Cryogenic single-molecule fluorescence annotations for electron tomography reveal in situ organization of key proteins in Caulobacter

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Both superresolution fluorescence imaging and CET are powerful techniques for uncovering the subcellular organization of biomolecules. Each approach has unique strengths and limitations. The combination of superresolution imaging, with its noninvasive and specific labels, and CET, with its high resolution, would enable the visualization of biomolecules of interest in the presence of their cellular context. However, correlating these two techniques relies on development of superresolution fluorescence imaging of samples at cryogenic temperatures. There are numerous approaches to superresolution fluorescence imaging, each with their own challenges when adapting them to cryogenic temperatures. Here, we will focus specifically on single-molecule active control microscopy (SMACM). This method requires precise localization of single molecules in a situation where molecular overlap is removed by using some active control mechanism to ensure only a subset of molecules are emitting in any one imaging frame. In this approach, resolution beyond the diffraction limit arises from a pointillist reconstruction of the single-molecule positions. Retaining active control mechanisms under cryogenic conditions remains a challenge to adapting SMACM for cryogenic temperatures due to the reduced thermal energy available for stochastic processes and/or restricted molecular conformations. Methods of controlling the emissive states of fluorophores at room temperature often rely on diffusional processes or large conformational changes (15, 16), both of which are limited in frozen-hydrated samples. Recent work has identified several photoactivatable fluorescent proteins that maintain their ability to photoactivate at cryogenic temperatures (17–20), and other methods have been shown to induce stochastic blinking of some fluorescent dyes when much higher excitation powers are used (20). Here, we demonstrate an efficient workflow for correlative cryogenic SMACM and CET using the red photoactivatable protein PAmKate (18). The limited number of PAmKate molecules that can be activated and imaged means that densely sampled structures remain a future goal. The primary utility of the single-molecule fluorescence localizations at the moment is to provide single-molecule ground truth annotations of specific molecules in CET reconstructions. We therefore call this technique correlative imaging by annotation with single molecules, or CIASM, pronounced “chasm.” We employ the CIASM workflow to investigate three different proteins in *C. crescentus*.

*C. crescentus* undergoes an asymmetric cell division to produce a motile “swarmer” daughter cell and a sedentary “stalked” daughter cell (21) (Fig. 1). Visualizing the subcellular organization of structural and regulatory proteins in these cells is crucial to understanding their life cycle. The cellular asymmetry and distinct characteristics of the two daughter cells are driven by the underlying asymmetric localization of critical signaling proteins. We have chosen to investigate three proteins whose asymmetric localization varies as a function of the cell cycle. The first, methyl-accepting chemotaxis protein A (McpA), is a member of the methyl-accepting chemotaxis proteins (MCPs). MCPs are anchored in the inner cell membrane and sense external stimuli through periplasmic ligand-binding domains. Upon binding, conformational changes occur which regulate the activity of a histidine kinase that regulates the flagellar motor machinery, resulting in changes in flagellar rotation upon stimulation (25). The chemoreceptor array forms on the dorsal surface of the inner membrane near the flagellated swarmer pole. The location of the array close to the base of the flagellum is crucial to its role in providing feedback to the flagellum motor machinery. The second protein, polar organizing protein Z (PopZ), forms space-filling microdomains at both the stalked and flagellated poles (26, 27). These PopZ microdomains are membraneless organelles that play a crucial role in selectively sequestering proteins to enhance chemical reactions and robustly maintain chemical gradients within the small volume of a bacterial cell (27, 28). The complement of proteins recruited to these microdomains differ at the two poles leading to their asymmetric sequestration in the daughter cells upon cell division. One such protein, SpmX, is the third protein investigated here. SpmX is sequestered to the nascent stalked pole PopZ microdomain during the swarmer to stalked cell transition (29). It is an intrinsically disordered integral membrane protein situated at the inner membrane with an N-terminal lysozyme homology region. SpmX is responsible for recruiting the histidine kinase DivJ, which contributes to the activity of scores of genes involved in cell cycle progression (27).

