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Photobleaching Enables Super-resolution Imaging of the FtsZ Ring in the Cyanobacterium Prochlorococcus

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Corresponding Author:	Qinglu Zeng, Ph.D. Hong Kong University of Science and Technology Clear Water Bay, Hong Kong HONG KONG
Corresponding Author's Institution:	Hong Kong University of Science and Technology
Corresponding Author E-Mail:	zeng@ust.hk
Order of Authors:	Yuanchao Zhan
	Qinglu Zeng, Ph.D.
	Yaxin Liu
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August 6th

Dear Editor Dr. Vineeta Bajaj,

Thank you for handling our manuscript.
We have read the review comments carefully and responded to the comments point-by-point in a separate document. As for the image acquisition and analysis, we provide to figures along with figure legend as supplemental material for script writing.

If you need more information, please let me know.

Thank you again for your time and effort on our manuscript.

Sincerely,

Qinglu Zeng
Assistant Professor
Department of Ocean Science and Division of Life Science,
The Hong Kong University of Science and Technology,
Hong Kong, China

香港九龍清水灣 Clear Water Bay, Kowloon, Hong Kong 電話 Tel: (852) 2358 7310 傳真 Fax: (852) 3693 4766

TITLE:

Photobleaching Enables Super-resolution Imaging of the FtsZ Ring in the Cyanobacterium *Prochlorococcus*

AUTHORS AND AFFILIATIONS:

Yuanchao Zhan[1], Yaxin Liu[1], Qinglu Zeng[1,2,3]

[1]Department of Ocean Science, The Hong Kong University of Science and Technology, Hong Kong, China

[2]Division of Life Science, The Hong Kong University of Science and Technology, Hong Kong, China

[3]HKUST Shenzhen Research Institute, Shenzhen, China

Corresponding Author:

Qinglu Zeng (zeng@ust.uk)

E-mail Addresses of the Co-authors:

Yuanchao Zhan (yczhan@ust.hk)

Yaxin Liu (yliuek@connect.ust.hk)

KEYWORDS:

STORM, photobleaching, cyanobacterium, *Prochlorococcus*, super-resolution imaging, three-dimensional, FtsZ ring, cell division

SUMMARY:

Here we describe a photobleaching method to reduce the autofluorescence of cyanobacteria. After photobleaching, stochastic optical reconstruction microscopy is used to obtain three-dimensional super-resolution images of the cyanobacterial FtsZ ring.

ABSTRACT:

Super-resolution microscopy has been widely used to study protein interactions and subcellular structures in many organisms. In photosynthetic organisms, however, the lateral resolution of super-resolution imaging is only ~100 nm. The low resolution is mainly due to the high autofluorescence background of photosynthetic cells caused by high-intensity lasers that are required for super-resolution imaging, such as stochastic optical reconstruction microscopy (STORM). Here, we describe a photobleaching-assisted STORM method which was developed recently for imaging the marine picocyanobacterium *Prochlorococcus*. After photobleaching, the autofluorescence of *Prochlorococcus* is effectively reduced so that STORM can be performed with a lateral resolution of ~10 nm. Using this method, we acquire the *in vivo* three-dimensional (3-D) organization of the FtsZ protein and characterize four different FtsZ ring morphologies during the cell cycle of *Prochlorococcus*. The method we describe here might be adopted for the super-resolution imaging of other photosynthetic organisms.

INTRODUCTION:

Super-resolution microscopies can break the diffraction limit of light and provide images within sub-diffraction resolutions (< 200 nm). They have been widely used in many organisms to study protein localization and subcellular structures. Major super-resolution microscopy methods include structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), STORM, and photoactivated localization microscopy (PALM). The mechanisms and applications of these super-resolution microscopes have been reviewed elsewhere[1,2].

STORM can achieve a resolution as high as 10 nm by spatial separation[3,4]. For STORM, only one molecule within a diffraction-limited region is activated ("on") and the rest of the molecules are kept inactivated ("off"). By an accumulation of rapid switch-on and -off of single molecules, a "diffraction-unlimited" image can be generated[3]. Meanwhile, many kinds of organic dyes and fluorescent proteins are applicable in STORM, allowing an easy upgrade from regular fluorescence microscopy to high-resolution microscopy[5,6].

STORM has not been widely applied in photosynthetic cells, such as cyanobacteria, algae, and plant cells with chloroplasts[7,8], which is due to the fact that STORM requires high laser intensity to drive photoswitching. The high-intensity laser unfavorably excites strong autofluorescence background in photosynthetic cells and interferes with the single-molecule localization in STORM imaging. In order to use STORM to investigate the subcellular structures or protein interactions in photosynthetic cells, we developed a photobleaching protocol to quench the background autofluorescence signals[9]. In a routine immunofluorescent staining procedure, specimens are exposed to white light of a high intensity during the blocking step, which lowers the autofluorescence of photosynthetic cells to meet the requirements for STORM. Thus, this protocol makes it feasible to investigate pigmented organisms with STORM.

Here, we describe the protocol to use STORM to image the FtsZ ring organization in the unicellular picocyanobacterium *Prochlorococcus*. FtsZ is a highly conserved tubulin-like cytoskeletal protein which polymerizes to form a ring structure (the Z ring) around the circumference of a cell[10] and is essential for the cell division[11]. Preserved *Prochlorococcus* cells are first photobleached to reduce the autofluorescent background and immunostained with a primary anti-FtsZ antibody, and then a secondary anti-Rabbit IgG (H+L) antibody is conjugated with a fluorophore (*e.g.*, Alexa Fluor 750). Eventually, STORM is used to observe the detailed FtsZ ring organizations in *Prochlorococcus* during different cell cycle stages.

PROTOCOL:

1. Sample Preparation and Fixation

1.1. Inoculate 1 mL of axenic *Prochlorococcus* MED4 to 5 mL of the seawater-based Pro99 medium[12]. Grow *Prochlorococcus* cultures at 23 °C under the light with an intensity of 35 µmol photons/m^{2}s. Five days later, collect 1 mL of culture into a 1.5-mL tube.

Note: Five days after the inoculation, *Prochlorococcus* MED4 will reach the late log phase, with approximately 10^8 cell/mL, which is appropriate for STORM imaging.

1.2. Add 100 µL of formaldehyde freshly prepared from 25% paraformaldehyde (PFA) (w/v) and 2 µL of 50% glutaraldehyde into the 1.5-mL tube to fix the culture. Incubate the sample in the dark at room temperature for 20 min.

Note: The sample was kept in the dark for fixation because glutaraldehyde is light sensitive.

1.3. Spin down the sample at 13,500 x *g* for 1 min; then, remove the supernatant and resuspend the cells in 100 µL of the Pro99 medium[12]. Store the sample at 4 °C until immunostaining.

