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Exploring Protein Superstructures and Dynamics in Live Bacterial Cells Using Single-Molecule and Superresolution Imaging

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Abstract

Single-molecule imaging enables biophysical measurements devoid of ensemble averaging, gives enhanced spatial resolution beyond the optical diffraction limit, and enables superresolution reconstruction of structures beyond the diffraction limit. This work summarizes how single-molecule and super-resolution imaging can be applied to the study of protein dynamics and superstructures in live *Caulobacter crescentus* cells to illustrate the power of these methods in bacterial imaging. Based on these techniques, the diffusion coefficient and dynamics of the histidine protein kinase PleC, the localization behavior of the polar protein PopZ, and the treadmilling behavior and protein superstructure of the structural protein MreB are investigated with sub-40-nm spatial resolution, all in live cells.

Keywords

Single-molecule imaging; Live-cell imaging; Live-cell PALM; Superresolution imaging; Superlocalization; *Caulobacter crescentus*

1. Introduction

Since its advent 20 years ago, single-molecule fluorescence imaging has given rise to a host of exciting experiments (1). Beyond enabling fundamental investigations of the physics of emissive molecules, one main advantage of this technique is its utility in biologically relevant, live-cell experiments. The optical fluorescence microscope is an important instrument for cell biology, as light can be used to noninvasively probe a sample with relatively small perturbation of the specimen, enabling dynamical observation of the motions of internal structures in living cells. Single-molecule epifluorescence microscopy extends these capabilities by achieving nanometer-scale resolution, taking advantage of the fact that one can precisely characterize the point spread function (PSF) of a microscope, allowing the center of a distribution, and thus the exact position of an emitter, to be localized with accuracy much better than the diffraction limit itself. This localization accuracy improves beyond the diffraction limit roughly as one over the square root of the number of detected photons (2).

Single-molecule imaging has shown utility in the investigation of live-cell images from a number of different cellular systems (3–9). More recently, single-molecule epifluorescence microscopy has been used to probe the inner workings of live bacteria. The small size of

Notes

Axial drift of the sample, as caused, for instance, by microscope objective settling or by sample drying, is particularly detrimental to superresolution experiments. These notes outline several strategies used by the experimenters to address this concern.

bacterial cells makes the optical diffraction limit particularly restrictive, which has stimulated the push toward superlocalization and superresolution to overcome this obstacle. As a result, the nascent field of bacterial structural biology has benefited greatly from single-molecule investigations of proteins in live cells. The overall shapes of such cells can be seen in a standard light microscope, but those interested in probing subcellular details, such as protein structure and localization, have typically had to resort to in vitro characterization combined with extrapolation to the cellular environment, as well as to indirect biochemical assays. While cryo-electron microscopy (EM) can provide extremely high spatial resolution, fixation or plunge freezing is essential, and in contrast to labeling by fluorescent protein (FP) fusions, methods for identifying specific proteins by cryo-EM are still lacking. As a consequence, bacterial cell biology is an area of study ripe for investigation with direct, noninvasive optical methods of probing position, coupling, and structure, with resolution below the standard diffraction limit.

Several groups have extended single-molecule imaging techniques to live bacterial samples (10–15). In this chapter, we focus on the application of single-molecule imaging and single-molecule-based superresolution studies of live cells to investigate the localization, movement, and structure of three proteins, PleC, PopZ, and MreB, that play important functions in *Caulobacter crescentus* cell cycle progression (10, 11, 14, 15). The *C. crescentus* cell cycle is characterized by dynamic changes in polar morphology and asymmetric cell division, and thus is a key model organism for study of bacterial developmental processes. Each round of dimorphic *C. crescentus* cell division gives rise to two distinct daughter cells: a motile, nonreplicating swarmer cell with a polar flagellum and a replicating stalked cell with an adhesive stalk (Fig. 1). This process of asymmetric division is partly driven by patterns of protein localization, which explains why study of specific proteins in this system is of great interest.

2. Materials

2.1. Growth Media

1. PYE: 2 g bactopectone, 1 g yeast extract, 0.3 g MgSO₄, and 0.0735 g CaCl₂ per L PYE. The mixture is autoclaved for 20 min and vented for 10 min to sterilize.
2. PYE solid media: Add 1.5% Bacto Agar (15 g/L); no CaCl₂ needed.
3. M2G minimal media: 930 mL water, 50 mL 20× M2 salts (17.4 g Na₂HPO₄, 10.6 g KH₂PO₄, and 5.0 g NH₄Cl per L), 1 mL 0.5 M MgSO₄, 10 mL 20% glucose, 1 mL 10 mM FeSO₄ (Sigma #F-0518), and 5 mL 0.1 M CaCl₂.