Slices from electron-tomographic reconstructions of *C. crescentus* (Fig. 1A) show the three different proteins in situ. Only in the case of McpA is a well-defined structure visible in the CET reconstructions, while the other two proteins, PopZ and SpmX, are not directly discernable. The chemoreceptor array, which contains McpA, is clearly visible under these imaging conditions. In this orientation the chemoreceptor array appears as two parallel lines in a given tomographic slice, but in a reconstructed volume is shown to be two parallel plates ~12 to 15 nm apart.
In this example, the plates are ~190 × 100 nm. The assignment of McpA to this array structure has been well established. Briegel et al. (24) showed that an overexpression of McpA-mCherry resulted in an increased production of these array structures and that under this overexpression the arrays could be found at either cell pole. Furthermore, diffraction-limited correlative light and electron microscopy was used to assign McpA-mCherry to the array structure (24). The array structures observed in *C. crescentus* are similar to chemoreceptor arrays found across numerous bacteria phyla (23). Because the chemoreceptor array is so clearly visible in CET, we used McpA to validate the cryogenic CIASM workflow before applying the workflow to PopZ and SpmX.

PopZ (19 kDa) and SpmX (46 kDa) are positioned at the cell pole in multiprotein complexes. Neither protein has been observed to form well-ordered structures in situ, making them difficult to identify with the current capabilities of CET. While PopZ is not directly discernable in CET, the amorphous polar microdomain it forms in *C. crescentus* can be inferred in CET reconstructions by the absence of electron-dense ribosomes in the polar region of the cell (27, 30). There is no means of inferring the position of SpmX from CET alone; however, previous fluorescence data have demonstrated that SpmX localizes in the polar region of the cell on one side of the stalk (31), but its exact location in relation to PopZ has not yet been visualized. In contrast to CET data, all these proteins are clearly visible in the room temperature SMACM reconstructions when fused to PAmKate (Fig. 1B). Despite the high precision of the SMACM localizations (~13 nm), the superstructures formed by the three proteins have similar size and shape in the SMACM reconstructions. Without the complexity of multicolor fluorescence, the typical cellular context provided by the optical microscopy is a simple cell outline provided by manually annotating bright-field images of the fluorescently imaged bacterial cell. The combination of SMACM localizations and CET using CIASM will provide the best of both imaging modalities. It will clearly assign McpA positions to the chemoreceptor array and will reveal the subcellular locations and organization of SpmX in relation to PopZ and the neighboring cellular environment.

### Results

The process of correlating cryogenic SMACM localizations and CET can be divided into the four-stage workflow shown in Fig. 2: sample preparation, cryogenic single-molecule fluorescence imaging, CET, and image registration and visualization. Sample preparation and CET, with slight exceptions (*Methods*), follow established protocols (7). The cryogenic fluorescence imaging as well as the registration and visualization methods have aspects unique to this publication and will be described in detail below.

To prepare the sample, a small amount of bacterial cell culture containing cells expressing PAmKate fusion constructs is deposited on a holey carbon electron microscopy grid and plunge frozen. Next, the grid is loaded onto a cryogenic fluorescence microscope stage mounted on a home-built upright microscope. PAmKate molecules are photoactivated with 405-nm light, and the resulting active fluorophores are excited with 561-nm illumination at a low intensity (50 to 100 W/cm²) to avoid sample devitrification (*SI Appendix, Fig. S2*). The reduced quantum yield of photobleaching at cryogenic temperatures improves localization precision by permitting the total collection of an average of ~11,800 photons per emitter (*SI Appendix, Fig. S3*), but this reduction in photobleaching paired with the low damage thresholds for devitrification at 77 K leads to long experiment times of ~3 h (18). During these long experiments, axial and lateral drift caused by the large thermal gradients inherent in the imaging system as well as the replenishing of liquid nitrogen to the cryogenic stage pose a challenge. In order to avoid manual drift correction and simplify data acquisition, the point spread function (PSF) has been made astigmatic with the addition of a cylindrical lens prior to the image plane. Custom LabVIEW software runs a real-time fitting routine of a user-selected PSF generated from 40-nm-diameter fluorescent polystyrene beads and automatically adjusts a mechanical stage holding the objective to keep the PSF in focus (18).