2. Precoating of the Coverslip with Polystyrene Beads

Note: Polystyrene beads are considered as the fiducial marker for drift correction.

2.1. Vortex the original vial of polystyrene beads and prepare a 1:20,000 dilution with 50% ethanol as working slurry.

2.2. Turn on the hot plate, set the temperature to 120 °C, and place coverslips onto the hot plate.

2.3. Load 100 µL of working slurry onto each coverslip. Incubate the coverslip on the hot plate for 10 min and, then, carefully transfer the coverslips to Petri dishes for storage at room temperature.

Note: The side with the beads attached to it should face up, and the cells should be attached on the same side. The beads are immobilized on the coverslips and used for correcting the sample-drifting in real-time during STORM imaging. After immobilizing the beads, the coverslips cannot be flamed.

3. Coating of Poly-L-lysine onto the Bead-coated Coverslip

Note: This is done for the immobilization of cyanobacterial cells.

3.1. Add 100 µL of poly-L-lysine (1 mg/mL) to the center of the coverslip with the bead-coated side facing up. Let it sit for 30 min at room temperature.

3.2. Use a pipette to carefully aspire the unattached poly-L-lysine and transfer it to a 1.5-mL tube. Save the poly-L-lysine at 4 °C for reuse.

3.3. Rinse the coverslip with 10 mL of ultra-filtered water in a 60-mm Petri dish and then briefly dry the coverslip on a paper towel.

3.4. Store the coverslip in a Petri dish (100 mm) for later usage. To prevent the attachment of the coverslip to the dish, line the Petri dish with a layer of parafilm.

Note: The coverslip, precoated with beads and poly-L-lysine, can be stored at room temperature for at least half a year. Therefore, preparing a batch of coverslips in advance is highly recommended.

4. Immobilization of Cells on the Coverslip

4.1. Transfer a precoated coverslip to a new Petri dish with parafilm at the bottom, which will be referred to as the staining dish henceforth. Load 100 µL of the fixed sample on the coverslip and let it sit for 30 min to allow cell attachment.

4.2. Transfer the coverslip to a well of a 12-well plate, which will be referred to as the washing dish henceforth. Add 1 mL of phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) to the well to wash the coverslip. Remove the PBS buffer and add a new PBS buffer to wash the coverslip again.

5. Permeabilization of Cyanobacterial Cells

5.1. Remove the PBS buffer using a pipette. Add 1 mL of freshly prepared permeabilization buffer to the well of the washing dish, which contains 0.05% non-ionic detergent-100 (v/v), 10 mM EDTA, 10 mM Tris (pH 8.0), and 0.2 mg/mL lysozyme.

5.2. Incubate the washing dish at 37 °C for 20 min. Then, remove the permeabilization buffer.

5.3. Add 1 mL of PBS buffer and place the washing dish on a shaker which gently shakes the washing dish for 5 min. Remove the PBS buffer. Repeat this step 3x to wash the coverslip.

6. Photobleaching of the Chlorophyll Pigments in a Blocking Step

6.1. Transfer the coverslip to the staining dish. Add 50 µL of blocking buffer, which contains 0.2%(v/v) non-ionic detergent-100 and 3%(v/v) goat serum, on the top of the coverslip.

6.2. Place the whole staining plate on ice and move it under the xenon light source for photobleaching for at least 60 min, with a light intensity at 1,800 µmol photons/m2·s.

6.3. After photobleaching and blocking, carefully remove the blocking buffer by adsorbing it with the edge of a paper towel.

Note: The intensity of xenon light is so high that a pair of proper sunglasses is needed for eye protection. Meanwhile, wrap the light source and the plate using aluminum foil to avoid the leaking of light, which may cause eye damage. Check the coverslip every 15 min to make sure that the coverslip remains moist. If the coverslip is dry, add 50 µL of blocking buffer.

7. Antibody Binding

7.1. Dissolve anti-*Anabaena* FtsZ antibody in 200 µL of water according to the manufacturer's instructions.

7.2. Dilute 1 µL of anti-FtsZ antibody into 99 µL of blocking buffer. Add 50 µL of diluted primary antibody on the coverslip and incubate it at room temperature for 30 min.

7.3. Transfer the coverslip to the washing dish. Add 1 mL of PBS buffer and place the washing dish on a shake. Gently shake the washing dish for 5 min. Repeat this step 3x to wash the coverslip.

7.4. Transfer the coverslip to the staining dish. Dilute 1 µL of a secondary antibody into 500 µL of blocking buffer. Add 50 µL of diluted secondary antibody onto the coverslip and incubate it for 30 min in the dark at room temperature.

7.5. Transfer the coverslip to the washing dish. Wash it with 1 mL of PBS buffer on a shaker. Gently shake the washing dish for 5 min. Wrap the washing dish with aluminum paper to make it light-proof. Repeat this step 3x.

7.6. Add 500 µL of formaldehyde freshly prepared from 4% paraformaldehyde (PFA) (w/v) to the well. Incubate the coverslip for 15 min in the dark at room temperature.

7.7. Remove the formaldehyde and repeat the washing step 3x.

7.8. Store the coverslip in PBS buffer at 4 °C in the dark until imaging.

8. Preparation of the STORM Imaging Buffer

8.1. Prepare 1 mL of imaging buffer according to **Table 1**, immediately before the STORM imaging.

Note: As the shelf life of the imaging buffer is about 2 h, prepare it fresh, right before usage. Avoid vortexing after the addition of glucose oxidase and catalase.

CAUTION: Methyl viologen is poisonous material. Please handle it with particular care. Cyclooctatetraene is classified as a carcinogen Cat. 1 chemical. Please avoid inhalation and skin contact and make the cyclooctatetraene stock solution and imaging buffer in a chemical hood.

9. Image Acquisition of STORM Data

9.1. Load the coverslip in the loading chamber (**Figure 1**). Load the freshly prepared imaging buffer gently into the chamber to avoid washing off the cyanobacterial cells. Place a rectangular coverslip on the top of the imaging buffer to prevent its reaction with oxygen in the air. Avoid trapping air bubbles underneath the coverslip.

9.2. Turn on the camera, the LED light, and the laser. Open STORM software for image acquisition, centroid position determination, and sample drifting correction[13].

9.3. Add half a drop of immersion oil on top of the lens. Load the chamber and make sure the lens of the objective makes contact with the coverslip.

9.4. Examine the signal with a 750-nm laser.

Note: Always include a second antibody-minus-staining negative control to ensure that the autofluorescence is diminished.

9.5. Identify a sample area that contains both cells and fiducial markers. Start the software for sample drifting correction.

Note: In general, the ideal sample area contains 10 - 30 cells. Meanwhile, the cells should be separated well to avoid any overlapping cells.

