Optical tweezers assisted imaging of the Z-ring in *Escherichia coli*: measuring its radial width

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Using single-beam, oscillating optical tweezers we can trap and rotate rod-shaped bacterial cells with respect to the optical axis. This technique allows imaging fluorescently labeled three-dimensional sub-cellular structures from different, optimized viewpoints. To illustrate our method we measure $D$, the radial width of the Z-ring in unconstricted *Escherichia coli*. We use cells that express FtsZ-GFP and have their cytoplasmic membrane stained with FM4-64. In a vertically oriented cell, both the Z-ring and the cytoplasmic membrane images appear as symmetric circular structures that lend themselves to quantitative analysis. We found that $D \approx 100$ nm, much larger than expected.

1. Introduction

Over the last 20 years, a wide variety of optical microscopy techniques has been developed, ranging from confocal [1] and two-photon microscopy [2] to photoactivated localization microscopy (PALM) and stimulated emission depletion microscopy (STED) [3, 4]. In biology, such techniques have allowed imaging sub-cellular structures at resolutions below the Rayleigh diffraction limit. Moreover, some of them were designed to obtain three-dimensional (3D) images of microscopic objects. For example, confocal microscopy uses $z$-axis scanning...
Figure 1. The optical system: $M_1$, $M_2$ and $M_3$—mirrors; $M_4$—dichroic mirror; GM—galvanometric mirror; FC—filter cube; $L_1$, $L_2$—telescope lenses that conjugate the planes of the galvanometric mirror and the objective back aperture.

(perpendicular to the plane of view) to image $z$-slices of the sample that are subsequently reconstructed into the full 3D image. The depth of field of the objective limits the scanning step size in the $z$-direction. Since the axial resolution is about three times lower than the lateral resolution, for cells that are thinner than 1 µm, e.g. *Escherichia coli*, only a small number of optical sections are significant for 3D image reconstruction. In this paper, we describe a different approach using oscillating optical tweezers to rotate the cell and allow imaging from optimal viewpoints (figure 1). The most straightforward implementation of our method is for the case of rod-shaped cells and herein we illustrate its ability to render quantitative 3D information on the structure of the Z-ring in *E. coli*.

The core of the bacterial division machinery is the so-called Z-ring, consisting of polymers of the tubulin-like protein FtsZ and acting as an internal scaffold that correctly localizes synthetic enzymes [5–9]. The Z-ring is attached to the inner side of the cytoplasmic membrane (CM) and lies at mid-cell. There are about 13 different proteins that assemble on the Z-ring before the onset of septation. The emerging structure that consists of the Z-ring and the division proteins is known as the divisome.

The formation of the Z-ring is tightly controlled in both space and time [6, 7, 9]. It was shown that in *E. coli* it is located at mid-cell with remarkable accuracy, ~4% [10]. This accuracy is the result of the combined effect of two independent mechanisms, namely, MinCDE oscillations [11, 12] and nucleoid occlusion [13]. The Min system consists of the MinC, MinD and MinE proteins. MinC inhibits the polymerization of FtsZ, MinD recruits MinC to the CM and MinE drives the MinC-MinD off the CM. As a result, the MinC–MinD complex oscillates between cell caps and is mostly absent in the range around the cell center [14–16]. The second Z-ring positioning mechanism, the nucleoid occlusion, postulates that the formation of the Z-ring is inhibited in the vicinity of the nucleoid. It was shown that the inhibitory action of the nucleoid is mediated by Noc (YyaA) in *B. subtilis* [17] and SlmA in *E. coli* [18].

In the time domain, Z-ring formation represents the first event of the division process. It takes place via the condensation of FtsZ oligomers. Several possible scenarios describing this process have been proposed [19–21]. It was believed for some time that the FtsZ oligomers condensate directly from the cytoplasm. More recently, however, helical FtsZ structures were observed on the CM along with Z-ring formation [22]. This led to the suggestion that FtsZ first forms membrane helices that later condense into a ring [5, 23].
In vitro, FtsZ forms single-stranded filaments that, on average, contain about 30 monomer units and are \( \sim 125 \text{ nm} \) long \([24, 25]\). It is believed that the Z-ring consists of an assembly of similar filaments. It is initially attached to the CM via FtsA and ZipA membrane binding proteins \([12]\). Of the total FtsZ in the cell, only \( \sim 30\% \) is contained in the Z-ring while the rest is in the cytoplasm \([25]\). Fluorescence recovery after photobleaching experiments indicate that there is a rapid exchange of FtsZ monomers between the ring and the cytoplasm with a half-time of \( \sim 10 \text{ s} \) \([25]\). On the one hand, using electron cryo-tomography on Caulobacter crescentus, FtsZ filaments were found to be distributed around a 16 nm distance from the CM \([26]\). Moreover, there were on average \( \sim 3 \) filaments per cell typically forming arcs rather than complete rings. On the other hand, recent work that used PALM to view the Z-ring of E. coli \([27]\) showed that there are significantly more FtsZ filaments in the Z-ring than were found by Li et al \([26]\) and that it is organized as a tight helix. It was suggested that the FtsZ distribution has a certain radial spread that is significantly larger than what would correspond to a single FtsZ layer.