Despite these challenges, cryogenic SMACM can provide accurate and precise localizations for proteins of interest in a manner compatible with CET, yielding annotations of the CET volume with single-molecule positions from fluorescence. There are two primary errors that impact the ability to annotate CET reconstructions with fluorescence localizations: localization error and registration error. The first, localization error, is a random error for each emitter and describes the precision with which any emitter can be localized. The second, registration error, is a systematic error for all localizations within a registered field of view and impacts the accuracy between the fluorescence annotations and the CET reconstruction. We will first describe how precise localizations are obtained from the fluorescence data and then how those localizations are accurately registered with the CET reconstructions.

Fig. 3 shows representative cryogenic SMACM data from *C. crescentus* cells expressing inducible PAmKate–PopZ fusion constructs. The integrated fluorescence intensity from one cell pole (Fig. 3B) exhibits stepwise dynamics indicative of single emitters. From the intensity trace, it is clear that there are often...
overlapping emitters within a diffraction-limited region. However, due to the long on-times, fluorescence from a single emitter will span numerous 1-s imaging frames, and this temporal information can be leveraged to separate fluorescence contributions from spatially overlapping emitters. To localize an emitter of interest, frames that are either just prior to the emitter of interest’s activation event or just after the emitter of interest’s photobleaching event are manually identified. These preframes or postframes are averaged to estimate the background image generated from other emitters active in the same frame. This background image is then subtracted from the frames in question, thus isolating the single emitter of interest has been isolated provides a localization as an estimate of the true emitter’s position. The precision of that estimate is the SEM of the frame localizations and is represented as the radius of a circle centered on the mean position. This process of isolating the contributions from single emitters of interest can be repeated for the entire SMACM time series, and following registration to CET data, the merged localizations provide single-molecule annotations, which can be overlaid onto a tomographic slice (Fig. 3D).

Registration of the SMACM localizations and the CET data are achieved through a series of image transformations starting with mapping the fluorescence to a low-magnification electron micrograph (3,600x, 38.8 Å/pixel) centered on the region of CET data collection. This low-magnification micrograph bridges the fluorescence and CET data. It is important that both the holes in the holey carbon grid and the gold beads used for CET alignment are clearly visible in this low-magnification micrograph, as both the holes and gold beads will be used in the registration process. First, the fluorescence data are registered with the low-magnification micrograph using the locations of 12 to 16 holes as control points for a projective transformation using custom Matlab software (Fig. 4A and SI Appendix, Note S1). We found this approach to be superior to using fluorescent beads as control points because it was challenging to achieve sufficiently high bead concentrations for accurate registration while simultaneously avoiding overlap with the biological structures of interest. Several automated methods for the identification of hole centers were explored, including template matching using an averaged hole image, centroid estimates, and Hough transformations that were used previously (36). Ultimately, software-assisted manual identification of hole centers proved to be the most robust method (SI Appendix, Fig. S5 and Note S2). Following registration of the fluorescence data to the low-magnification electron micrograph, the micrograph was registered with the higher-magnification CET data. (A) Heavily saturated average fluorescence data showing the identified centers (dark blue asterisks) of the holes (cyan circles) in the holey carbon grid. These hole centers will be used as control points for a projective transformation to register fluorescence and electron microscopy images. (B) Low-magnification electron micrograph of the same region imaged in A with the same holes and hole centers identified. The Inset shows 15-nm gold beads labeled with black arrows. Gold beads neighboring the cell of interest outlined in white are used as control points for a similar coordinate transformation that will register this low-magnification micrograph with the high magnification CET data. (C) Resulting CET and McpA–PAmKate SMACM localizations (red) for the cell of interest outlined in white in B. The agreement between the position of the chemoreceptor array and the single-molecule localizations validates the image registration process.