9.6. Acquire one wide-field image as a reference, with the camera electron multiplication (EM) gain at 300 and an exposure time of 30 ms (**Figure 2A**).

9.7. Increase the 750-nm excitation laser intensity to a higher power, approximately 4.5 kW/cm^2. Once the fluorophores have transitioned into a sparse blinking pattern, acquire one super-resolution image by collecting 10,000 frames at 33 Hz (**Figure 2B**).

Note: If the fluorophores are not isolated, wait for a couple of minutes until more fluorophores are switched to the dark state and the isolated single molecules flicker sequentially. The number of frames described here is suitable for our sample and needs to be optimized for other targeted structures.

10. Reconstruction of Super-resolution Images from Raw Data

10.1. Use a plugin called **QuickPALM** (**Table of Materials**)[14] in ImageJ to reconstruct a 3-D color super-resolution image.

Note: A preliminary chromatic image (**Figure 2C**) along with a color-coded scale bar (**Figure 2C**) will appear. The color represents the depth on the z-axis.

10.2. To demonstrate the 3-D structure in a video, construct a stack of super-resolution images with z-axis sectioned every 10 nm using **QuickPALM**[14]. Adjust the brightness of the stacks and duplicate the stack of one target cell. Use a plugin called **3D Viewer** (**Table of Materials**)[15] in ImageJ to generate the 3-D image of the cell. Record the rotation of the 3-D structure for demonstration purposes.

REPRESENTATIVE RESULTS:

STORM achieves super-resolution imaging by activating individual photoswitchable fluorophores stochastically. The location of every fluorophore is recorded and a super-resolution image is then constructed based on these locations[4]. Therefore, the precision of the fluorophore location is important for the super-resolution image reconstruction. The absorption spectra of *Prochlorococcus* peak at 447 nm and 680 nm, and *Prochlorococcus* has a minimum absorption at wavelengths above 700 nm[16]. However, *Prochlorococcus* MED4 cells still emitted high autofluorescence when exposed to an extremely high intensity of the 750-nm laser (**Figure 3A**), which is required for STORM imaging. Thus, the high autofluorescence background of *Prochlorococcus* cells heavily interferes with super-resolution imaging.

In order to utilize STORM to investigate protein organizations in *Prochlorococcus*, we developed a photobleaching method. After exposure to a white light of high intensity for 30 min, the autofluorescence of *Prochlorococcus* MED4 cells decreased (**Figure 3B**), although several cells with autofluorescence were still detected. We further elongated the photobleaching time to 60 min and found that the majority of the cells lost their autofluorescence (**Figure 3C**). These results indicate that the photobleaching method we developed here can greatly decrease the autofluorescence in photosynthetic organisms.

After photobleaching, we were able to use STORM to visualize the cell division protein FtsZ in *Prochlorococcus* MED4 cells. The cyanobacterium *Prochlorococcus* is the smallest and the most abundant photosynthetic organism on earth[17]. The diameter of a *Prochlorococcus* cell is only 500 - 700 nm[18]. With such a small cell size, it is impossible to visualize the FtsZ ring organization in *Prochlorococcus* cells using conventional wide-field fluorescence microscopy (**Figure 2A**). However, STORM has a spatial resolution of 9.6 nm in the xy-plane and 41.6 nm in the z-axis. Using STORM, we were able to reveal a detailed morphology of the FtsZ ring in *Prochlorococcus* (**Figures 2B** and **4**). By rotating 3-D STORM images, we identified four different types of FtsZ ring morphologies: clusters (**Figure 4A**, **Movie S1**), an incomplete ring (**Figure 4B**, **Movie S2**), a complete ring (**Figure 4C**, **Movie S3**), and double rings (**Figure 4D**, **Movie S4**). Gaps were observed in the complete FtsZ rings of *Prochlorococcus* (**Figure 4C**, **Movie S3**), which is similar to that found in *Caulobacter cresentus*[19]. These four types of FtsZ ring morphologies showed the assembly process of the FtsZ ring during the cell cycle of *Prochlorococcus*, and this study helped us to understand the role of the FtsZ ring during the cell division of *Prochlorococcus*[9].

FIGURE AND TABLE LEGENDS:

Figure 1: Components of the loading chamber and the assembled chamber with the coverslips and imaging buffer for STORM imaging. The coverslip with the sample was placed on the groove of the bottom part first. The upper part was then carefully screwed on to the bottom part. The imaging buffer was added in the loading chamber and then carefully covered with a square coverslip. Bubbles should be avoided to minimize any movement that may influence the accuracy of the measurement.

Figure 2: Representative wide-field, 2-D STORM, and 3-D color STORM images of FtsZ in *Prochlorococcus* MED4. The images were taken from the same field of view using regular (**A**) wide-field microscopy, (**B**) 2-D STORM, and (**C**) 3-D color STORM. The colors in panel **C** indicate

the depth of the fluorescent signals on the z-axis. The numbers in panel **C** indicate the cells of interest. The scale bars = 1 µm.

Figure 3: Photobleaching of *Prochlorococcus* MED4. Fixed *Prochlorococcus* MED4 cells were (**A**) not photobleached, or they were photobleached for (**B**) 30 min and (**C**) 60 min. The XD-300 xenon light was used at an intensity of 1,800 µmol/m^2·s. Cells were excited by a 750-nm laser at the same intensity as was used for STORM imaging. The autofluorescences were imaged with the same filters as used in STORM to make sure the presence of minimal autofluorescence to affect STORM. The scale bars = 2 µm.

Figure 4: Representative STORM images of four FtsZ ring morphologies. Four different FtsZ ring morphologies were observed in *Prochlorococcus*: (**A**) clusters, (**B**) an incomplete ring, (**C**) a complete ring, and (**D**) double rings. For the same cell, images of (i) wide-field fluorescence microscopy, (ii) STORM on the xy-plane, and (iii) STORM after rotation were shown. The 3-D movies corresponding to the cells in panels **A**, **B**, **C**, and **D** are shown in Movies S1, S2, S3, and S4, respectively. The scale bars = 500 nm.

Table 1: Recipe for the imaging buffer.

Movie S1: Clusters of FtsZ proteins observed in *Prochlorococcus* MED4 cells.

Movie S2: An incomplete ring of FtsZ proteins observed in *Prochlorococcus* MED4 cells.

Movie S3: A complete ring of FtsZ proteins observed in *Prochlorococcus* MED4 cells.

Movie S4: A double-ring of FtsZ proteins observed in *Prochlorococcus* MED4 cells.

DISCUSSION:

In this protocol, we described a procedure to significantly reduce the autofluorescence of the cyanobacterium *Prochlorococcus* (**Figure 3C**) and, then, immunostain the proteins in the cells, which enabled us to utilize STORM to study the 3-D FtsZ ring morphologies in *Prochlorococcus* (**Figure 4**). This protocol might be adopted for super-resolution imaging in other photosynthetic organisms.