The difficulty in resolving the apparent contradiction between the results of \([26]\) and \([27]\) is partly due to the choice of imaging viewpoint. The standard imaging mode for E. coli is in the horizontal orientation, namely, with its long cell axis in the plane of view (figure 2(A), left panel). However, the Z-ring lies in a plane perpendicular to the long cell axis corresponding in this cell orientation to a less than ideal viewpoint (figure 2(C), left panel). Here, we use optical tweezers to rotate E. coli cells to the vertical orientation where the Z-ring lies in the plane of view. This allows imaging the Z-ring as a symmetrical circular structure and accurately measuring its radial width. Moreover, oscillating the laser beam by means of a galvanometric mirror at about 100 Hz leads to an effective linear trap that allows aligning the trapped cell in the horizontal orientation. Fast switching between the two orientations allows extracting information from the two imaging modes within a time window of a few seconds. We found that, for cells that have not yet started to constrict, the Z-ring extends about 100 nm inwards from the CM. This result is consistent with the qualitative picture suggested by Fu et al \([27]\). Preliminary results of this work were previously published elsewhere \([28]\) and later confirmed by a 3D PALM study where the Z-ring of C. crescentus was found to be of a similar width \([29]\).

2. Materials and methods

2.1. Experimental setup

Cells were imaged using an Olympus IX70 microscope together with a CoolSNAP ES2 camera (Photometrics). In our setup, the pixel size corresponds to a length of 41 nm. Exposure time for fluorescence imaging was 0.5 s. For the optical tweezers, we use a diode laser of 150 mW (SDL) that is first collimated and then focused by the 100× objective (UPLFLN 100XO2PH, 1.3 NA, oil immersion). Before the beam enters the objective it is reflected from a galvanometric mirror and expanded by the telescope lenses L1 and L2 (figure 1). Lenses L1 and L2 also conjugate the plane of the galvanometric mirror to that of the objective back aperture. In this configuration, tilting the galvanometric mirror does not shift the beam as it enters the objective, but rather it tilts it with respect to the optical axis. Therefore, the truncation of the beam due to the objective is kept constant as the mirror rotates and the trap structure is preserved while it scans the image plane \([30]\). In our system, the beam is truncated at about 2.7\( \sigma \) of the Gaussian profile.

The trapping force of optical tweezers is larger in the \((x, y)\) plane (perpendicular to the optical axis) than along the optical axis, \(z\). Thus, the optical trap aligns elongated objects,
Figure 2. Horizontal (right) and vertical (left) images of an *E. coli* cell from a HIL experiment. The length scale is the same for all images. Bar = 1 µm. (A) Phase contrast image, (B) the CM (red), (C) the Z-ring (green), and (D) overlay of panels (B) and (C). Note that the yellow ring has a red outer rim suggesting that the radius of the Z-ring is smaller than the radius of the cell. (E) The maximal intensity contour of the horizontal CM image in panel (B) (red) overlaid with three intensity levels of the horizontal Z-ring image in panel (C) (green). (F) The maximal intensity contour of the vertical CM image in panel (B) (red) overlaid with the maximal intensity contour of the vertical Z-ring image in panel (C) (green).

such as rod-shaped *E. coli* cells, with their long axis in the z-direction (figure 2). Oscillating the galvanometric mirror at a frequency of about 100 Hz generates an effective steady, linear trap along the x-axis, similar to the one caused by a cylindrical focusing lens [31]. We use a function generator and a sinusoidal voltage function, $V(t)$, to drive the galvanometric mirror. The amplitude of $V(t)$ determines the length of the linear trap. Whenever the trap length equals the length of the cell, $L$, the *E. coli* aligns with its long axis oriented in the x-direction. Reducing the trap length below $L$ rotates the cell out of the image plane such that it aligns at the desired angle with the optical axis [32, 33]. In both the horizontal and the vertical orientations cells are sequentially imaged in either phase contrast or fluorescence including changing filter sets for the different fluorophores. In our setup, we can switch between imaging modes within a few seconds, faster than the minimal time required for the Z-ring to change its structure [25]. The telescope system, lenses $L_1$ and $L_2$, of which $L_1$ is mounted on an X-stage, is used to adjust the height of the optical trap without affecting the imaging path in the system. This procedure allows us to focus the image of trapped cells.

2.2. Bacterial strains and growth conditions

To study the structure of the Z-ring we have used *E. coli* strain EC488 (courtesy of DS Weiss) [34]. It expresses FtsZ-GFP from the chromosome and its wild-type *ftsZ* gene is replaced
with the ftsZ84(ts) allele. Under moderate induction conditions, it was shown that the fraction of FtsZ-GFP is between 30 and 40% of the total FtsZ in the cell. Although FtsZ-GFP is not fully functional, EC488 was found to display normal growth and division behavior. In our experiments, cells were grown at 37 °C in Luria broth (LB) until OD_{600} \approx 0.2 in the exponential regime. To induce the expression of FtsZ-GFP, IPTG was added during the last 1 h of culture growth at a concentration of 40 µM. We find that cells behave normally and their growth pattern is not affected by the presence of IPTG. In what follows, we refer to such experiments as low induction level (LIL).