Fig. 3. Representative cryogenic SMACM data from PAmKate–PopZ fusion constructs in C. crescentus. (A) Overlay of diffusion-limited fluorescence from the average of the 405-nm photoactivation frames (gray) showing cellular autofluorescence and the average of the 561-nm excitation frames (heat map) showing the overlapping diffusion-limited locations of PAmKate–PopZ molecules. (B) Fluorescence intensity trace from the integrated region indicated by the white dashed box in A. The fluorescence contributions from one emitter (green area) are obtained by subtracting a background (gray area) estimated by taking the average of several frames prior to the activation of the emitter. (C) Localizations from the single emitter shown in B across multiple frames (black dots). Each frame provides a noisy estimate for the true emitter’s position. These frame localizations are merged into a single estimate of the emitter’s location (green circle) with a precision given by the SE on the mean of the frame localizations represented as the radius of the green circle. (D) Overlay of the PAmKate–PopZ merged localizations (green) on a tomographic slice from their corresponding cell with the ribosome excluded region outlined with a dashed black and white line. Each green circle identifies the position and uncertainty in position of a different PAmKate–PopZ emitter.
data using the gold beads. To do this, a projection of the CET data was taken across the axial dimension following tomographic reconstruction, and 15 to 25 beads were manually selected using custom Matlab software. Then, using these bead locations as control point pairs, a similarity transformation that only allows for rotation and scaling was calculated. Once completed, the merged fluorescence localizations can be carried to the CET image space first by the application of the projective transformation and then the similarity transformation. In order to assess the quality of the registration process, a complete set of CET data were collected on a 40-nm polystyrene fluorescent bead (SI Appendix, Fig. S6). The distance between the center of the bead as measured in the CET data and the bead localization by fluorescence after registration is ∼30 nm. Further validation is provided by registering McpA–PAmKate localizations because the chemoreceptor array, which is partially composed of the labeled McpA, is visible in the CET data (Fig. 4B). It is clear from the overlay that SMACM localizations are superior for identifying and labeling a structure to traditional diffraction-limited correlate relative light and electron microscopy, which for this red fluorophore and imaging system would yield a fluorescent spot ∼350 nm in diameter (Fig. 4C and SI Appendix, Fig. S7).

While the holey carbon substrate provides control points for the lateral registration of the fluorescence and CET data, our approach currently lacks control points for axial registration between the two imaging modalities. However, with the addition of astigmatism to the fluorescence microscope for axial drift correction during acquisition, there is axial localization information in the single-molecule PSFs. The resulting axial precision of these localizations is worse than expected by approximately a factor of 2 (SI Appendix, Fig. S8). This reduced precision is attributed to the residual effects of overlapping emitters. Despite lacking axial control points, absolute positioning of the SMACM localizations relative to the reconstructed tomograms can be performed by aligning the average position of the features observed in each imaging modality (Fig. 5 and Movie S2). Assuming that the axial registration error is dominated by the sparse SMACM localizations and that the SMACM localizations randomly sample the structure observed in CET, the error in the accuracy of the registration will follow SE statistics and decrease with an increased number of localizations. For example, the SD of the PAmKate–PopZ axial localizations shown in Fig. 5B is 90 nm. This results in a 27-nm SEM for the 11 localizations. Once axial registration has been performed, the SMACM localizations can be viewed in the full three-dimensional (3D) context provided by CET. While the axial registration error using this approach is comparable to that of the lateral registration error, this approach only works for structures that are visible directly in the CET data, somewhat defeating the purpose of correlation. Improvements in fiducials for axial registration are needed to make 3D correlations generalizable, and this is an area of ongoing development.

