

3D super-resolution microscopy of bacterial division machinery

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Abstract. Super-resolution microscopy is a promising tool for the field of microbiology, as bacteria sizes are comparable to the resolution limit of light microscopy. Bacterial division machinery and FtsZ protein in particular attract much attention of scientists who use different super-resolution microscopy techniques, but most of the available data on FtsZ structures was obtained using two-dimensional (2D) super-resolution microscopy. Using 3D single-molecule localization microscopy (SMLM, namely dSTORM) to visualize FtsZ, we demonstrate that this approach allows more accurate interpretation of super-resolution images and provides new opportunities for the study of complex structures like bacterial divisome.

1. Introduction

Single-molecule localization microscopy (SMLM) is a powerful fluorescence microscopy technique, surpassing the diffraction limit of resolution by an order of magnitude [1]. SMLM appears to be a flexible tool to study bacterial division machinery and provides possibilities to investigate complex mechanisms, including arrangement of proteins which are involved in bacterial division.

Cytokinesis in bacteria is based on formation of a ring-like structure (Z-ring) in the middle of dividing cell which is composed of several proteins. One of these proteins – FtsZ – is a prokaryotic tubulin homologue that plays a key role in cell division [2, 3]. The Z-ring guides septum formation and thereby allows to segregate future daughter cells, thus it is crucial to ensure proper Z-ring localization. There are several regulation systems that provide correct Z-ring positioning in the cell [4]. In *Escherichia coli* two partially overlapping systems are involved in this process – Min-system and nucleoid occlusion that restrict Z-ring formation to the middle of the cell by direct interactions with FtsZ.

FtsZ was the first identified cytoskeletal protein among prokaryotes and is considered to be an ancestor of tubulin, but unlike tubulin, FtsZ does not form hollow tubes [5]. FtsZ is a highly conserved protein and is present in most bacteria and archaea. It binds to inner plasma membrane via accessory proteins (FtsA, ZipA in *E.coli*) and attracts several downstream proteins which are involved in cell

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wall synthesis and carry out septum formation, thus Z-ring is considered to be a kind of scaffold in this process.

FtsZ functions have been extensively investigated both *in vitro* and *in vivo* [4, 6]. Using *in vitro* approach it was shown that FtsZ in physiological conditions is able to polymerize into filaments with length up to 200 nm and thickness of 1 subunit [6]. FtsZ bundling ability varies from one species to another and depends on conditions. In appropriate conditions FtsZ is capable of forming longer and thicker filaments, including bundles, tubules, ribbons and other higher-order structures, although usually these conditions are far from those present in cells [7]. To explain how FtsZ which forms short filaments *in vitro* could form relatively large Z-ring, two alternative models were proposed. The first model suggests that the Z-ring is a relatively disordered array of short FtsZ filaments that weakly interact with each other and possibly form bundles. The second model depicts the Z-ring as an ordered structure (continuous ring or helix), formed by annealed short filaments [6].

To investigate FtsZ organization in the Z-ring and understand which of two models better fits *in vivo* situation, two main visualization approaches were used, first based on fluorescence microscopy (FM), including super-resolution, and second – cryo-electron tomography (CryoET). Super-resolution FM (SMLM, SIM, STED) allowed to obtain images of the Z-ring which seems to demonstrate uneven or discontinuous structure in several species [8-11]. At the same time data obtained using CryoET supports continuous Z-ring arrangement and thereby is in clear contradiction with FM data [12]. It seems to be important to gather more data on the Z-ring structure under different conditions and using different methods to resolve this controversy.

In the current work 3D SMLM in combination with immunofluorescence labeling was utilized to obtain images of Z-rings during exponential growth in *E.coli*.

2. Materials and methods

All experiments were conducted using Top10 *E. coli* strain transformed with pGEX-4T-2 plasmid. Cells were grown overnight in LB medium supplemented with ampicillin (100 µg/ml) at 37°C, then passaged to fresh medium and grown until OD₆₀₀≈0.5. Then cells were fixed directly in culture by addition of sodium-phosphate buffer (pH 7.4, 30 mM), formaldehyde (2.6%) and glutaraldehyde (0.04%) for 10 minutes at room temperature followed by 50 minutes on ice.

Cells were harvested by centrifugation, washed in PBS three times and immobilized on poly-L-lysine coated coverslips. Then cells were permeabilized using 0.1% Triton X-100 in PBS, followed by 10 µg/ml lysozyme in GTE buffer (glucose 50 mM, Tris 32.5 mM pH=7.5, EDTA 10 mM), each for 5 minutes.

Next cells were blocked using 2% w/v BSA in PBS (PBS-BSA) for 1 hour and then incubated overnight with rabbit anti-FtsZ polyclonal antibodies (Agrisera) diluted 1:200 in PBS-BSA. Cells were washed 5 times with 0.01% Tween-20 in PBS, incubated with goat anti-rabbit secondary antibodies (Alexa 647 conjugated F(ab')₂ fragments of goat anti-rabbit antibodies (LifeTechnologies)) diluted 1:100 in PBS-BSA for 1 hour at room temperature and washed another 5 times as described above.