For improved contrast, we also performed experiments with a significantly higher induction level, at 500 µM IPTG. Such experiments will be referred to as high induction level (HIL). While the cell growth rate was lower in HIL experiments than in LIL experiments, we found no differences in the structure and dynamics of the Z-ring that were due to the changes in the induction level.

To image the CM we used the FM4-64 fluorescent stain (Molecular Probes) [35] at 1 µM concentration. Moreover, we imaged the cell cytoplasm using E. coli strain BL21(DE3) transformed with plasmid pEGFP (pBR322 origin), encoding EGFP protein under the lac promoter. This strain was also grown in LB until reaching an OD_{600} of 0.3 at 37 °C and was induced for 1 h with IPTG. Finally, to measure the radial width of the Z-ring, we used doubly labeled cells that expressed FtsZ-GFP and were labeled with FM4-64 in the CM.

2.3. Image analysis

The E. coli cell shape is less involved before the onset of constriction than afterwards. We therefore study cells that are between \( \tau_z \), the time when Z-ring assembly has been completed, and \( \tau_c \), when the cell starts constricting. To select cells belonging to this period of the cell cycle, each trapped cell was imaged in both the horizontal and vertical orientations. In each orientation, the cell was imaged in three different modes: (i) phase-contrast, (ii) fluorescence using the filter set for the CM stain, FM4-64 and (iii) fluorescence with the filter set for GFP. While the horizontal GFP image reveals the existence of additional FtsZ structures, e.g., helices [22], the corresponding FM4-64 image is an indicator of the beginning of the constriction [36]. In this study, only cells that showed no constriction and a clear Z-ring without additional structures were included. Moreover, to exclude the possibility of a recently initiated septum too small to resolve [36], we included in our analysis only cells that were significantly shorter than the average cell length at the onset of division.

In addition to the information provided by the horizontal images about the stage of the cell cycle and the distribution of the FtsZ-GFP on the CM, vertical images allow determining whether the formation of the Z-ring has ended, \( t > \tau_z \). For the cell shown in figure 2, the vertical Z-ring image displays an almost perfect circular symmetry indicating that its Z-ring is complete. However, we also observe cells for which the Z-ring shows a relatively normal appearance in the horizontal orientation, but are clearly incomplete when viewed in the vertical orientation. In figure 3 we compare two different Z-rings, each imaged in both the horizontal and vertical orientations. Although their horizontal images, where the ring manifests as two bright spots, suggest that the ring on the right (figure 3(B)) is less symmetric than the ring on the left (figure 3(E)), the source of this asymmetry is not clear. However, the corresponding vertical images reveal that the ring on the right is incomplete (figure 3(D)). A partial Z-ring is an indication that the cell has not reached \( \tau_z \) and thus should be excluded from our analysis.
Figure 3. Two different Z-rings imaged in the GFP fluorescence mode, in both the vertical (panels (A) and (D)) and the horizontal (panels (B) and (E)) orientations, together with the corresponding plots of the maximal intensity contours corresponding to the images in panels (A) and (D) (panels (C) and (F), respectively). All images are on the same scale. Bar = 0.1 \( \mu \)m. Left: an almost perfectly symmetric Z-ring (the same as in figure 2). Right: an incomplete Z-ring.

To distinguish the partial rings from the complete rings we use the maximal intensity contour of the vertical Z-ring image (figures 2(F), 3(C) and (F)). It corresponds to the maxima of radial intensity profiles as described below. Incomplete rings display a much larger variability in the radial position of the maximal intensity contour relative to that of complete rings. We classify a ring as incomplete whenever at least one of the points of its maximal intensity contour is closer to the cell center than to the average radius of the contour.

The vertical image of the Z-ring also allows measuring its width in the radial direction (inwards from the CM). The first step in our analysis consists of finding the centers of symmetry for the images of both the CM (figure 2(B), right panel) and the Z-ring (figure 2(C), right panel). To this end, the three parameters that define a circle are fitted to maximize the fluorescence intensity along the circumference of the circle. To test the performance of this method, we compared its results with those of another algorithm that searches for two perpendicular axes of maximal symmetry with respect to reflection [37]. We found that the two methods agree with each other within the experimental error.

