware requirements (i.e., z piezo, high NA objective) and requires only tens of images per timepoint, allowing one to easily investigate dynamic 3D structures[6].

There have been an increasing number of approaches to study the organization of bacterial cells in 3D structures. These include approaches conceptually similar to this that take advantage of high quality, 3D fluorescence images[30-32]. This approach requires well isolated cells and makes no *a priori* assumptions about cellular geometry. However, to move into dense cellular aggregates or biofilms, the cells are assumed to be rod-like. This lower resolution view still enables investigating packing arrangements of the cells, although the high density of cells in the biofilm prevents analysis of the subcellular localization of specific factors.

In the future, it may be interesting to develop a framework to integrate the single molecule and wide-field approaches with this 3D reconstruction technique. Moreover, it may be possible to include this forward convolution approach with machine vision segmentation tools[32] to allow for reconstructions of more dense cell clusters.

Why cells evolved into specific shapes is a complex issue that must reflect the complex environment in which they live. Understanding the evolution and function of cell shapes requires taking precise and accurate measurements of those shapes, which is what this method provides.

ACKNOWLEDGMENTS:

We would like to thank Jeffrey Nguyen and Joshua Shaevitz for helping to develop this method. Funding: RMM – NIH F32 GM103290-01A1, BPB – Glenn Centers for Aging Research and National Science Foundation PHY-1734030.

DISCLOSURES:

The authors have nothing to disclose.