3. Methods

3.1. Sample Preparation

Single-molecule fluorescence imaging of proteins in living cells can be accomplished via a genetically encoded fusion of the protein of interest to a photostable fluorescent protein emitting at long enough wavelengths to avoid cellular autofluorescence. However, single-molecule imaging requires a low copy number of fluorophores in the cell at any one time so that the ~250-nm diameter image spots from single molecules do not overlap. This low concentration can be achieved in several ways:

1. The gene encoding the protein–FP fusion is integrated into the chromosome in lieu of the gene encoding the native protein. In such strains, the initial FP concentration is generally too high for single-molecule imaging, so the emitting FP population must be reduced via photobleaching before measurements are made (10).

2. The gene encoding the protein–FP fusion is integrated into the chromosome under the control of an inducible promoter while maintaining the gene encoding the untagged wild-type protein at its native chromosomal locus. This has the advantage of allowing the experimenter to control the level of tagged protein while maintaining a significant background of the native protein in order to minimize any physiological effects of the tagging (11, 14).
3. A large number of protein–FP fusions are expressed in the cell, and the number of emitting FPs in any imaging frame can be subject to optical control, for instance by using a photoactivatable FP or by bleaching all of the FPs and then photoreactivating a sparse subpopulation (15).

In order to resolve the emission from single FPs above background, cell and sample autofluorescence must be minimized. In *C. crescentus*, this is accomplished by the following:

1. After initial growth in rich PYE medium, cells are grown in minimal M2G medium for >24 h.
2. Blue and green FP mutants are avoided in favor of yellow and red FPs. At these longer wavelengths, cell autofluorescence is greatly diminished. The experiments discussed below all use the enhanced yellow fluorescent protein EYFP (S65G, V68L, S72A, T203Y) which can be excited at 514 nm.

The high resolution provided by single-molecule imaging also requires the cells to lie still on a substrate. This is accomplished by preparing an agarose pad onto which the cells are deposited for imaging.

1. Agarose is added to M2G to a final concentration of 1.5–2% by mass.
2. The agarose/M2G mixture is heated in the microwave for 1–2 min until boiling, shaking every 30 s.
3. 1 mL warm agarose solution is pipetted onto a clean slide and then covered by a clean coverslip to ensure a flat surface.
4. Just prior to imaging, the coverslip is removed, 5 μ L cells are deposited on the agarose pad, and the cells are then covered by a clean coverslip.
5. The sample is sealed with wax (melted on a hot plate) to prevent drying (see Note 1).

The sample of cells on agarose must also contain fiduciary markers that can quantify stage drift over the imaging time. Fiducials can be any bright, nonbleaching emitters, for example quantum dots (11) or fluorescent beads (15); these are added to the cells in nM concentration (see Note 2).

3.2. Imaging

Single-molecule imaging of bacterial cells is accomplished in a standard wide-field epifluorescence microscopy configuration, and the general imaging considerations have been described in detail (16). Confocal or total internal reflection methods are often not necessary because the entire cell itself is mostly within the depth of focus of a high numerical aperture (NA) microscope (~500–1,000 nm). Because the photon emission rate

¹Sealing the cells-on-agarose-pad with wax prevents excessive drying of the agarose pad over time. This is important in order to maintain constant cell sample thickness.

²Despite sealing in wax, samples settle over the course of the experiment. It is, therefore, important to ensure that the coverslip is in close contact with the microscope stage and not hanging through an opening in the microscope stage.

from a single molecule is typically ten orders of magnitude smaller than the number of scattered photons per second irradiating one pixel of the detector, appropriate filtering is necessary to reject scattered laser light. To optimize the number of detected photons, the single-molecule fluorescence microscope should incorporate a high-NA oil-immersion objective, sharp and carefully chosen filters, and a sensitive detector capable of detecting single photons. In modern experiments, this detector is generally an electron-multiplying charge-coupled detector (EMCCD) (11, 14, 15), though single-molecule imaging has also been accomplished in live cells with an intensified CCD (10).

For imaging of EYFP–protein fusions (see Note 3),

1. The sample is excited with a 514-nm Ar⁺ ion or solid-state laser with similar wavelength.
2. The excitation beam is converted from linear to circular polarization with a $\lambda/4$ wave plate in order to avoid the preferential detection of certain chromophore orientations (which often cannot be directly correlated with the orientation of the protein under investigation due to the FP fusion).
3. In the detection pathway, a 525-nm longpass dichroic filter and a 530-nm longpass filter are used to reject scattered laser light with an optical density of OD 8–10. Bandpass filters may also be used when necessary to reject other sources of background.
4. In experiments where photoswitching is useful, photoreactivation of EYFP to control the emitting fluorophore density is performed with a 407-nm laser. This light is also circularly polarized and is coupled into the excitation pathway with a dichroic mirror. Superresolution imaging based on single-molecule fluorescence and photoreactivation is accomplished by capturing different sparse subsets of emitters in each imaging frame. Upon photobleaching of a sparse subset, a new subset is generated by photoreactivation and the imaging cycle continues. The final image is generated based on the sum of localizations from many single-molecule imaging frames (15, 17–20).