In the second step of the analysis, the intensity levels were computed along 360 radial rays emerging from the center of symmetry at angles that increase by 1° from one ray to the next. Along each ray, we sampled the fluorescence intensity with a 20 nm step size. To reduce
the error in the measured intensities due to the finite pixel size, we used linear interpolation between the fluorescence intensities of the three nearest pixels. In what follows, the intensity values along each ray will be referred to as radial profiles. The position of the maximum for each of these profiles was used to obtain the maximal intensity contours for the corresponding image (figures 2(F), 3(C) and (F)). Moreover, we average the radial profiles for each of the circularly symmetric images resulting in a smooth function, the average radial profile, \( I(r) \). The \( I(r) \) profile describes the fluorescence intensity variation along the radial direction. In figure 4(A), we show the \( I(r) \) profiles of both the CM and the Z-ring vertical images for the same cell as in figures 2 and 3(A)–(C). As can be noted in figure 2(D), right panel, and figure 2(F), here as well, the maximum of the CM average radial profile is further from the cell axis than the corresponding maximum of the Z-ring. The difference between the radii of the two maxima for this cell, \( \Delta r \), is 44 \pm 11 nm. As we will show in what follows, the value of \( \Delta r \) is closely related to the radial width of the Z-ring.

2.4. Image modeling

To analyze the geometry of the cellular structures that correspond to the observed images, we model the image of the different possible object geometries and compare the model images with those obtained from experiment. To this end, we use the theoretical 3D point spread function (3D PSF) appropriately adjusted for our large NA objective lens [38]. The amplitude PSF is given by

\[
U(r, z) = \frac{2\pi i}{\lambda} \int_0^{\alpha} \sqrt{\cos \theta} J_0(kr \sin \theta \exp(-ikz \cos \theta)) \sin \theta d\theta,
\]

where \( n \sin(\alpha) \) is the numerical aperture of the objective lens, \( k \) and \( \lambda \) are the wave number and wavelength of the fluorescent source, respectively, and \( J_0 \) is the zero-order Bessel function.

To test the theoretical PSF we compared it to images of small fluorescent beads, \( \sim \)100 nm in diameter. In the focal plane, we found good agreement between the PSF of (1) and the measured intensity distribution (figure 5). Using the 3D PSF, the image of a 3D distribution of incoherent fluorescent sources, \( O(x,y,z) \), can be obtained by convolution

\[
I(x, y, z) = \iiint |U(x', y', z')|^2 O(x'+x, y'+y, z'+z) \, dx' \, dy' \, dz'.
\]

Since the expression of the 3D PSF in (1) cannot be further simplified, the values of the PSF were obtained by numerical integration. We computed the \( U(r,z) \) function at points that were 10 nm apart in a 3 x 3 x 3 \( \mu \)m volume centered at the origin. Subsequently, the image function, \( I(x,y,z) \), was computed by numerically integrating (2) with the same 10 nm step size.

2.5. Image quality

To reduce the influence of experimental noise in the CM and Z-ring images, we have optimized several of the imaging parameters. Firstly, we need to ensure that trapped cell images are properly focused. This is particularly important for imaging the Z-ring in the vertical cell orientation that is used for quantitative measurements. Since the fluorescence emission light and the trapping laser beam pass through the same objective (figure 1), the distance between the position of the focal plane and that of the optical trap along the optical axis cannot be adjusted by raising or lowering the objective. Instead, focusing is achieved by means of the \( L_1 \) and \( L_2 \)
Figure 4. Average radial profiles. (A) The profiles of the CM (red circles) and the Z-ring (green triangles) for the same cell as in figures 2 and 3(A)–(C), \( r_{CM} = 407 \pm 6 \text{ nm} \) (red arrow), \( r_{GFP} = 363 \pm 9 \text{ nm} \) (green arrow) and \( \Delta r = 44 \pm 11 \text{ nm} \); (B) the same as in panel (A), but here the profiles are obtained from the numerical simulation of the cylindrical model (inset) using (1) and (2). Correspondingly, \( r_{CM} = 410 \text{ nm} \), \( r_{GFP} = 398 \text{ nm} \) and \( \Delta r = 12 \text{ nm} \). (C) The same as in panel (B) but for the toroidal model (inset). Here, \( r_{CM} = 410 \text{ nm} \), \( r_{GFP} = 362 \text{ nm} \) and \( \Delta r = 48 \text{ nm} \). (D) Comparison of the toroidal model of panel (C) (crosses) with the FtsZ-GFP \( I(r) \) for a Z-ring in the shape of a flat disc at
Figure 4. (Continued) Mid-cell with the same radial width as that of the torus (circles). An enlarged view of the range around the maximum is shown in the inset. (E) The cylindrical model for different lengths of the cylindrical Z-ring, \( \Delta z \): \( \Delta z = 50 \text{nm} \) (circles), \( \Delta z = 100 \text{nm} \) (crosses, the same as in panel B), \( \Delta z = 200 \text{nm} \) (triangles) and \( \Delta z = 300 \text{nm} \) (squares). Inset like in panel (D). (F) The toroidal model for different values of \( r_1 \): \( r_1 = 35 \text{nm} \) (circles), \( r_1 = 45 \text{nm} \) (crosses, the same as in panel (C)) and \( r_1 = 55 \text{nm} \) (triangles). Inset like in panel (D).