To prevent sample drift during data acquisition, custom active stabilization system was used. To track sample position, polystyrene microspheres (2.1 µm, SpheroTech) were immobilized on the coverglass surface.

All images were obtained using custom set-up based on AxioImager.Z1 (Carl Zeiss) motorized microscope which was described previously [13]. Images were acquired by EM-CCD camera (Andor iXon 897) at 100× total magnification. One CCD pixel corresponded to 108 nm in the focal plane. LF635/LP-B-000 (Semrock) filter set was used to visualize Alexa 647 fluorescence (excitation filter was removed to enable dye activation using a 405 nm laser).

Raw images were acquired using MicroManager [14] in PBS-Tris buffer with pH 7.5, containing 10% w/v glucose, 10 mM 2-mercaptoethylamine combined with 50 mM 2-mercaptoethanol, 2 mM cyclooctatetraene and an oxygen scavenging system (2.5 mM protocatechuic acid and 50 nM protocatechuate 3,4-dioxygenase) [15]. Alexa 647 fluorescence was excited using 635 nm diode laser with a power density of approximately 1 kW/cm² at the focal plane and 2000-10000 frames containing

individual Alexa 647 molecule images were acquired. To measure axial positions of individual molecules, a weak cylindrical lens (ESCO, 8 m focal length) was introduced to the optical path between the objective and the tube lens.

Data processing was performed using ImageJ, SMLM reconstruction was carried out using ThunderSTORM plugin for ImageJ [16].

3. Results and discussion

SMLM previously allowed us to obtain two-dimensional (2D) images of Z-ring on different division stages and to demonstrate that Z-ring thickens during constriction [17]. We were not able to detect Z-ring thickening using conventional fluorescence microscopy, and this fact emphasizes the usefulness of SMLM for the study of bacterial division. However, images obtained using 2D SMLM represent projections, and this fact substantially complicates interpretation of visualized structures. To implement 3D SMLM, a weak cylindrical lens was introduced into the optical path between the objective and the tube lens.

SMLM with artificial astigmatism allowed us to obtain 3D images of Z-rings in *E.coli* cells. The method used in this work allows to visualize area about 1 μm in depth what is comparable with bacterium outer diameter. Obtained images demonstrate hollow ring-like structure in the middle of some cells. Z-rings on these images demonstrate uneven structure reminiscent of beads on a string (Fig. 1). These structures are in strong agreement with data obtained using 2D-SMLM and most other methods [8-12, 17]. However, we were not able to show that observed discontinuity is inherent to actual FtsZ distribution since several stochastic effects of the method used could sufficiently influence obtained images.

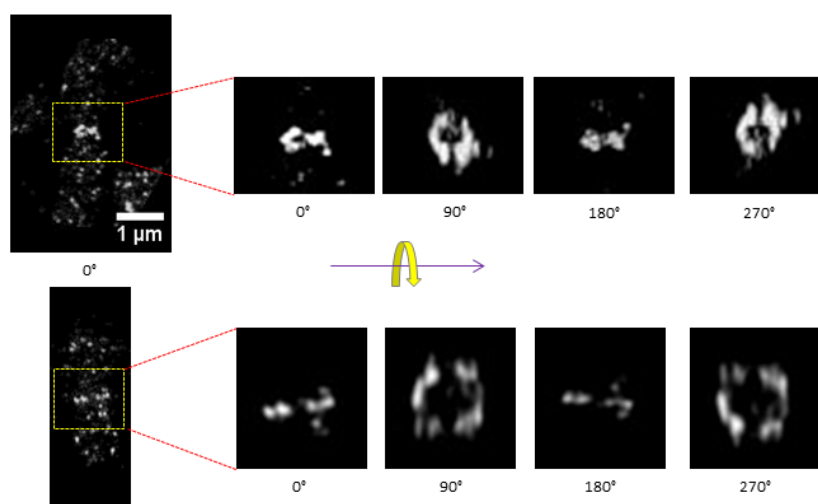


Figure 1. 3D reconstruction of FtsZ ring structure (Z-ring) in *E. coli* dividing cells. 2D image of cell (left panel) and a fragment of the same cell image (in yellow rectangle) obtained using rendering with four different angles (from 0 to 270 degrees).

To rule out the possibility that method used could distort actual FtsZ distribution in Z-ring, further work is needed. For instance, to decrease effects of labeling stochasticity, other labeling techniques which provide one label to one target molecule ratio should be used (e.g. fluorescent fusion proteins). Utilization of photoactivatable or photoconvertible dyes may allow to reduce probability of multiple detections of a single molecule which also affect obtained images.

4. Conclusions

In this work 3D-SMLM in combination with immunofluorescence labeling was successfully used to visualize Z-ring in fixed *E.coli* cells. Obtained images demonstrate uneven FtsZ distribution in the Z-ring which is in agreement with data obtained using other FM methods, but contradicts the results of cryo-electron tomography. To verify if the Z-ring is actually organized in that way, further study is needed.

3D-SMLM expands opportunities of localization microscopy and allows to investigate complicated structures like bacterial divisome. SMLM In combination with immunofluorescent staining can be especially useful in the cases of microorganisms for which effective genetic manipulation protocols haven't yet been developed (e.g. Mycoplasma).

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