EMCCD cameras can operate at rates as fast as ~50 frames/s for a fairly large field and even faster for subimages. The integration time is chosen based on the experimental system.

1. A longer integration time (100 ms) increases the signal-to-noise ratio when detecting quasi-stationary or slow-moving FPs. These long integration times are applied to the slowly diffusing mobile protein PleC (10) and to MreB monomers incorporated into filaments (11, 15). The long-integration-time imaging cannot easily observe fast-moving emitters.
2. On the other hand, a shorter integration time is employed to resolve freely or quickly diffusing FPs. Typical fast integration times used in *C. crescentus* are 15.4–32.2 ms for the quickly diffusing unpolymerized proteins, PopZ and MreB (11, 14).

3.3. Data Processing

Single molecules of the protein–FP fusion are identified by several criteria, including:

1. Digital (one-frame) photobleaching
2. Appropriate number of detected photons

³The microscope objective settles over time, changing the instrument focus and blurring the acquisition. We have, therefore, found it necessary to let the microscope settle after initial focusing, and then use a PIFOC P721 piezo scanner/objective positioner for any needed further focus adjustments.

3. A diffraction-limited emission PSF

The position of a nanometer-sized isolated emitter is at the center of the PSF; this center can be identified by eye as the brightest pixel in the emission spot (10, 14) or the molecule can be more accurately localized by fitting the PSF to a 2D, symmetric Gaussian function (11, 15). High-density superresolution images based on multiple cycles of single-molecule imaging frames require the fitting software to be automated (15), and it is essential to carefully analyze the data in terms of numbers of photons detected and localization precision in order to represent the position determinations in an unbiased manner (20).

Absolute protein positions and direction of movement are determined relative to fiducials and to bright- or dark-field images of the *C. crescentus* cells. The midpoint and endpoints of each cell are observable, the stalked end is recognized by its stalk, and the cell cycle stage can be identified from the cell morphology and the presence or absence of a stalk. It would also be possible to use DIC images, but the changes in microscope optics compared to those for single-molecule fluorescence imaging would have to be carefully calibrated. The techniques described in this chapter measure images in only two dimensions, but 2D measurements of diffusion on the surface of the bacterial cell membrane can be corrected appropriately by simulating 3D diffusional movement along an appropriately curved membrane (10, 11).

3.4. Dynamic Localization of the PleC Histidine Protein Kinase

A short region of the PleC histidine kinase, near its N-terminus, is inserted in the inner (cytoplasmic) membrane while the major portion of the protein resides in the cytoplasm. In *C. crescentus*, the dynamic localization of PleC regulates polar organelle biogenesis, motility, and asymmetric cell division by localizing to the flagellar pole at specific points of the cell cycle (Fig. 1). The swarmer cell must differentiate into a stalked cell, shedding its flagellum and building a stalk, in order to replicate and divide, and during this differentiation process, PleC becomes delocalized. As the stalked cell progresses into a predivisional cell, a new flagellum is formed opposite the stalk and PleC is localized to the new flagellar pole (21). Three different mechanisms have been postulated for the crucial process of PleC localization: free diffusion of PleC through the inner membrane followed by capture and immobilization at a binding site (22, 23), directed insertion of PleC into the inner membrane at the flagellar pole at the time of translation (24, 25), and active transport of PleC to the pole by a motor protein. Deich et al. used single-molecule microscopy to distinguish among these putative mechanisms by measuring the diffusion coefficients and movement directionality of PleC molecules in *C. crescentus* cells as a function of cell type and position within the cell (10).

1. Strain EJ148 of *C. crescentus* was created with PleC-EYFP fusions replacing the wild-type PleC on the chromosome and expressed under control of the PleC promoter.
2. The EYFP tags are exposed to 514-nm illumination until all but a sparse subset remained in the emissive state.
3. These isolated single-molecule emitters are imaged and their positions recorded as a function of time in order to measure the movement of PleC molecules in the cells.