With the successful registration of SMACM localizations and CET for biomolecules of general interest in the two lateral dimensions, we turned our attention to the interaction between PopZ and SpmX by visualizing their subcellular organization. Fig. 6A shows the SpmX and PopZ localizations for individual cells. To overcome the sparsity of localizations recovered from individual cells, the localizations from all cells for which there are correlative data can be aligned based on the position of the base of the stalk and outer membrane, which are clearly observable in CET (SI Appendix, Fig. S9). Once aligned, a more detailed SMACM reconstruction can be overlaid by pooling all localizations onto a representative tomographic slice (Fig. 6B). Interestingly, while SpmX is localized to the stalked pole of the bacterium, it is not localized symmetrically about the stalk. Rather, the localizations are clustered on either the dorsal or ventral side of the bacterium when viewed from the top. This pattern of asymmetric localizations has been previously reported (31), albeit at a lower resolution, and is seen in all stalked poles for which correlative CET data were collected. The resulting visualization of both SpmX and PopZ shows SpmX is localized to the base of the PopZ microdomain (Fig. 6C). The extent of SpmX along the inner membrane closely matches that of PopZ. This shared extent of PopZ and SpmX can be in the aligned cell data shown in Fig. 6C as well as in individual cells (SI Appendix, Fig. S10). The spatial extent of PopZ and SpmX can be quantified in relation to observables from the CET data, such as the manually annotated ribosome exclusion region that normally defines the PopZ microdomain. We see good agreement between the CET and SMACM localizations, suggesting that PopZ is uniformly distributed throughout the ribosome exclusion volume as was previously assumed.

**Discussion**

We have established a four-stage workflow that correlates SMACM localizations with CET reconstructions to provide ground truth annotations of specifically labeled biomolecules of interest. This workflow achieves localizations with an average precision below 10 nm, resulting from the large number of photons being collected from each emitter due to the reduced quantum yield of photobleaching at cryogenic temperatures. These large numbers of photons collected over multiple frames make it essential that localizations be merged into a single estimate for an individual emitter both to prevent overcounting of the number of emitters and to achieve the highest possible localization precision. For these precise localizations to be useful as annotations for correlative imaging, they must be accurately registered with the CET data. Our workflow accomplishes this by using the centers of the holes in holey carbon grids as fiducials visible in both imaging modalities and achieves registration with an accuracy of ~30 nm as assessed by the registration of a fluorescent polystyrene bead. These precise and accurate localizations identify the
which shows good agreement with the PAmKate manual annotation of the ribosome exclusion region from the CET data, and PopZ localizations along the cell axis, where zero is defined as the base showing the relative positions of SpmX and PopZ. (Fig. 6–A)

The correlative imaging of McpA arrays provides a strong validation of this workflow due to the visibility of the chemoreceptor array in CET. This validation gives confidence in the registration of stalked pole specific factors such as bactofilins and peptidoglycan binding proteins is mediated through SpmX in Asticcacaulis biprosthecum, a close relative of C. crescentus, and so interactions with other proteins may be driving SpmX’s asymmetric location within the PopZ microdomain (37). Understanding how SpmX oligomerization and interactions with other proteins lead to its subcellular organization and ultimately its biological function remains an exciting and active area of research in our laboratory.

The results presented here provide the clearest picture to date of the subcellular localization of the PopZ–SpmX signaling complex in C. crescentus, and a method for correlating CET and fluorescently tagged proteins in any cell. Currently, the most restrictive limitation in the quality of correlative images produced by our approach is the incomplete photophysical control over the emissive states of molecules at low temperatures using low excitation power. While PAmKate can be efficiently photoactivated at cryogenic temperatures, it is not possible to efficiently switch the emitters back to an off-state. Instead, we rely on stochastic processes to either permanently photobleach emitters or shelve them into long-lived dark states. These stochastic processes have very low quantum yields at cryogenic temperatures, and while these low yields permit the collection of more photons than room temperature studies, thus improving localization precision, the long on-times are actually limiting resolution by restricting the number of emitters that can be localized. Improved photophysical control over the fluorescent labels could possibly be obtained through new photochemistry that is robust to cryogenic temperatures or by exploiting the rugged cryogenic energy landscape to produce long-lived dark states not observed at room temperature. No matter how it is achieved, the ability to both activate and inactivate emitters efficiently is necessary for improved cryogenic super-resolution imaging compatible with CET. Other aspects of this method that limit the quality of registered images include the axial precision, axial registration methods, and devitrification at low excitation powers.