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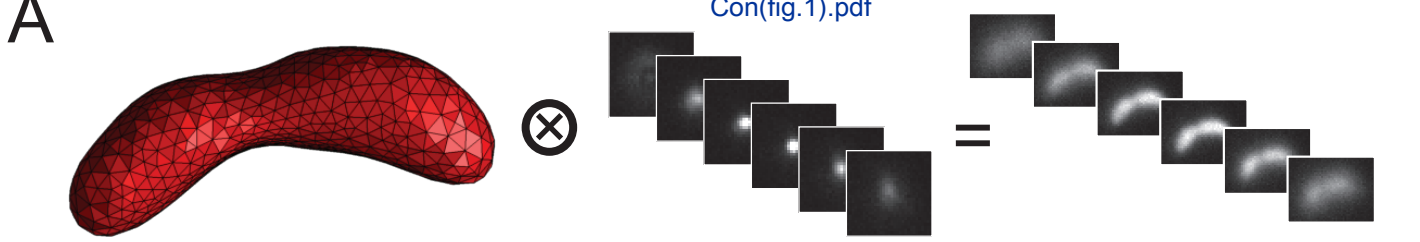
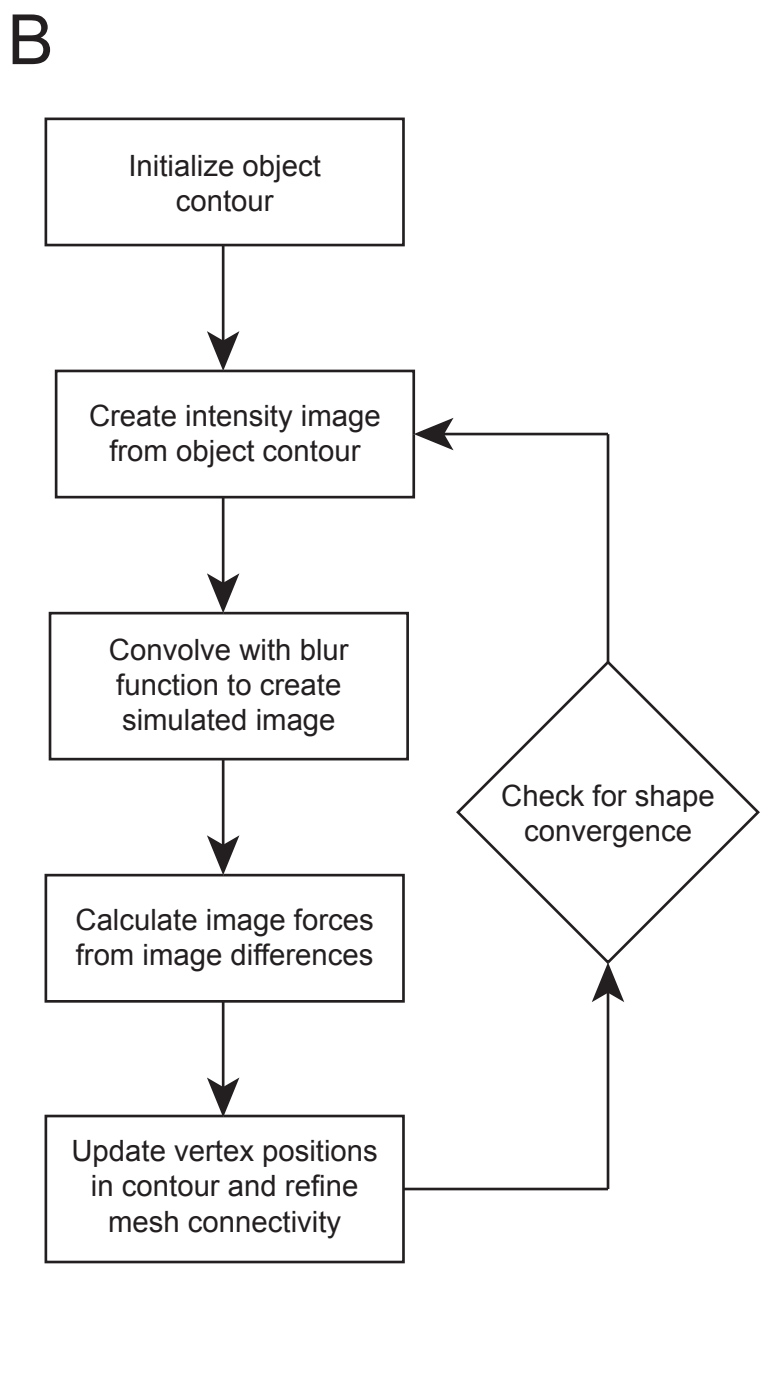
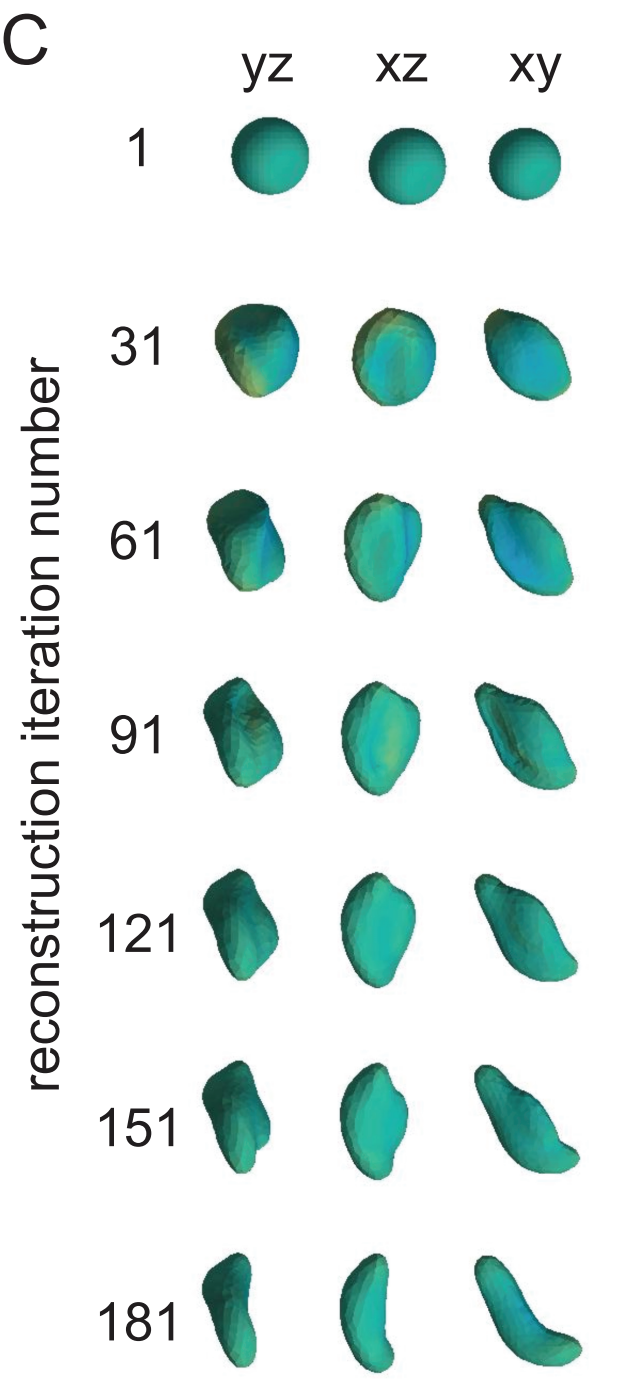
Figure 1