Figure 2 shows representative fluorescence images of this system. The first image in each of the series in Fig. 2a–c is a dark-field image illustrating the position, size, and orientation of the cell under investigation. The other images in each series represent successive 100-ms imaging frames of the same cells in which each punctate spot is the fluorescence image of a single PleC-EYFP molecule. Figure 2a shows images acquired uninterruptedly (every 100 ms). Due to the relatively slow dynamics of the membrane-bound PleC, such continuous

imaging bleached the molecules before significant motion could be observed. Indeed, the lower molecule in Fig. 2a is bleached during the last frame, after only 600 ms of imaging. Time-lapse (TL) imaging was, therefore, utilized to increase the information attained before photobleaching. Figure 2b, c shows two different cells in which a 100-ms imaging frame is acquired every 1 s. In this way, more movement is observed before photobleaching.

The single-molecule investigation of PleC in *C. crescentus* demonstrated several key aspects of PleC dynamics. Bulk microscopy indicates the presence of a bright, fixed PleC locus at the flagellar pole of swarmer and predivisional cells, but by resolving PleC-EYFP on a single-molecule level, a population of mobile PleC molecules could be identified in these cells. Mobile PleC molecules were also seen in the stalked cells, which show no evidence of localization in bulk microscopy. The trajectories of 400 such mobile molecules were further investigated by tracking the PleC-EYFP molecules, and the measured mean square displacement (MSD) of the molecules at different time lags is shown in the triangles in Fig. 2d. Because the observed motion is a 2D projection of 3D motion along a membrane surface, the actual distance moved by the molecule is larger than the observed movement. By simulating diffusion on a cell surface, modeled as a cylinder with spherical ends, the measured MSD is converted to a geometry-corrected MSD (circles in Fig. 2d). The geometry-corrected MSD is linear with the time lag, Δt , consistent with Brownian diffusion with a diffusion coefficient, $D = \text{MSD}/(4\Delta t)$, of $12 \pm 2 \mu\text{m}^2/\text{s}$.

A normalized coordinate system was created in which the variable s ($0 \leq s \leq 1$) indicates the position of a molecule along the long axis of a *C. crescentus* cell. The position $s = 0$ represents the flagellar pole, and $s = 1$ the stalked pole. The geometry-corrected coefficients D for the mobile PleC-EYFP molecules were classified based on the cell type in which the molecules were imaged (stalked, swarmer, or predivisional) and their position in the cell along the s axis. No measurable difference in D was observed for the different cell types. The geometry-corrected coefficient D was also not found to vary significantly as a function of s , except for a small reduction in D at the cell flagellar pole of swarmer and predivisional cells, perhaps attributable to immobilization at the binding site during the course of observation. Finally, Fig. 2e shows the direction of PleC-EYFP motion along the s axis, where $-s$ indicates motion toward the flagellar pole, as a function of position and cell type. Here, stalked cells, which do not have localized PleC, are in black, and swarmer and predivisional cells, which have localized PleC in addition to the mobile PleC under consideration, are in gray, and each cell is divided into ten equal bins, so length bin 2 includes data for molecules starting with $0.1 < s < 0.2$. All molecules were equally likely to move in the $+s$ or $-s$ direction, regardless of cell type or PleC position.

The motion of PleC in the membrane appears to be governed by Brownian diffusion and is the same at all times in the cell cycle and all positions in the cell. In addition, the absence of directed motion argues against the existence of an active transport mechanism. Overall, the results of single-molecule investigations of PleC in *C. crescentus* were consistent with the diffusion and capture model of PleC localization at the flagellar pole.

3.5. Diffusion Dynamics of the Polar Protein PopZ

The ability of single-molecule microscopy to track individual intracellular proteins was subsequently applied to the proline-rich protein PopZ in live *C. crescentus* cells. PopZ is required to anchor the *C. crescentus* chromosome origins to the cell poles via the DNA-binding protein ParB, an anchoring that is critical for the control of its temporal and spatial localization in the cell cycle. PopZ is positioned at the flagellar end of nonreplicating swarmer cells (Fig. 1). Upon differentiation into a stalked cell, a second concentration of PopZ begins to accumulate at the opposite pole (distal from the stalk) with an intensity that increases as the cell cycle progresses. This second polar accumulation of PopZ is, thus, in

place to tether the newly replicated chromosomal origin when it completes its transit across the cell. The mechanism of PopZ localization was investigated on a single-molecule level (14).

1. The merodiploid strain GB175 of *C. crescentus* was created with an unperturbed *popZ* locus and *popZ-eyfp* under control of the vanillate promoter at the *vanA* chromosomal locus.
2. Leakage of the vanillate promoter ensures the expression of 1–10 copies of PopZ-EYFP per cell in a background of untagged PopZ molecules.
3. These isolated single-molecule emitters are imaged and their positions recorded as a function of time.