None of these challenges is the result of fundamental physical limitations; rather, improvements in all of these areas can be obtained with additional engineering and experimentation. Given the importance of combined single-molecule sensitivity and specificity with high-resolution cellular context, we are confident that these challenges will be overcome and that CIASM has a bright future. The strengths and weaknesses of the SMACM and CET complement each other such that the resulting images are more informative than either technique is individually. We expect that this method will be useful for highlighting the large number of cellular protein complexes that cannot be directly discerned in CET due to either their small size or lack of periodic structure (such as the locations of the members of the divisome), and for identifying proteins in electron-dense structures of unknown composition.

Materials and Methods

Generation of C. crescentus PAmKate Fusion Strains. McpA–PAmKate. To construct C-terminal PAmKate fusions in a single step under a native locus, we used the vector pYFPC-2 (or under the xylose locus,
The YFP gene was cut out using EcoRI and Nhel enzymes, and the resulting backbone was purified by separation on an agarose gel. The PAmKate gene was amplified from Addgene plasmid #32691 (38) and inserted into the above backbone using Gibson assembly, resulting in the pPAmKateC-2 and pXyIPamKateC-2 vectors. The McpA coding region was amplified with appropriate overhangs from genomic DNA from NA1000 cells and gel purified. The pXpAmKateC-2 vector was linearized by digesting with Ndel, and the McpA gene was inserted using Gibson assembly. The linker used for the McaP fusion was GTSRSNPSNVHRDSQ-

Room Temperature Superresolution Imaging. C. crescentus cells were induced with 0.3% xylose for 3 h (McpA-PAmKate, PAmKate-PopZ) or cultured with endogenous expression (SpmX-PAmKate) and placed on agarose pads. These molecules either form relatively fixed structures or do not move dramatically during live cell imaging; highly mobile molecules are rejected as bad fits. Imaging was performed with a custom epifluorescence microscope. Fluorescence emission under >680 W/cm² of 561 nm excitation (Coherent OBIS) was collected using an oil immersion, supercorrected objective (Olympus PLANON60xOSC, 60×/N.A. 1.4) mounted in a Nikon Diaphot 200 microscope and imaged onto an EMCCD camera (Andor iXon) at 20 Hz. Occasional pulses (<1 s) of 405-nm photoactivation light (Coherent OBIS) were used to maintain sufficient concentrations of active emitters. In total, 7,000 to 9,000 frames were collected for each dataset. The acquired data were processed with ThunderSTORM (35) by fitting identified particles with a standard Gaussian model and filtering out bad fits (σ of Gaussian, >150 nm, intensity, <200 photons/frame; or uncertainty, >18 nm; <10% of localizations were removed by these filters).

Correlative Imaging: Sample Preparation. McpA-PAmKate and PAmKate-PopZ cell cultures were induced with 0.3% xylose for 3 h prior to plunge freezing. The SpmX–PAmKate strain was endogenously expressed. A small aliquot of the bacterial cultures expressing PAmKate fusion constructs was mixed with 30% (vol/vol) ethylene glycol, which serves as a cryoprotectant, and aliquot of the bacterial cultures expressing PAmKate-PopZ or cultured with endogenous expression (SpmX–PAmKate) and placed on agarose pads. These molecules either form relatively fixed structures or do not move dramatically during live cell imaging; highly mobile molecules are rejected as bad fits. Imaging was performed with a custom epifluorescence microscope. Fluorescence emission under >680 W/cm² of 561 nm excitation (Coherent OBIS) was collected using an oil immersion, supercorrected objective (Olympus PLANON60xOSC, 60×/N.A. 1.4) mounted in a Nikon Diaphot 200 microscope and imaged onto an EMCCD camera (Andor iXon) at 20 Hz. Occasional pulses (<1 s) of 405-nm photoactivation light (Coherent OBIS) were used to maintain sufficient concentrations of active emitters. In total, 7,000 to 9,000 frames were collected for each dataset. The acquired data were processed with ThunderSTORM (35) by fitting identified particles with a standard Gaussian model and filtering out bad fits (σ of Gaussian, >150 nm, intensity, <200 photons/frame; or uncertainty, >18 nm; <10% of localizations were removed by these filters).