Previous studies on photosynthetic organisms mainly used SIM to achieve super-resolution imaging because SIM does not require lasers of high intensity. However, the spatial resolution of SIM is only ~100 nm[1], which is much lower than that of STORM. In this study, the spatial resolution of STORM images could reach 9.6 nm in the xy-plane and 41.6 nm in the z-axis[9]. The improved resolution provided by photobleaching and STORM imaging allows researchers to study photosynthetic organisms in unprecedented detail.

Photobleaching prior to STORM is a critical step that enables researchers to observe the location and dynamics of proteins in autofluorescent organisms. Besides cyanobacteria, we have demonstrated that autofluorescence of the flowering plant *Arabidopsis thaliana* could also be quenched after 60 min of photobleaching[9]. The photobleaching has been widely used, for instance for improving the signal-to-noise ratio and the visualization of multiple biomarkers sequentially[20]. It was also demonstrated that photobleaching before imaging is advantageous when observing an autofluorescence sample under Raman spectroscopy[21]. Therefore, it might be feasible to apply this photobleaching method to reduce the autofluorescence of other photosynthetic organisms, including eukaryotic algae, vascular plants, and organisms containing proteorhodopsin and bacteriochlorophyll.

Photosynthetic organisms contain different pigments that have different absorption spectra; therefore, organic dyes and fluorescent proteins need to be prudently selected. For example, in this study, a dye with a maximum excitation wavelength of around 750 nm was used. Although two channels (750 nm and 650 nm) are available for the STORM presented here, *Prochlorococcus* autofluorescent signals could still be observed after being exposed under a 650-nm laser, even after prolonged photobleaching. This is probably because 650 nm is one of the major absorption wavelengths for *Prochlorococcus*. On the other hand, some *Synechococcus* and red algae contain phycoerythrin as their pigment[22], which has an absorption spectrum from 488 nm to 560 nm and a minimum absorption at 650 nm[23]. Therefore, it might be feasible to use a 650-nm channel instead of a 750-nm channel to study the proteins within these phycoerythrin-containing autotrophs.

In summary, a proper combination of photobleaching and STORM provides a powerful approach to understand the protein organization in photosynthetic cells in detail, which will further reveal their dynamics and potential function.

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DISCLOSURES
The authors have nothing to disclose.

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Figure 1

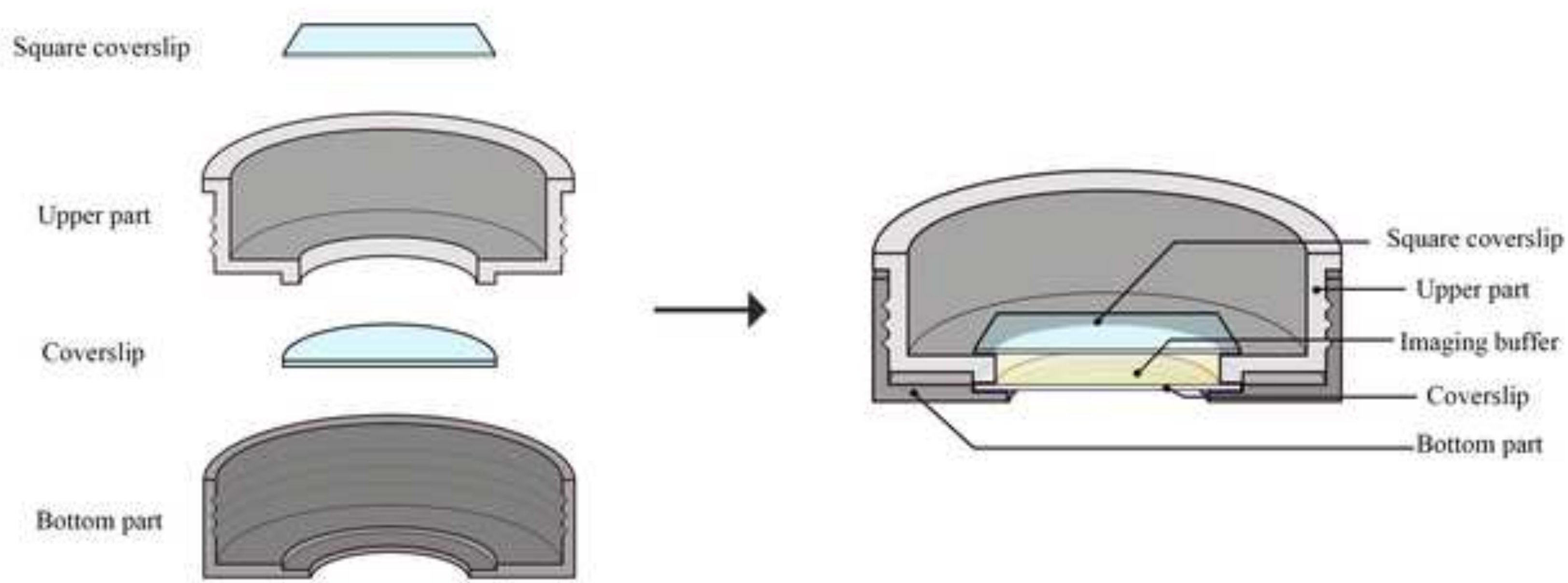
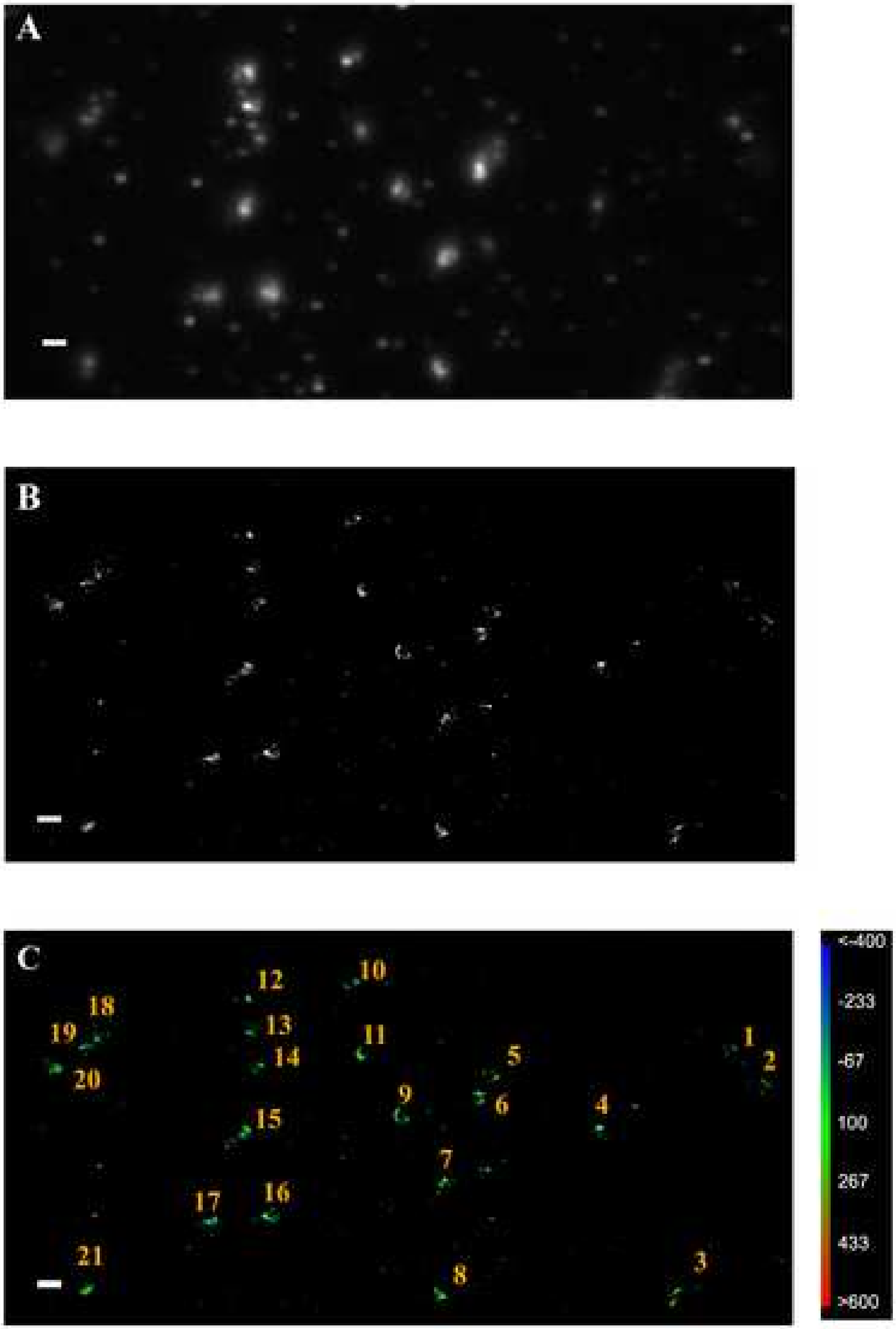