Two subpopulations of PopZ were, thereby, identified: pole-localized molecules that remained fixed within the measurement localization accuracy (60 nm) and mobile molecules that were resolved with fast (32.2 ms) imaging frames. Figure 3a, d shows the motion of two molecules tracked in one cell under investigation. In Fig. 3a, the tracks are overlaid on a transmitted white-light image of the cell with the cell outline shown (dotted line) while in Fig. 3d, the longitudinal positions of the same two molecules are recorded as a function of imaging time. In Fig. 3a, d, the gray traces (indicated by the white arrow in Fig. 3a) show the position of a polar-localized molecule, and the black traces show the trajectory of a mobile molecule that explores a large portion of the cell interior. Figure 3b–f shows the trajectories of 12 fixed (gray) and mobile (black) PopZ-EYFP molecules in five cells.

The mobile PopZ-EYFP molecules exhibited random, diffusive movement in the cytoplasm during most of the time they were tracked, though on some occasions, they remained fixed at a pole (e.g., Fig. 3d at 3–4 s). This movement is consistent with a diffusion and capture mechanism for PopZ localization at the cell pole. The spatial dynamics of single PopZ molecules would not have been observable without single-molecule imaging.

3.6. Movement of the Structural Protein MreB

The structural protein MreB mediates polarity, chromosome segregation, and cell shape in *C. crescentus* (26–28). Bulk-level imaging and biochemical studies of the cytoplasmic MreB protein are consistent with the formation of a dynamic superstructure made up of actin-like filaments. This structure provides the machinery for key cellular processes, and the dynamics of MreB molecules were investigated on a single-molecule level (11).

1. The merodiploid strain LS3813 of *C. crescentus* was generated by integrating a single *P_{xyI}::eyfp-mreB* construct into the *xyIX* locus in a *C. crescentus* chromosome already containing a wild-type, unlabeled copy of MreB under its endogenous promoter (26).
2. Cells with only a small number of isolated EYFP-MreB molecules are prepared by incubating the cells in the M2G minimal medium with 0.0006–0.003% xylose for 4 h.
3. The cells are imaged with 15.4-ms imaging frames in order to resolve the tagged MreB molecules.

Figure 4a1 shows one such frame, in which three single molecules were captured. The image is smoothed by applying a low-pass filter (3×3 kernel of 0.0625, 0.125, 0.0625, 0.125, 0.25, 0.125, 0.0625, 0.125, and 0.0625) in Fig. 4a2 for enhanced visibility. In this cell, two subpopulations of MreB were distinguished from one another: slow-moving molecules and fast-moving molecules (arrowheads and arrow, respectively, in Fig. 4a1). The trajectory of the fast-moving molecule in Fig. 4a1, obtained by fitting the fluorescence image in every

frame to a 2D Gaussian function, is plotted in Fig. 4a3. This molecule diffused rapidly and explored a large portion of the cell. Four hundred and fifty successive frames were summed to form the fluorescence image in Fig. 4a4. After this 7-s integration time, the fluorescence from the two slow-moving molecules was still evident, but emission from the fast-moving molecule was smeared out over many pixels and did not appear. The white line in Fig. 4a shows the outline of the *C. crescentus* cell.

Because the dynamics of the moving molecules were unchanged in the presence of the MreB-depolymerizing drug A22, the fast-moving EYFP-MreB molecules were ascribed to a free, unpolymerized population. The behavior of the unpolymerized single molecules was further characterized based on 111 trajectories. The observed MSD of the fast-moving molecules is plotted as a function of time lag (Δt) in the open circles of Fig. 4b. Based on the first four points, a diffusion coefficient of $D = \text{MSD}/(4\Delta t)$ of $1.11 \pm 0.18 \mu\text{m}^2/\text{s}$ was extracted. This value of D is smaller than expected for a free cytoplasmic protein, but is consistent with the motion of a membrane-associated protein. Since the observed motion represents a 2D projection, simulations were again performed to correct for the three-dimensionality of the cell, and the geometry-corrected MSD of the molecules is plotted in the filled circles of Fig. 4b. The geometry-corrected MSD is linear with the time lag, Δt , consistent with diffusion along the cell membrane with $D = \text{MSD}/(4\Delta t)$ of $1.75 \pm 0.17 \mu\text{m}^2/\text{s}$. The velocity autocorrelation function, $C_V(\tau)$, was also calculated for these molecules. As shown in Fig. 4c, for the fast-moving molecules, $C_V(\tau)$ dropped to zero at the very first time step, indicating a nondirected random walk.