Figure 5. The squared amplitude of the theoretical PSF of (1) at \( z = 0 \) (squares) is compared to the intensity distribution in the images of small fluorescent beads (100 nm diameter, circles). The beads were imaged using the GFP filter set and the theoretical PSF was computed at the corresponding wavelength.

telescope lenses. Moving the X-stage on which \( L_1 \) is placed along the optical axis modifies the height of the optical trap without affecting the position of the focal plane. We found that there is no detectable variation in the focus of the vertically imaged Z-ring for different cells. Moreover, since the galvanometric mirror is imaged by the telescope on the back aperture of the objective (figure 1), small shifts of the trap in the \( xy \) specimen plane are decoupled from small \( z \) shifts (see [30] for a detailed discussion of this issue). This allows using the same procedure as for vertically oriented cells to also focus them when horizontally oriented.

Secondly, the fluorescence intensity significantly varies between the cells that were imaged. It depends on the level of expression of FtsZ-GFP, on the quantity of the FM4-64 stain present in the CM and on the extent of bleaching of the fluorophores. To test the effect of the fluorescence intensity on the behavior of the average radial profiles we have gradually reduced the intensity of the signal from individual cells by repeated exposure. Since our measurement only involves the maxima of the radial profiles, we have tracked the variation in their position for a series of ten sequential 0.5 s exposures of individual cells. We found that the stability of the radial profile maximum as the cell is progressively bleached depends on the contrast between the intensity at the maximum, \( I_{\text{max}} \), and that at the cell center, \( I_0 \). Accordingly, we determined the contrast level, \( C \), where

\[
C = (I_{\text{max}} - I_0)/I_0, \quad (3)
\]
and found that, as long as $C > 0.1$, the maximum of the average intensity profile is independent of the intensity level apart from random fluctuations. We therefore only analyzed cells for which the average radial profiles of both the CM and the Z-ring displayed contrast levels, $C$, larger than the 0.1 threshold.

3. Results

As discussed in section 2, we selected cells that (i) had already formed their Z-rings, $t > \tau_z$, (ii) had not yet started to constrict, $t < \tau_c$, (iii) had no FtsZ structures on the CM aside from the Z-ring itself and (iv) displayed contrast levels, $C$, in the vertical orientation above 0.1 for both the CM and Z-ring images. Out of more than 30 cells that were analyzed, only seven cells satisfied all these requirements. We only found cells that satisfied the contrast requirement in HIL experiments. However, the results that were obtained in the LIL experiments where the contrast was relatively good were similar to those of the HIL experiments only with larger errors. To obtain the position of the peak in the average radial profiles, these were computed in the range $0 \text{ nm} < r < 750 \text{ nm}$ with 10 nm step size. Subsequently, we refined the positioning accuracy using third order interpolation in the vicinity of the maximal value of the sampled $I(r)$. Testing the precision of this procedure by computing $I(r)$ with a step size of only 1 nm, we found that it renders the position of the maximal average radial profile with an error of less than 2 nm. In what follows, $r_{FM}$ and $r_{GFP}$ denote the radial positions of the FM4-64 and the GFP $I(r)$ maxima, respectively, and $\Delta r \equiv r_{FM} - r_{GFP}$. The value of $\Delta r$ for cells that satisfied all the four imaging criteria was about 50 nm. Although the range of values of $r_{FM}$ for these cells was relatively large, between 407 and 530 nm, the corresponding $\Delta r$ displayed low variability, $\Delta r = 44 \pm 11, 63 \pm 11, 49 \pm 11, 50 \pm 11, 49 \pm 11, 78 \pm 11$ and 46 $\pm 11$ nm. For these cells, the average $\Delta r$, $\langle \Delta r \rangle$, is $54 \pm 6$ nm. On this scale, the widths of the CM, $\sim 6$ nm, and that of the FtsA and ZipA layer that links the CM and the Z-ring, a few nanometers, are practically negligible. This suggests that $2\Delta r$ represents a good approximation to the radial width of the Z-ring, $D$. In what follows, we will present further support for this relation.

We have identified three dominant sources of error in our experiments. Specifically, errors are due to fluctuations of cells in the optical trap, imperfect focusing, and the fluctuations of the non-averaged radial profiles, $I(r, \theta)$, as a function of $\theta$. The errors due to the Brownian motion of trapped cells were calibrated by changing the trapping power. For laser beams varying between 18 and 61 mW at the exit from the objective (experiments were performed at 37 mW) we found that $\Delta r$ fluctuates with a standard deviation of 7 nm. Errors due to focusing were calibrated using cells fully embedded in agar that were found to be vertically oriented. For such cells, we imaged the Z-ring while focusing at different heights above or below its focal plane within a few $\mu$m range. The corresponding values of $\Delta r$ are distributed with a standard deviation of about 5 nm. Finally, the variability of the $I(r, \theta)$ profiles contributes another 7 nm to the error in $\Delta r$.