Square coverslip
Upper part
Coverslip
Bottom part
Square coverslip
Upper part
Imaging buffer
Coverslip
Bottom part

Figure 2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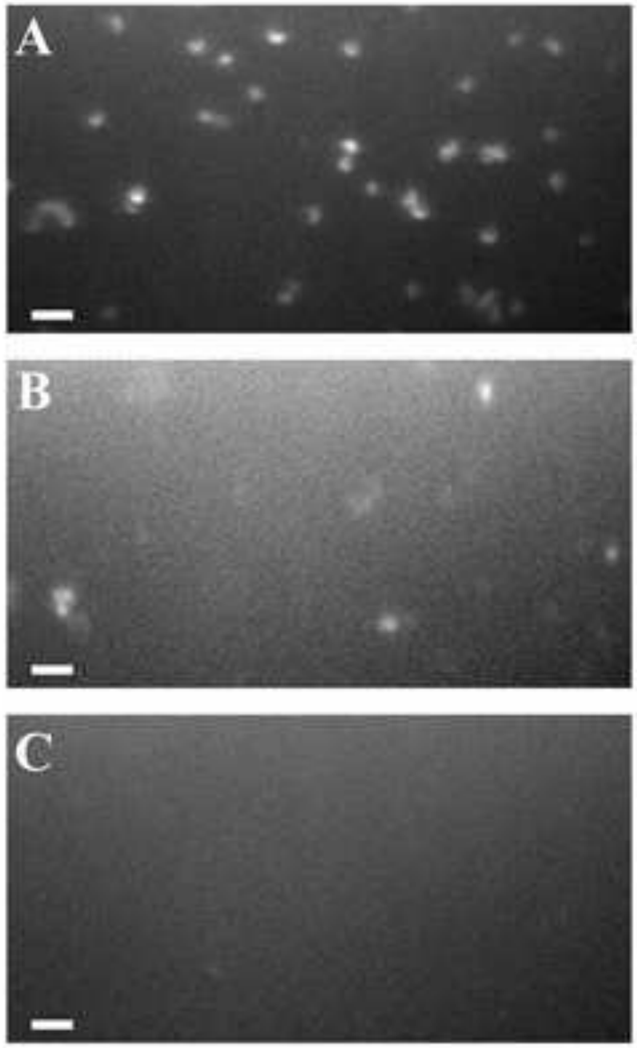
A
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C

Figure 4

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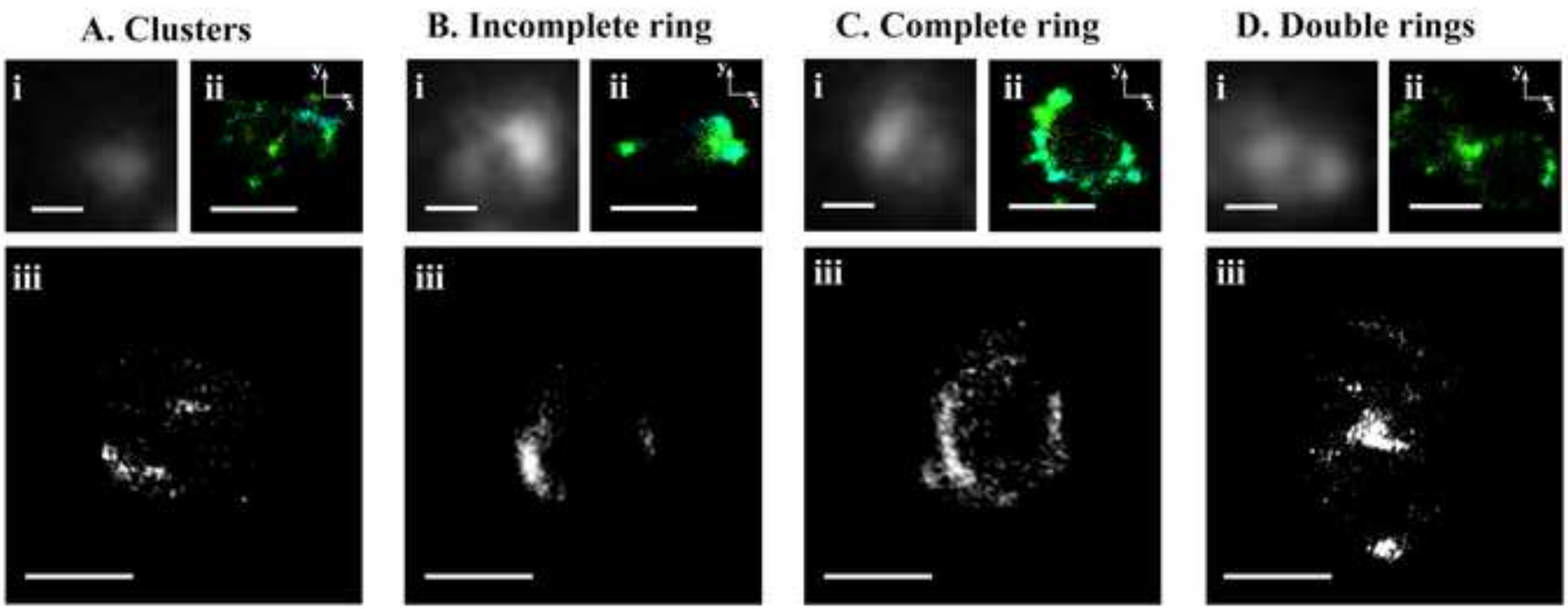