Further experiments addressed the behavior of the slow-moving MreB. Because slow-moving EYFP-MreB molecules were not observed in the presence of the MreB-depolymerizing drug A22, the slow-moving molecules represent polymerized MreB. The dynamics of 120 single members of this subpopulation were carefully examined. Since the molecules were stationary within the 30-nm resolution of the measurement over the course of the 7-s integration time shown in Fig. 4a4, time-lapse imaging was used. Specifically, 100-ms imaging frames are separated by 9.9 s. In this way, the slow movement of polymerized MreB molecules could be followed over a longer time period before photobleaching. Figure 4d shows eight such fluorescence images in reverse contrast, where a single molecule is tracked for 220 s. The molecule moves from left to right, and then turns and moves right to left at a downward angle, shown by the arrow. The observed MSD of the slow-moving MreB molecules is plotted as a function of Δt in Fig. 4e. Here, the MSD was characterized by a quadratic dependence on time lag, as is typical of directed motion. Also consistent with directed motion is the computed velocity autocorrelation function, $C_V(\tau)$, shown in Fig. 4f, in which $C_V(\tau)$ remains positive until $\tau = 80$ s.

The slow, directed motion of polymerized MreB was further explored by analyzing the trajectories of each individual molecule in the context of two putative models for the motion of an MreB molecule in an MreB filament: (1) the filaments into which the monomers are incorporated were moving or (2) the monomers themselves were treadmilling through largely stationary filaments, analogous to the motion of actin. If the motion of polymerized MreB is due to whole filament movement, then the observation time for the slow-moving single molecules should be limited by the photobleaching time of the EYFP, independent of experiment timescale. The total emission time of EYFP-MreB before photobleaching under continuous emission was measured, with an average on-time of 4.6 s. However, the true irradiation time before photobleaching of the molecules imaged with 9.9-s time lapses was found to be only 0.8 s. Most of the fluorescence from the polymerized MreB molecules, therefore, disappeared before photobleaching occurred likely as a result of dissociation from the end of a filament and the onset of fast diffusive motion not resolvable with the 100-ms

imaging frames. This result indicates that MreB molecules treadmill through filaments with fixed ends.

Given that the polymerized MreB molecules exhibited directed motion, a speed value was extracted from each single-molecule trajectory. The average speed was 6.0 ± 0.2 nm/s. From its crystal structure, the length of an MreB monomer is 5.4 nm (29); the average speed, therefore, corresponded to 1.2 monomer additions per second in steady-state fixed MreB filaments. The average length traveled by a polymerized MreB molecule before dissociation, which corresponds to the filament length, was also measured by considering only MreB molecules that became polymerized after the start of imaging and that dissociated before the end of imaging. The average length of 128 MreB filaments was 392 ± 23 nm – quite small relative to the average cell length of 3.5 μ m. Most of the trajectories moved perpendicular to the long axis of the cell. A smaller number of trajectories were oblique. These nonperpendicular trajectories were characterized as moving toward the swarmer pole or toward the stalked pole, but no preferred orientation was observed.

3.7. Cell Cycle-Dependent Superresolution Structure of MreB

By fitting the PSF of isolated nanoscale emitters with 30-nm localization accuracy and following these superlocalized molecules over time, the previous experiment attained superresolution via single-molecule tracking, as displayed in Fig. 4d. That is, as a single labeled MreB treadmills through a filament, the track shows the shape of the filament. This approach was, however, limited by the isolated fluorophore requirement to ~1–3 tagged MreB molecules, and therefore ~1–3 trajectories per cell. To address this upper bound and more fully observe the full superstructure in one cell, photoactivated localization microscopy (PALM) was used to extend the investigation of EYFP-MreB to cells expressing high concentrations of these molecules, and thus to visualize the superstructure formed by a collection of MreB filaments in these cells (15). In particular, bulk visualization of MreB in *C. crescentus* indicates that MreB forms a cell cycle-dependent structure (26, 28, 30). Prior to the onset of the division process, MreB is arranged in a helix-like shape along the longitudinal axis of the cell. As the cell begins cytokinesis, MreB is assembled into a ring at the division plane. The helical structure is then reassembled in the two daughter cells (Fig. 1). Single-molecule-based superresolution imaging was applied to measure these structures.

1. *C. crescentus* cells expressing 100–1,000 copies of EYFP-MreB are created by incubating the strain LS3813, described above, in the M2G minimal medium with 0.006–0.075% xylose for 4 h.
2. The cells are imaged with 100-ms imaging frames in order to resolve only the slow-moving, polymerized MreB molecules. The initial density of fluorescent tags in these cells was initially too great for isolated, single-molecule imaging, but this could be addressed based on the fact that apparently bleached EYFP molecules can be reactivated with violet light (31).
3. After the bleaching of all emissive EYFP-MreB molecules, a sparse subset is reactivated with 407-nm irradiation, where the reactivation intensity of 10^3 – 10^4 W/cm² is chosen such that at most one EYFP molecule is reactivated in each diffraction-limited region.
4. The positions of these isolated molecules are determined by PSF fitting.
5. The reactivation and imaging process was repeated 20 times until a superresolution image could be reconstructed from the sum of the localizations.