To verify the possibility of experimental artifact in determining $\Delta r$, we have addressed several potential problems. Firstly, we have tested the effect of chromatic aberrations. Since the Z-ring and the CM are imaged using different fluorophores that emit at different wavelengths, we need to exclude the possibility that this affects the value of the measured $\Delta r$. Specifically, the emission spectrum of GFP is centered at 507 nm and that of CM bound FM4-64 at 615 nm. To this end, we have used pairs of fluorescein coated micro-beads that are attached to the glass bottom of our sample imaging them with both the GFP and the FM4-64 filter sets.
Measuring the distances between a pair of beads in each of the two images, we found that these are equal within the error of our positioning algorithm (<10 nm). Secondly, we have considered the possibility that the trapping laser may damage the E. coli cell and influence the outcome of our measurements. Photodamage in E. coli due to optical traps was carefully quantified by Ayano et al [39]. They showed that whenever the total energy delivered to the cell over its lifetime is below 0.36 J, normal cell growth and division are not affected. In our experiment, the laser power at the exit from the objective is 37 mW, allowing a time window of about 10 s during which the effect of photodamage is negligible. Therefore, for each cell, we first capture the two vertical fluorescence images that are required for the quantitative measurement, namely, establishing the radial width of the Z-ring. Since this part of the imaging sequence is performed within the 10 s time window, we expect that the damage due to optical trapping has a negligible effect on the measured values of D. Although the duration of a complete imaging sequence (figure 2) extends to about 60 s, we observed no changes in the cell morphology or in the structure of the Z-ring during this time. Thirdly, it may be objected that the FM4-64 CM stain could quench GFP via Förster resonance energy transfer (FRET). Since the range of FRET is only \( \sim 5 \) nm, its effect on the maximum of the FtsZ-GFP \( I(r) \) should be smaller than the experimental error. To test this estimate, we have measured the position of the FtsZ-GFP \( I(r) \) peak both in cells that were stained with FM4-64 and in cells that were not. We found that the difference between the corresponding averages (over seven cells) of the FtsZ-GFP \( I(r) \) peak positions is less than 1 nm, suggesting that in our study FRET between FM4-64 and GFP can be neglected.

We now proceed to justify the interpretation of \( \Delta r \) as being equal to half the width of the Z-ring, D. This relies on the premise that the FM4-64 \( I(r) \) is maximal at the position of the CM itself that is negligibly thick (\( \sim 6 \) nm), while the maximum of the FtsZ-GFP \( I(r) \) is located at a value of \( r \) that corresponds to the radial center of the wide Z-ring. The latter is particularly doubtful considering that 70% of the total FtsZ in the cell is homogeneously dispersed throughout the cytoplasm. It is possible that this fraction of FtsZ-GFP contributes to the corresponding \( I(r) \) a component that is largest at the cell center and, therefore, is biasing the position of the presumed radial center of the Z-ring towards smaller \( r \) values. To illustrate the behavior of the \( I(r) \) for a uniformly stained cytoplasm, we have imaged cells that express EGFP homogeneously throughout the cytoplasm (figure 6(A)). As expected, the corresponding average radial profile monotonically decays from the cell center outwards (figure 6(B)). Accordingly, a more careful analysis of the relation between the geometry of the objects and the corresponding images is required in order to support our proposed relation between \( \Delta r \) and D. For this purpose, we have used the approach described in section 2.4 to simulate the images of the different possible geometries for the Z-ring and have compared the outcome with the experimental images.

First, we have analyzed a model that has a similar Z-ring structure to the one proposed by Li et al [26]. In this model, a 100 nm wide ribbon attached to the CM and placed at mid-cell corresponds to the Z-ring. Moreover, 70% of the total amount of FtsZ in the cell is homogeneously distributed in the cytoplasm. The cell length, L, is 3 \( \mu \)m and its radius, R, is 430 nm. The latter is such that the \( I(r) \) of the model CM is maximal at \( r_{FM} = 410 \) nm, almost the same radial position as for the FM4-64 \( I(r) \) of the cell shown in figures 2, 3(A)–(C) and 4(A). In what follows, we refer to this cell model as the cylindrical model. The spatial distribution of FtsZ and that of the CM were numerically convoluted with the appropriate PSFs to obtain the corresponding images. We used a PSF computed at the wavelength where the GFP emission
is maximal for the FtsZ distribution and an analogous FM4-64 PSF for the CM distribution. Extracting the average integral profiles from model images and locating their respective maxima we obtained \( r_{\text{GFP}} = 398 \text{ nm} \) and \( r_{\text{FM}} = 410 \text{ nm} \), corresponding to \( \Delta r = 12 \text{ nm} \), much smaller than the measured \( \Delta r \) (figure 4(B)). This suggests that our measurements are not compatible with the cylindrical model and the findings of Li et al [26]. Accordingly, we propose an alternative model, namely, the toroidal model where the Z-ring is represented as a torus that is about 100 nm wide.