S1

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Table 1. Recipe for imaging buffer

Chemicals	Stock solution	Final concentration
glucose	N/A	10% (w/v)
Tris-Cl (pH 8.0)	0.5 M	50 mM
Ascorbic acid	100 mM	1 mM
Methyl viologen	100 mM	1 mM
Cyclooctatetraene	200 mM	2 mM
TCEP	0.5 M	25 mM
Glucose oxidase	56 mg/ml	0.56 mg/ml
Catalase	4 mg/ml	40 μg/ml

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Polystyrene particles	Spherotech	PP-20-10	2.0-2.4 µm
Coverslip	Marienfeld	0111580	18 mm ∅, Thickness No. 1
Ethanol	Scharlau	ET00021000	
Poly-L-lysine hydrobromide	Sigma-Aldrich	P9155	mol wt 70,000-150,000
Paraformaldehyde	Sigma-Aldrich	158127	
Glutaraldehyde solution, 50%	Sigma-Aldrich	340855	
PBS	Sigma	P3813	
Triton X-100	Sigma	T8787	
EDTA Disodium Salt, 2-hydrate	Gold biotechnology	E-210-500	
Trizma base	Sigma	T1503	
Lysozyme	Sigma	L6876	
Goat serum	Sigma	G9023	
anti-*Anabaena* FtsZ antibody	Agrisera	AS07217	
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody	Life Technologies	A-21039	conjugated with Alexa Fluor 750
D-Glucose Anhydrous	Fisher Scientific	D16-1	
L-Ascorbic Acid	Sigma-Aldrich	A5960	
Methyl Viologen	Sigma-Aldrich	856177	
Cyclooctatetraene	Sigma-Aldrich	138924	
tris(2-carboxyethyl)phosphine (TCEP)	Sigma-Aldrich	646547	
Glucose Oxidase	Sigma-Aldrich	G2133	
Catalase	Sigma-Aldrich	C9322	
XD-300 Xenon light source			250 W
STORM microscope	NBI	SRiS microscope	
Rohdea	NBI	SRiS 3.0	software for imaging acquisition
Luna	NBI	SRiS 3.0	software for drifting correction
QuickPALM			https://code.google.com/archive/p/quickpalm/wiki

is

Home

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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CORRESPONDING AUTHOR

Name:	Qinglu Zeng
Department:	Department of Ocean Science
Institution:	The Hong Kong University of Science and Technology
Title:	Assistant Professor

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1. SUMMARY:

The format has been followed.

Commented [A1]: Formatted please check.

2. SUMMARY: After photobleaching, STORM is used to obtain 3D super-resolution images of the cyanobacterial FtsZ ring.

"STORM" had been expanded as stochastic optical reconstruction microscopy. And STORM is not a trademark or commercial name.

Commented [A2]: Please expand during first time use. Please ensure that STORM is not a trademarked or commercial name.

3. Section 1.1: Grow the axenic *Prochlorococcus* strain MED4 in the seawater-based Pro99 medium[12]. Maintain *Prochlorococcus* cultures at 23 °C under the light with an intensity of 35 µmol photons/m^{2}s.

The volume (5 ml) has been updated in section 1.1. Temperature and light intensity had been included in section 1.1. Rotation is not needed; therefore, we do not mention it.

Pro99 medium is not a commercial medium. It is widely used to culture cyanobacteria. The paper we cited describes the recipe of Pro99 medium in detail.

The revised Section 1.1 is this: "Inoculate 1 ml axenic *Prochlorococcus* MED4 to 5 ml of the seawater-based Pro99 medium. Grow *Prochlorococcus* MED4 at 23 °C under the light with an intensity of 35 µmol photons/m^{2}s."

Commented [A3]: Grow how? how much in what volume? What is the temperature used, rotation if any? We need all the details for filming purpose.

Commented [A4]: Is this commercial/trademarked term? If yes we will need a generic name.

Commented [A5]: Please use this format for the units throughout the manuscript.

4. Section 1.2: Measure the concentration of the cultures by flow cytometry. When the concentration reaches approximately 10^8 cell/mL, collect 1 mL log-phase culture into a 1.5 mL tube.

Since the flow cytometry is not the major part of this protocol, we don't want to introduce too much about the details of flow cytometry. Instead, we'd like to provide the time it takes to reach an appropriate number of cells. And section 1.2 has been combined with section 1.1. Meanwhile, a note has been added.

"1.1 Transfer 1 ml axenic *Prochlorococcus* MED4 to 5 mL of the seawater-based Pro99 medium. Grow the *Prochlorococcus* MED4 at 23 °C under the light with an intensity of 35 µmol photons/m^{2}s. Five days later, collect 1 mL culture into a 1.5 mL tube.

Note: Five days after inoculation, *Prochlorococcus* MED4 will reach the late phase, with approximately 10^8 cell/ml, which is appropriate for STORM imaging."

Commented [A6]: How? Need details if this needs to be filmed,

Commented [A7]: How much time does it takes. What is the starting amount?

5. Section 1.4: Spin down the sample at 13,500 x g for 1 min, then remove the supernatant and re-suspend the cells in 100 µL Pro99 medium [12]. Store the sample at 4 °C until immunostaining.

No. The recipe of the Pro99 was described in the paper we cited.

Commented [A8]: Is this commercial?

6. Section 5.1: Remove the PBS buffer. Add 1 mL freshly prepared permeabilization buffer into the well of washing dish, which contains 0.05% non-ionic detergent-100 (v/v), 10 mM EDTA, 10 mM Tris (pH 8.0) and 0.2 mg/mL lysozyme.