To illustrate the photoreactivation process, Fig. 5a–f shows the fluorescence emission from EYFP-MreB molecules in a single cell over time. Each punctate white spot is the emission

from a single molecule, and the fluorescence images (acquired with 100-ms integration times) are superimposed on a reversed-contrast white-light image of the cell. After the initial bleaching step, two single EYFP-MreB molecules were observed in the cell (Fig. 5a). Additional imaging with 514-nm light bleached all the fluorophores (Fig. 5b). A 2-s dose of 407-nm laser illumination returned some EYFP-MreB to an emissive state (Fig. 5c, e), though all fluorophores are apparently bleached prior to the 407-nm pulse (Fig. 5d, f).

1. The positions of these molecules are calculated from a fit of the fluorescence images to a 2D Gaussian function and are recorded for every such frame.
2. At the end of a 4-min acquisition period, these localization events are summed.
3. To form a final superresolution image, each localized molecule is depicted in the reconstruction as a unit area Gaussian with fixed width equal to the average statistical localization accuracy of 30–40 nm.

This technique, termed “Live-Cell PALM,” enabled the imaging of the EYFP-MreB superstructure in *C. crescentus*. In particular, it resolved several MreB bands spanning the length of stalked cells and a tightly focused midplane ring in predivisional cells. Importantly, understanding superresolution features derived from many image acquisitions requires careful consideration of the emitter photophysics and the dynamics, if any, of the underlying structure. Since, as described above, polymerized MreB molecules treadmill slowly along MreB filaments, time-lapse imaging, as described above, was incorporated into live-cell PALM to increase the number of localization events given the fact that the maximum labeling concentration in live-cell experiments is limited due to changes in the cell morphology at high concentrations of fusion protein. Specifically, a 900-ms delay was introduced between each 100-ms imaging frame, and the sample was only illuminated with 514-nm light during the short acquisition time. 407-nm reactivation pulses were again applied after photobleaching to generate novel sparse subsets of emissive EYFP-MreB.

Figure 5g–j presents the results from TL-PALM imaging of EYFP-MreB in *C. crescentus*. Two distinct MreB superstructures were identified in the cells: a quasi-helical arrangement in a stalked cell (Fig. 5g, h) and a midplane ring in the predivisional cell (Fig. 5i, j). In Fig. 5h, j, the TL-PALM reconstruction is superimposed on a reversed-contrast, white-light image of the cell in which the EYFP-MreB was visualized. The images obtained by TL-PALM were more continuous than those acquired without the introduction of dark periods. Furthermore, the use of TL increased the number of localization events (487 in Fig. 5g and 330 in Fig. 5j) to the point, where the Nyquist criterion for 40-nm resolution was satisfied (32), giving rise to a true superresolution reconstruction of the MreB superstructure in *C. crescentus*.

4. Summary and Outlook

Single-molecule imaging permits high-resolution measurements of fluorescent emitters in time and space. This noninvasive, non-perturbative technique is quite useful for the study of single protein molecules in live cells. By applying the techniques of single-molecule microscopy to EYFP-tagged proteins in live *C. crescentus* cells, the diffusion dynamics, localization patterns, and structure of three key proteins, PleC, PopZ, and MreB, could be explored. By providing a noninvasive tool for high-resolution imaging that is beginning to approach the spatial resolution of cryo-electron microscopy, single-molecule, superresolution imaging can enable investigations of many more intracellular processes and has become an important biophysical tool.

Because the single-molecule-based imaging techniques described here are based on wide-field microscopy, they can further provide the basis for more sophisticated experiments.

Specific challenges that are being addressed include improving the axial resolution of single-molecule microscopes (33–35), incorporating multiple excitation and emission wavelengths (36, 37), and imaging thick samples (38).

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Fig. 1.

Schematic of the dynamic localization of the proteins PleC (*stars*), PopZ (*circles*), and MreB (*lines*) over the course of the *Caulobacter crescentus* cell cycle. PleC is localized to the flagellar pole of the nonreplicating swarmer cell. As the swarmer cell differentiates into a stalked cell, PleC becomes delocalized. As the stalked cell progresses into a predivisional cell, a new flagellum is formed opposite the stalk and PleC is localized to the new flagellar pole. PopZ is also positioned at the flagellar end of swarmer cells. Upon differentiation into a stalked cell, a second concentration of PopZ begins to accumulate at the opposite pole (distal from the stalk) with an intensity that increases as the cell cycle progresses. Prior to the onset of the division process, MreB is arranged in a helix along the longitudinal axis of the cell. As the cell begins cytokinesis, MreB is assembled into a ring at the division plane. The helical structure is then reassembled in the two daughter cells.