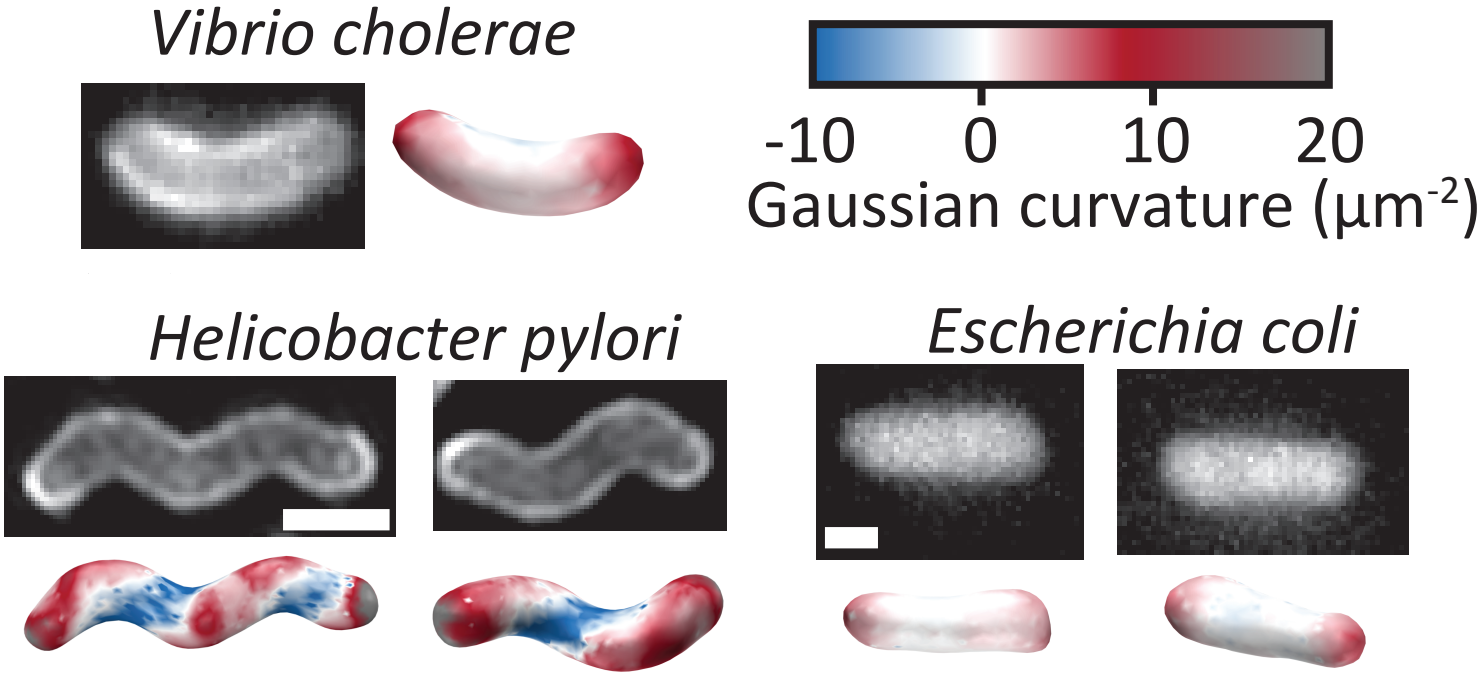
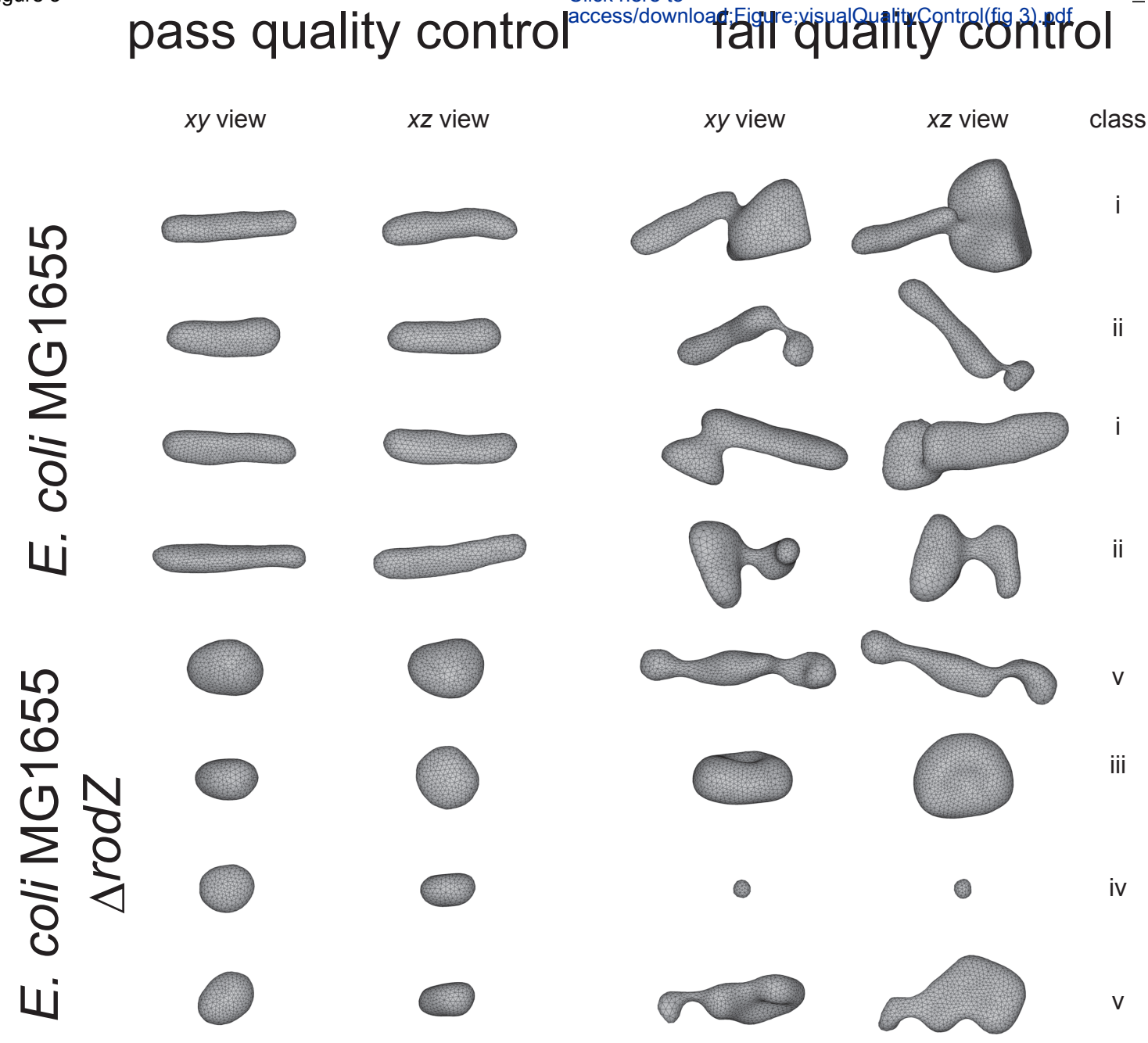