Yes. The sentence has been modified as follows:

"5.1. Remove the PBS buffer using a pipette. Add 1 mL freshly prepared permeabilization buffer into the well of washing dish."

Commented [A9]: How? using a pipette

7. Section 5.3: Add 1 mL PBS buffer and place the washing dish on a shaker. Gently shake the washing dish for 5 min, then remove the PBS buffer. Repeat this step three times to wash the coverslip.

Commented [A10]: Manually?

The washing dish is shaken on shaker automatically, not manually. The sentence has been modified as follows:

"Add 1 mL PBS buffer and place the washing dish on a shaker which gently shakes the washing dish for 5 min. Then remove the PBS buffer."

8. Section 9.2: Turn on the camera, the LED light and the laser. Open STORM softwares, Rohdea and Luna. Use Rohdea for image acquisition and centroid position determination. Use Luna for sample drifting correction with 1-nm accuracy[13].

Commented [A11]: We cannot use trademarked/commercial/copyrighted names in the manuscript. Please use generic term instead.

The Luna and Rohdea have been replaced by generic name "software". The sentence has been rewritten as follows:

"Turn on the camera, the LED light and the laser. Open STORM software for image acquisition, centroid position determination and sample drifting correction[13]."

9. Section 9.5: Identify a sample area that contains both cells and fiducial markers. Start Luna to remove sample drifting in real time.

Commented [A12]: How? please provide all the button clicks, graphical user interface to do so.

The graphical user interface has been provided in supplemental material (Figure S1). The sentence has been modified as follows:

"9.5. Identify a sample area that contains both cells and fiducial markers. Start the software for sample drifting correction."

10. Note: The ideal sample area needs to have adequate number of cells. Meanwhile, the cells should be separated well to avoid any overlapping cells.

Commented [A13]: This is redundant. Please quantify what is meant by adequate.

Based on our experience, 10-30 cells per view is an adequate number. The sentence is replaced as follows:

"Note: In general, the ideal sample area contains 10-30 cells. Meanwhile, the cells should be separated well to avoid any overlapping cells."

11. Section 9.6: Acquire one wide-field image as a reference, with camera electron multiplication (EM) gain at 300 and an exposure time at 30 ms (**Figure2A**).

Commented [A14]: Acquire how?

The interface and buttons are provided in supplemental material (Figure S1).

The sentence has been changed as "Acquire one wide-field image as reference with camera electron multiplication (EM) gain at 300 and an exposure time at 30 ms (**Figure 2A**)."

12. Section 9.7: Increase the 750-nm excitation laser to a higher power, approximately 4.5 kW/cm^2. Once the fluorophores have transitioned into a sparse blinking pattern, acquire one super-resolution image by collecting 10,000 frames at 33 Hz (**Figure2B**).

Commented [A15]: By button clicks or knob turns? Please provide all the graphical user interface and button clicks for the same.

The interface and buttons are provided in supplemental material (Figure S1). The sentence has been changed as:

"9.7. Increase the 750-nm excitation laser intensity to a higher power, approximately 4.5 kW/cm2. Once the fluorophores have transitioned into a sparse blinking pattern, acquire one super-resolution image by collecting 10,000 frames at 33 Hz (Figure 2B).

13. Section 10.1: Use a plugin called “QuickPALM” in ImageJ to reconstruct a three-dimension Color super-resolution image.

The interface and buttons are provided in supplemental material (Figure S2). The “QuickPALM” is an open source plugin. The reference of “QuickPALM” and related website have been cited.

The protocol has been revised as follows:

“10.1. Use a plugin called “QuickPALM” in ImageJ to reconstruct a three-dimension color super-resolution image.”

14. Section 10.2: To demonstrate the three-dimension structure in a video, construct a stack of super resolution images with z axis sectioned every 10 nm. Duplicate the stack of one target cell. Use a plugin called “3D Viewer” in ImageJ to generate the 3D image of the cell.

The interface and buttons are provided in supplemental material (Figure S2). The “3D Viewer” is an open source plugin. The reference of “3D Viewer” and related website have been cited.

“10.2. To demonstrate the three-dimension structure in a video, construct a stack of super-resolution images with z axis sectioned every 10 nm using “QuickPALM”. Adjust the brightness of the stacks and Duplicate the stack of one target cell. Use a plugin called “3D Viewer” in ImageJ to generate the 3D image of the cell. Record the rotation of the 3D structure for demonstration purpose.

15.Movie S1: Clusters of FtsZ proteins were observed within *Prochlorococcus* MED4 cells.
Movie S2: An Incomplete ring of FtsZ proteins was observed within *Prochlorococcus* MED4 cells.
Movie S3: A complete ring of FtsZ proteins was observed within *Prochlorococcus* MED4 cells.
Movie S4: A double-ring of FtsZ proteins was observed within *Prochlorococcus* MED4 cells.

Noted.

16. Discussion:

A summary is added as follows:

“In summary, a proper combination of photobleaching and STORM provides a powerful approach to understand the protein organization in photosynthetic cells in detail, which will further reveal protein dynamics and potential function.”

Commented [A16]: How ? please provide all button clicks and graphical user interface. Also if this plugin is not open source please remove the term and use generic term instead. Please refer to the term in the table of materials instead and cite it here.