The cylindrical and toroidal models are equivalent with respect to the cytoplasmic FtsZ and the cell dimensions, but the Z-ring of the latter is shaped like a torus (figure 4(C)). We assume that the torus is located at mid-cell, it extends inwards from the CM and is homogeneously filled with FtsZ. While its minor radius, \( r_1 \), is 45 nm, the major radius, \( r_2 \), is 385 nm. A similar model was used by Fu et al [27] to describe their results. Using the toroidal model, we obtained \( r_{\text{GFP}} = 362 \text{ nm} \) and \( \Delta r = 48 \text{ nm} \), in good agreement with our experimental result for the cell of figures 2, 3(A)–(C) and 4(A). We propose that the agreement between the predictions of the toroidal model and experiment represents strong evidence that the proposed relation between the distance between the maxima of the CM and FtsZ \( I(r) \) and the radial width of the Z-ring, \( D \approx 2\Delta r \), holds within the range of our experimental error. Notably, the 23 nm shift in the value of \( r_{\text{GFP}} \) from the position of the center of the torus due to the FtsZ distribution in the cytoplasm is almost balanced by a similar shift, 20 nm, in \( r_{\text{FM}} \) from the radial position of the CM, \( R = 430 \text{ nm} \). It is natural to expect that the latter may be due to the effect of the cell caps.

To test the conclusions that we drew from the comparison between the images of the cell models and those obtained from experiment, we have further analyzed the behavior of the theoretical FtsZ average radial profiles as a function of the model parameters. On the one hand, we find that for both the cylindrical and the toroidal Z-ring models the FtsZ \( I(r) \) and the corresponding \( r_{\text{GFP}} \) are almost independent of the extent of the Z-ring in the direction of the long cell axis (longitudinal direction, figures 4(D) and (E)). On the other hand, the same FtsZ \( I(r) \) and its \( r_{\text{GFP}} \) significantly vary when the radial extent of the Z-ring is modified (figure 4(F)). Specifically, in figure 4(D) we compare the FtsZ \( I(r) \) for the toroidal Z-ring model with that corresponding to a flat disc Z-ring that extends over the same radial range as the torus. We find that the corresponding \( I(r) \) are almost identical and their maxima are located at positions that are less than 1 nm apart. This suggests that the average radial profile is practically independent...
Figure 7. The FtsZ-GFP intensity distribution of the same horizontally oriented cell as in figures 2, 3(A)–(C) and 4(A). The image in panel (C) and the simulated image in panel (D) are on the same scale. Bar = 0.5 µm. (A) The FtsZ-GFP fluorescence intensity distribution after background subtraction. (B) The corresponding distribution as obtained from the toroidal model with \( r_1 = 45 \) nm and \( r_2 = 385 \) nm. (C) The image corresponding to the intensity distribution of panel (A). (D) The simulated image corresponding to the intensity distribution of panel (B).

of the longitudinal distribution of the Z-ring. A similar behavior is also found for the case of the cylindrical model. In figure 4(E), we show the \( I(r) \) of the cylindrical model for four different longitudinal widths of the Z-ring, \( \Delta z \), namely, 50, 100, 200 and 300 nm. As in figure 4(D), the corresponding \( I(r) \) are hardly distinguishable and their maxima are located at 399, 398, 398 and 397 nm, respectively. However, the behavior shown in figure 4(F) for the toroidal model is strikingly different. Here we vary the minor radius of the torus, \( r_1 \), while its major radius is adjusted accordingly such that the outer edge of the torus remains in contact with the CM. We show the behavior of \( I(r) \) for \( r_1 = 35, 45 \) and 55 nm and find that the corresponding maxima are located at 369, 362 and 350 nm, respectively. Since \( r_{\text{GFP}} \) is strongly dependent on the toroidal minor radius, \( r_1 \), it represents the optimal experimental quantity to measure in order to determine the radial width of the Z-ring, \( D \). Moreover, the values of \( \Delta r \) are 38, 45 and 57 nm, respectively, satisfying with good accuracy the relation between the width of the torus and the distance between the peaks of the CM \( I(r) \) and that of the FtsZ distribution, namely, \( D \equiv 2r_1 \approx 2\Delta r \). This shows that this relation holds in a range of \( r_1 \) values for model cells of similar dimensions and is not limited to a particular set of cell parameters.

Although imaging cells in the vertical orientation is most efficient for measuring the width of the Z-ring, this is not the traditional viewpoint used when imaging *E. coli*. It is therefore worthwhile to obtain a more quantitative analysis of the horizontal images of the Z-ring. In figure 7 we show the FtsZ-GFP fluorescence intensity distribution for the same
cell as in figures 2, 3(A)–(C) and 4(A) and compare it with the corresponding distribution as obtained from the toroidal model. Although the two distributions are apparently quite similar, a quantitative comparison is difficult due to the low precision in locating the position of the peaks in the experimental intensity distribution. On the one hand, we find that the distance between the maxima of the experimental distribution, \( W_{\text{exp}} \), is \( \sim 620 \) nm, while that corresponding to the toroidal model, \( W_{\text{tor}} \), is 675 nm. On the other hand, we expect that the error of \( W_{\text{exp}} \) is at least of the order of 60 nm (\( \sim \sqrt{2} \) pixel size). One of the main reasons why the error of \( W_{\text{exp}} \) is significantly larger than that of \( r_{\text{GFP}} \) is the lack of symmetry in the horizontally aligned Z-ring image, precluding the option of averaging. This further highlights the advantage of vertical imaging of the Z-ring and, in general, of aligned imaging.