Fig. 2. Dynamics of single PleC histidine kinase molecules. (a–c) *Leftmost image*: Dark-field image of the cell under investigation. Columns 2–7: Consecutive fluorescence images of single PleC-EYFP molecules spaced by different time intervals: (a) images acquired every 100 ms, (b–c) images acquired every 1 s. (d) Measured mean square displacement (*triangles*) and geometry-corrected mean square displacement (*circles*) vs. time lag. The corrected data slope of $0.049 \mu\text{m}^2/\text{s}$ corresponds to a diffusion coefficient, $D = 12 \pm 2 \mu\text{m}^2/\text{s}$. (e) Direction of motion in stalked cells without localized PleC (*black bars*) and swarmer and predivisional cells with localized PleC (*gray bars*), where $-s$ indicates motion toward the flagellar pole. Each bin represents one-tenth of the cell length, with the polar bins excluded. Reprinted with permission from ref. 10.

Fig. 3.

Visualization of single PopZ-YFP molecules in live cells. (a) Positions of two PopZ-EYFP molecules within a cell, with *heavy lines* tracking the distance moved between 32.2-ms frames. One molecule (*black*; indicated by *arrow* at *top right*) remains localized to the pole, and the other (*dark gray*) has increased mobility. The tracks are overlaid on a *white-light image* of the cell, which is outlined in *black*. (b–f) Time-dependent behavior of 12 single molecules in five cells. Stationary and mobile molecule positions are both tracked with *heavy lines*. The *heavy lines* are *dotted* during dark (blinking-off) periods. *Thin horizontal dotted lines* in (b) and (d) mark the positions of the poles opposite to the pole at which

stationary molecules are localized. The trajectories in **(d)** correspond to the longitudinal position of the molecules tracked in **(a)** as a function of time. Reprinted with permission from ref. 14.

Fig. 4.

Analysis of motion of EYFP-MreB. (a) 15.4-ms integration time fluorescence images of single EYFP-MreB molecules in a *C. crescentus* cell. White line shows the cell outline. (a1) 15.4-ms epifluorescence image showing three single EYFP-MreB molecules. The top and bottom molecules (arrowheads) are stationary on this timescale, and the middle molecule (arrow) is mobile. (a2) Smoothed image of (a1) obtained by applying a low-pass filter. (a3) Recorded trajectory of the mobile (middle) molecule in (a1). (a4) Summed image of 450 sequential imaging frames. The fluorescence from the two stationary molecules is still evident, whereas the middle molecule does not appear on this 6.93-s integration timescale (scale bar, 1 μm). (b) Measured (open circles) and geometry-corrected (filled circles) MSD of fast-moving MreB molecules. The solid line is a linear fit of the corrected data. (c) Velocity autocorrelation of fast-moving molecules; this quantity drops to zero at the very first time lag. (d) Inverted-contrast, 100-ms integration time fluorescence images of single slow-moving EYFP-MreB molecules in a *C. crescentus* cell at 30-s intervals. The arrows illustrate the directional movement of the molecules and the black line shows the cell outline. The superlocalized track of a single molecule leads to a superresolution image of the filament through which the molecule is treadmilling. (e) Measured MSD versus time lag for slow-moving MreB molecules. The solid line is a quadratic fit to the data, indicative of directional motion. (f) Velocity autocorrelation of slow-moving MreB; this quantity remains positive over at least 80 s. Reprinted with permission from ref. 11.

Fig. 5. Superresolution imaging of MreB superstructure. **(a–f)** Reactivation of single EYFP-MreB fusions in the same live *C. crescentus* cell using 407-nm light. Fluorescence images of single EYFP-MreB molecules from 514-nm excitation are overlaid on a reversed-contrast, *white-light image* of the cell. **(a)** Initial image showing two isolated emissive EYFP-MreB molecules. **(b, d, f)** After photobleaching by 514-nm irradiation, the cell contains no emissive EYFP-MreB. **(c, e)** Reactivated EYFP-MreB molecules are observed following a short 407-nm reactivation pulse (*scale bar*, 1 μ m). **(g–j)** TL-PALM images of EYFP-MreB in *C. crescentus* cells created by fitting molecule positions in imaging frames, such as those in **(a)**, **(c)**, and **(e)**. **(g, h)** Quasi-helical structure in a stalked cell. **(i, j)** Midplane ring in a predivisional cell. Fluorescence PALM images are shown in **(g)** and **(i)**. The PALM images in **(h)** and **(j)** are from the same cells as in **(g)** and **(i)**, respectively, overlaid on a reversed-contrast, *white-light image* of the cell (*scale bars*, 300 nm). Reprinted with permission from ref. 15.