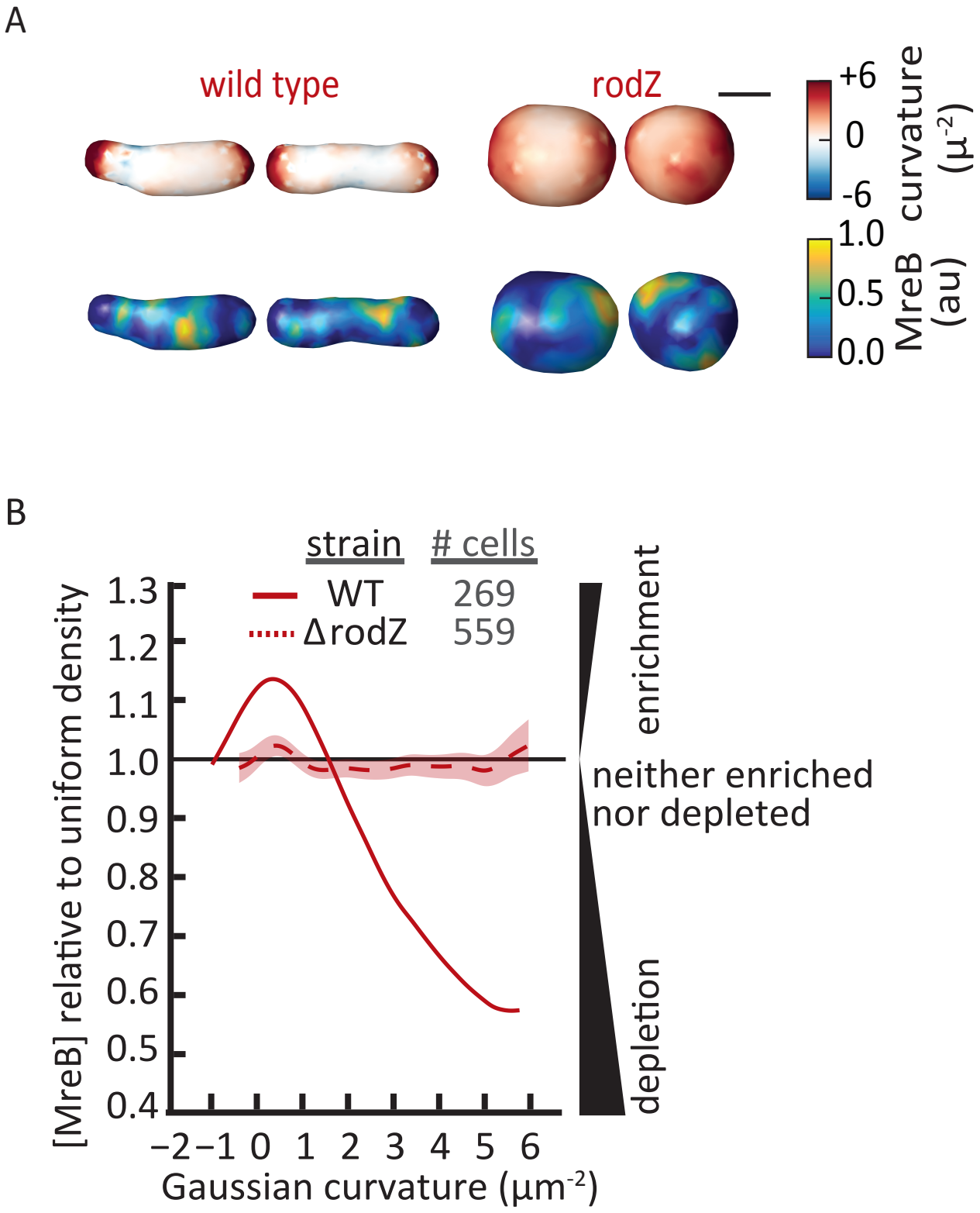