This intensity occurs rapidly and locally to the area of excitation. Photoactivation was achieved using short pulses (50 to 500 ms) of ~5 to 15 W/cm² of 405-nm light. Both the intensity and duration of the 405-nm pulses were adjusted to achieve sufficient activation while avoiding excess emitter overlap as much as possible. Fluorescence emission from 561-nm excitation was filtered from scattered excitation light using a dichroic mirror (Semrock Di03-5651-11-25x6e) as well as a bandpass and a notch filter (FF01-607/70-25, Chroma ZET561NF). A 1-m focal length cylindrical lens was mounted just prior to the camera (Andor iXon) to generate an astigmatic PSF that was used for axial localization and for live drift correction using custom Labview acquisition software (18). Drift correction was performed by fitting the PSF of 40-nm fluorescent polystyrene beads. Fluorescence frames were collected at 1 Hz, and ~10,000 frames were acquired for each SMACM dataset. These frames were drift corrected using cross-correlation and then binned in time by a factor of 2 to improve the signal-to-noise ratio and reduce data volume. These drift-corrected frames are then used for subsequent data analysis including the localization process described in Fig. 3 and accompanying text.

Correlative Imaging: Cryogenic Electron Tomography and Microscopy. CET data were collected using a 300-kV electron microscope (Titan Krios Thermo Fisher) with a direct detector (K2 Gatan) and an energy filter (Bioquantum Gatan). The tilt series was acquired with either 3° steps for PopZ imaging or 1° steps for McpX and McpA imaging. The tilts were acquired in a bi-directional manner (42) beginning with the sample at 21°. For tilt series with 1° steps the stage positions were [21, 20, 19 . . . –45, 22, 23, 24 . . . 45], and for 3° steps the stage positions were [21, 18, 15 . . . –45, 24, 27, 30 . . . 45]. The total dose for each tilt series was 100 eÅ². Smaller steps sizes enabled easier alignment of the tilt series during reconstruction using the Etomo package in IMOD (43). Tilts were acquired with a pixel size of 7.28 Å and 10 μm of defocus. These imaging parameters were chosen so that an entire C. crescentus cell could be acquired in a single tomogram. At these relatively low magnifications, resolving high-resolution structures would not be feasible, and so the defocus was set to improve low-resolution contrast. The additional low-magnification image required for image registration was taken following CET data collection and was centered on the cell in the CET data. These drift-corrected frames had a pixel size of 38.8 Å. At this magnification, a sufficient number of holes, typically 12 to 16, were visible for registration to the fluorescence data, and the 15-nm gold beads used for alignment to the CET data were also visible (Fig. 4).

Correlative Imaging: Visualization. CET data were reconstructed using the Eтомo package from IMOD (43) using 15-nm gold beads as fiducials. The resulting tomograms were binned by four and, in the case of annotation, were annotated by hand or with the assistance of a neural network in EMAN2 (14). Fluorescence data in most cases were displayed as merged single-emitter localizations. The pooled reconstructions shown in Fig. 6 were displayed as histograms of the merged localizations with a pixel size of 14.6 nm.

Data Availability Statement. Tomographic reconstructions and single-molecule localizations have been made publicly available in the Electron Microscopy Data Bank (accession no. EMD-21706) (44).

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