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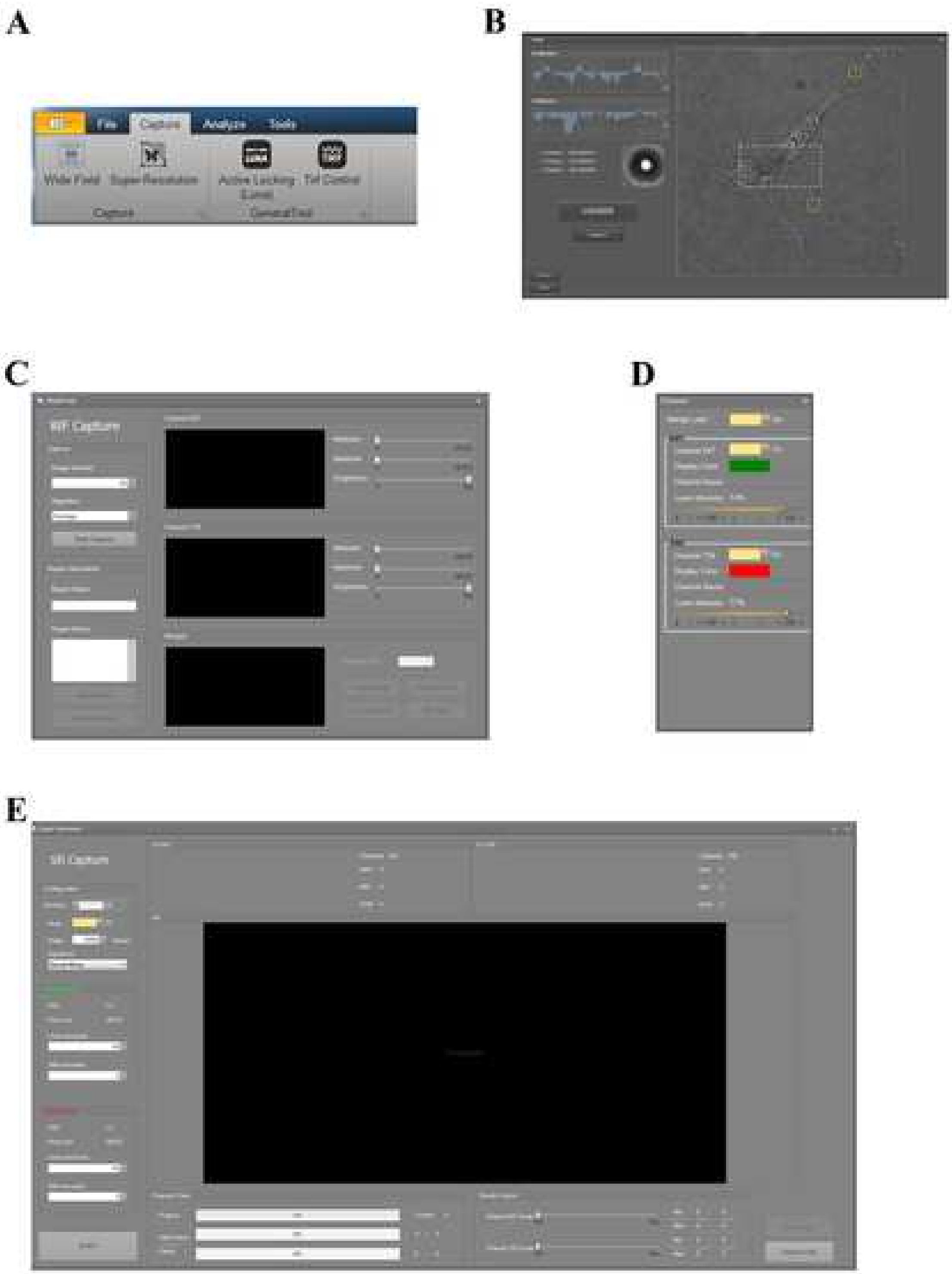

Figure S1. The graphical user interface for STORM imaging acquisition and processing. (A) The main operation panel for imaging acquisition. (B) The sample active locking operation panel. (C) The user interface for wide field image acquisition. (D) Control panel for laser intensity adjustment. (E) The user interface for super-resolution image acquisition.

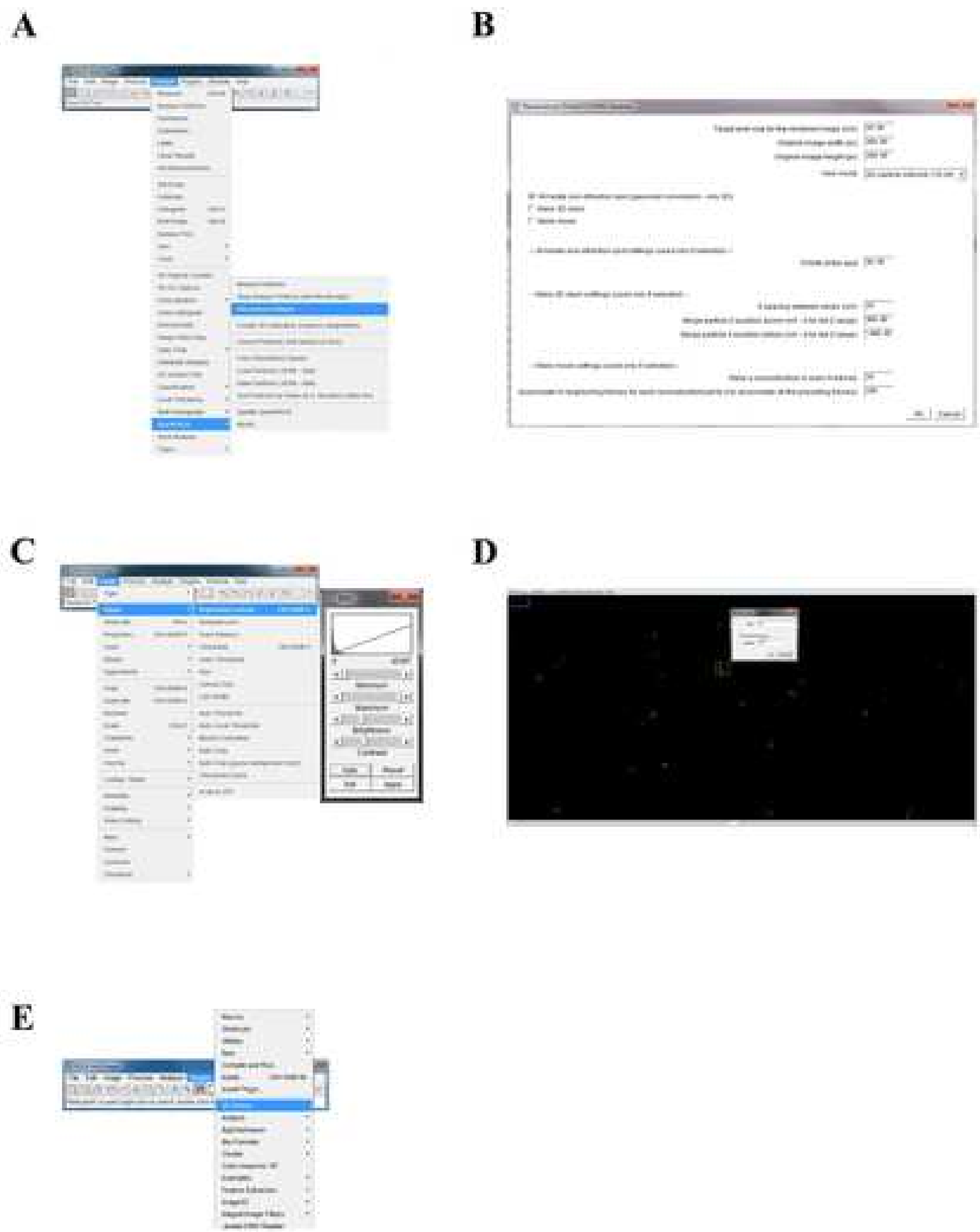

Figure S2. The construction of STORM image and 3D image using Fiji ImageJ. (A) The process of reconstructs dataset. (B) The main interface of "Quick PALM" reconstruct dataset. (C) The graphic user interface for brightness adjustment. (D) The graphical user interface for stack duplication. (E) The process of 3D imaging construction.