Figure 3





Name of Material/Equipment	Company	Catalog Number	Comments/Description
50nm fluorescent beads	Invitrogen	F8795	these are used to measure the blurring function of the mi
Agarose	sigma-Aldrich	A9539	
Cotton Swab	Puritan Medical Products Company LLC	S304659	used to appy VaLaP
Cover Slips	VWR	16004-302	
Fiji	ImageJ	https://fiji.sc	used to cro cells
FM4-64	Invitrogen	LST3166	membrane dye used to stain cells
Huygens Software	Scientific Volume Imaging	Huygens essential or professional	Use to measure blurring function of microscope
Lanolin	Sigma-Aldrich	L7387	combine with paraffin and petroleum jelly to make VaLaP
LB growth medium	BD Difco	DF0446173	
M63 medium	US Biological	M1015	
MATLAB	Mathworks		Needed to run forward convolution scripts
Microscope Slides	Fisher	12-550-133	
NIS Elements	Nkon		
Paraffin	Sigma-Aldrich	327212	
Petroleum Jelly	Equate	49035-038-27	
piezo z stage	Nikon	77011589	
pipet tips -p200	USA Scientific	1111-0730	
pipet tips- p10	USA Scientific	1111-3730	

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Author(s):	Benjamin P. Bratton, Brody Barton, Randy M. Morgenstein

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Institution:	Oklahoma State University
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This has been changed

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For example: Agarose M63, VaLaP, etc.
Agarose and VaLaP are not commercial names. They are in the table of materials.

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5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
The text of the protocol section has been changed to the imperative tense and notes have been added.

6. The Protocol should contain only action items that direct the reader to do something.
The protocol has been modified throughout.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
The protocol has been modified throughout.

8. Please include a single line space between each step, substep, and note in the protocol section.
The protocol has been modified throughout.

9. Please use complete sentences throughout.
The protocol has been modified throughout.

10. Please ensure you answer the “how” question, i.e., how is the step performed?
The protocol has been modified throughout.

11. 1.1: What kind of cells are used in this experiment? How do you engineer to express fluorescent proteins? How do you grow? What temp, and other conditions and for how long? How do you subculture? Any O.D checks if any?
The protocol has been modified throughout.

12. 2.6: How do you perform the dye addition?
This has been added to the protocol.

13. 2.12: Do you check the slides under the microscope at this stage? What dilution? Dilute to what dilution?
At the concentration used in this experiment dilution is not necessary; however, a note has been added to indicate what to do if higher density cells are used.

14. 3: How do you focus on the image? How do you use the piezo stages? How do you collect the images? What is the magnification used? Please include all the knob turns in the instrument and all click by click instructions of how to use the associated software?
The protocol has been modified to include this information.

15. 4: How is this done?
A more detailed protocol has been provided.

16. 5: How is this done? Please provide step by step description of your protocol and how to use the software and scripts. How do you perform the cell reconstruction? Please include all the specific details associated with your protocol. How do you perform reconstruction? What statistical analysis is performed? How do you screen the reconstructed images to ensure it is correct?
A more detailed protocol has been provided.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

18. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. , e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
We have modified the results section in lines 598-604 to make this more clear. Also, figure 4 shows sub-optimal experimental results.

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This has been fixed.

21. 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 in separate columns in a .xlsx file. Once done please sort the materials table in the alphabetical order.
The table has been updated.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Bratton et al describes procedure to acquire and reconstruct 3D images of fluorescently stained bacterial cells. The method is potentially very useful for studies of sub-cellular organization in bacteria. There are frequent occasions where 2D image is not sufficient; some of these are also mentioned in the manuscript.