In principle, one may expect that the error in the measurement of \( W_{\text{exp}} \) could be further reduced by appropriately interpolating the intensity distribution in the vicinity of the maxima. However, we found that this approach is not useful due to the relatively large level of noise in the image. In addition to random noise, we also find some asymmetry between the maxima of the experimental intensity distribution. This indicates that there is more FtsZ-GFP in the upper half of the cell than in the lower one which, in turn, is likely to further contribute to the discrepancy between \( W_{\text{exp}} \) and \( W_{\text{tor}} \).

### 4. Conclusions and discussion

We have shown that we can trap and align rod-shaped bacterial cells using oscillating optical tweezers and obtain preferred imaging viewpoints of particular sub-cellular structures. This approach together with image analysis was used to measure the radial width of the Z-ring in *E. coli*, \( D \). We used unconstricted cells with a mature Z-ring that was visualized via FtsZ-GFP and stained the CM with FM4-64. In a vertically oriented cell, both the Z-ring and the CM images appear as symmetric circular structures that lend themselves to quantitative analysis. We found that \( D \) is about 100 nm. The relatively large width is consistent with the observations of others [27, 29]. Moreover, simulation of the experimental FtsZ distribution using the theoretical 3D PSF was strongly in favor of a toroidal rather than a thin cylindrical model of the Z-ring.

The accuracy of our measurement of the radial width of the Z-ring, \( D \), was about 20 nm, \( \Delta D \approx 20 \) nm. This error is remarkably small relative to the optical resolution, \( \sim 240 \) nm, at the wavelengths corresponding to GFP emission. The sub-resolution accuracy in measuring \( D \) is due to the angular averaging of the radial intensity profiles from the FM4-64 and FtsZ-GFP vertical images (figures 2(B) and (C), right panels). This approach allows to accurately determine the radial position of the maximal intensity values for the respective average radial profiles, \( r_{\text{FM}} \) and \( r_{\text{GFP}} \), and \( D \approx r_{\text{FM}} - r_{\text{GFP}} \). Since the average radial profiles are rather smooth, one would expect that the information on the width of the Z-ring could alternatively be obtained directly from the FtsZ-GFP vertical images. Unfortunately, this alternative approach is significantly less accurate leading to extremely large errors in the measured values of \( D \). The low accuracy of the direct approach is related to the difficulty of measuring the size of an object smaller than optical resolution. For such objects, their position can be determined much more precisely than their size. The simplest example is that of fluorescent beads with diameters smaller than optical resolution. While these can be easily tracked with several nm precision, it is practically impossible to obtain a similar accuracy for their diameter by standard optical imaging.

The significantly reduced error for our two-color measurement of \( D \) compared to the error from the direct approach further underlines the strength of our method. It is therefore
worthwhile comparing the two-color approach to the procedure and results of the direct Z-ring width measurement. The direct procedure consists of two steps. First, we separate the Z-ring contribution to the FtsZ-GFP image from that of the cytoplasm. This is achieved by subtracting the appropriately normalized cytoplasm average profile of figure 6 from the measured FtsZ-GFP \( I(r) \). In the second step, we assume that the Z-ring is sufficiently thin along the cell axis for its contribution to the vertical FtsZ-GFP image to be well approximated as that of a 2D object. Considering the result of figure 4(D), this is a natural assumption. Therefore, we model the Z-ring as a flat disc that extends a distance \( D \) from the CM inwards. Convoluting such objects with the Gaussian approximation for the 2D PSF and searching for the best fit to the ring part of the FtsZ-GFP average radial profile allows establishing the values of \( D \) and \( \Delta D \). A typical result that was obtained using this approach on a cell with no staining of the CM is \( D = 171 \pm 94 \) nm. As expected, \( \Delta D \) is \( \sim 55\% \) of the value of \( D \) and almost five times larger than the error from the two-color approach. In addition, the value of \( D \) itself is significantly larger than those obtained from the two-color approach. This reveals a second, more technical, weakness of the direct method of measuring \( D \). While the position of the maximum for the average intensity profiles is only weakly affected by focusing errors and small fluctuations, these factors significantly contribute to the width of the \( I(r) \), leading to a systematic overestimate of \( D \) (compare figures 4(A) and (C)).

Since the amount of FtsZ in the Z-ring is limited, our findings suggest that the Z-ring consists of a sparse, multilayered network of FtsZ filaments. Such a network leaves ample empty volume for the future constriction. As the Z-ring shrinks, the FtsZ filaments slide into the empty space condensing the network. It is natural to expect that the radial width of the Z-ring will decrease as the constriction proceeds in order to prevent it from blocking passage between the two cell halves. The future challenge of our technique is to monitor the width of the Z-ring throughout the septation process. To this end, we will have to replace FM4-64 staining with, for example, FtsA-mCherry. The latter will allow tracking the dynamics of the constricting edge of the CM. Moreover, a similar approach may allow locating the positions of the different divisome proteins on the Z-ring and their dynamics during cell division.

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