Major Concerns:
1) While addressing an important problem, the approach focuses on trivial aspects of the procedure leaving the challenging parts uncovered. Most labs are perfectly capable of making agar pads (yellow lines 112-132) and taking Z-stack of images (yellow lines 146-155). Who needs to watch movie on this? There are just two more yellow lines (164-165) that describe screening of results. This seems also a rather common sense step.
We have updated the highlighted text to show more of the computational steps, however we do not think that pad preparation is common sense to most labs and have left that section in.

The difficult part is how to use correctly the software on github that the authors have uploaded. There is no documentation uploaded in github nor it is described in the manuscript. Without clear instructions and documentation how to use the software the whole procedure is useless. Authors should have detailed documentation available for their Matlab code.
What needs to be explained is "5.3 Run forward convolution MATLAB scripts." Currently it is just one sentence in the manuscript.
Thank you. The protocol has been expanded in the sections describing how to run the MATLAB scripts.

2) Explain also how to process fluorescent protein data.
This has been added to the protocol at line 492.

3) Some limitations of forward convolution technique should be mentioned in the manuscript. Many users will take the results otherwise on their "face" value even though the procedure is likely to produce artifacts.
Please discuss in the manuscript what controls can be used to test validity of the outcome.
We have added lines 671-681 to discuss limitations and controls. Please see fig. 4 as examples for quality control.

Minor Concerns:
1) Fluorescent beads used to determine blurring function of the microscope should be listed.
This has been added to the table of materials.

Reviewer #2:
Manuscript Summary:
Review:Three-dimensional imaging of bacterial cells for accurate cellular representations and precise protein localization

The authors describe a method for 3D imaging of bacterial cells, including irregularly shaped (i.e. deviating from cocci or rods) cells. This method is potentially of interest for a large community of bacterial cell biologists.

Unfortunately, the study shows largely published results, and is very vage, when it becomes interesting. A large part of the protocol deals with sample preparation, which is very trivial and has been described in a myriad of previous publications. The part that is interesting, namely 3D image generation, is only very vaguely described.
*We are glad that you find the 3D **reconstructions** interesting, but to be clear this method is not a novel way to generate 3D images (optical sectioning) but rather a way to make 3D reconstructions of cells. See comments to the next point.*
To make this clearer we have modified the protocol to include more detail on the computational steps.

Also, 3D deconvolution has been described in detail before, but has frequently lead to artefacts. In which way is the described protocol/algorithm better than commercially available or other custom made deconvolution software?
We are not doing deconvolution, this method is for forward convolution. Lines 64-66 and 693-697 were added to help distinguish these methods. In the discussion we talk about pros and cons of these methods. To reiterate we did not write new or any deconvolution software, we wrote new forward convolution software.

Major Concerns:
the developed method is frequently compared with other methods (Line 58: the "best method"), but there is no way to judge whether is it indeed better. Please prove that the described method is the best way by showing appropriate comparisons.

- Figures shown are largely left overs from previous publications, and controls/benchmarking is missing.
Only one of the figures has been published before, and this is the first time we have published a 3D helicobacter cell.

- At the least, please provide a small scheme of the algorithm
This has been added to figure 1.

Minor Concerns:
- please state average processing times per cell
This has been added.
- Figure 2 footnote (lines from 197-199) and Line 109 please rephrase
We have reworded this section
- point 5.4: please give objectives of how "correct" is distinguished from "incorrect"
This has been added and see figure 4
- please spell out "versus" or use "vs" instead of "v"
This has been fixed
- please include space between numbers and units
This has been fixed
- please prove that a number of 200 to 400 samples is representative/statistically significant
We have reworded this section. The number of cells needed depends on the underlying variability of the sample of interest. One estimate for the number of cells that needs to be measured is when the within sample error for estimates of the mean is similar to the between sample variability of the means. Increasing the number of cells within a sample increases the precision of the mean estimator, decreasing the within sample error (smaller error bars). Increasing the number of cells in each sample does little to change the between samples

variability. For MreB geometric localization in E. coli, this crossover point happens around 100 cells.

- a number of 50 nm or under point 4) 100 nm is stated to be necessary for efficient reconstruction, please clarify
Thank you, we have added a note about to address this on line 308-310