Multi-color localization microscopy of fixed cells as a promising tool to study organization of bacterial cytoskeleton

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Abstract. Localization microscopy allows visualization of biological structures with resolution well below the diffraction limit. Localization microscopy was used to study FtsZ organization in Escherichia coli previously in combination with fluorescent protein labeling, but the fact that fluorescent chimeric protein was unable to rescue temperature-sensitive ftsZ mutants suggests that obtained images may not represent native FtsZ structures faithfully. Indirect immunolabeling of FtsZ not only overcomes this problem, but also allows the use of the powerful visualization methods arsenal available for different structures in fixed cells. In this work we simultaneously obtained super-resolution images of FtsZ structures and diffraction-limited or super-resolution images of DNA and cell surface in E. coli, which allows for the study of the spatial arrangement of FtsZ structures with respect to the nucleoid positions and septum formation.

1. Introduction.
Localization microscopy is a new method of fluorescence microscopy that increases its resolution about ten-fold and well below the diffraction limit. The main idea behind this method is the temporal separation of individual molecules fluorescence, their separate detection and subsequent localization [1, 2]. Increased resolution is especially valuable for the study of prokaryotic organisms as the sizes of most of them are comparable to the diffraction limit of optical microscopy which makes localization microscopy a method of choice for the investigation of bacteria and archaea internal organization [3].

Bacterial division is a robust and complex process with a number of cytoskeleton-like proteins involved in it. They play crucial roles in determination of division site and management of the following division stages. FtsZ, one of prokaryotic homologs of eukaryotic tubulin, plays central role in the division of most bacteria, being the first actor in the formation of the Z-ring at the middle of dividing cells (Figure 1,A) [4, 5]. Previously structures formed by FtsZ in Escherichia coli were visualized with the use of FtsZ fluorescent chimeras [6, 7], but chimeric proteins were unable to substitute wild-type FtsZ in these cells. It suggests that fluorescent labelling may perturb native FtsZ structures. It is therefore important to compare these results with the ones obtained using an alternative immunolabelling method, which lacks some of the drawbacks of fluorescent protein labelling.
Proper FtsZ positioning in the cell is controlled by two major systems: Min-system and nucleoid occlusion (Figure 1,B). Oscillating MinCDE system inhibits FtsZ polymerization to prevent septum formation at the cell poles [8]. Nucleoid occlusion protein SlmA binds to DNA and inhibits FtsZ polymerization over nucleoid therefore preventing improper septum positioning that may lead to DNA breakdown [9]. It is therefore of great interest to investigate spatial relations between structures formed by FtsZ, nucleoid and cell surface. Immunofluorescent labeling of fixed cells provides numerous possibilities of DNA, cell surface and protein visualization using wide selection of dyes, allowing for dual-color and possibly three-color super-resolution microscopy of FtsZ, DNA and cell surface, which shall provide deeper understanding of FtsZ functioning and regulation in *E. coli*.

### 2. Materials and methods.

In all experiments Top10 *E. coli* strain transformed with pGEX-4T-2 plasmid was used. The use of a plasmid encoding an ampicillin resistance protein was aimed to simplify bacteria handling and prevent possible contamination by the means of ampicillin resistance selection. Cells were inoculated from the frozen stock into fresh LB medium with added ampicillin (100 µg/ml), grown for 5 hours at 37°C to OD≈0.3 and fixed directly in culture medium by the addition of sodium-phosphate buffer (pH 7.4, final concentration 30 mM), formaldehyde (final concentration 2.6%) and glutaraldehyde (final concentration 0.04%) for 10 minutes at room temperature followed by 50 minutes on ice. To quench autofluorescence cells were incubated for 10 minutes in 30 mM sodium borohydride in PBS. After that cells were washed 3 times with PBS and immobilized on poly-L-lysine coated coverslips with nonspecifically adsorbed polystyrene microspheres (2.35 µm, Bangs Laboratories). Then cells were

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**Figure 1. E. coli division machinery.** A) Cross-sectional scheme of the division machinery, depicting major proteins that form the Z-ring. FtsZ is a central division protein that polymerizes to form filaments that further assemble to form the Z-ring. ZipA anchors the Z-ring to the inner membrane. FtsA is an ATP-binding protein that stabilizes the Z-ring and links it to the transmembrane protein FtsI. B) Nucleoid occlusion (NO) and Min-system are the two inhibitory systems that assure proper Z-ring positioning by preventing the Z-ring assembly at random positions along the cell. NO prevents Z-ring formation over nucleoid by the means of DNA-binding protein SlmA. Min-system, comprised of MinC, MinD and MinE proteins, prevents Z-ring formation at cell poles by oscillating along the cell axis.
permeabilized with 0.1% Triton X-100 in PBS and 10 µg/ml lysozyme in GTE buffer (glucose 50 mM, Tris 32.5 mM pH=7.5, EDTA 10 mM), each for 5 minutes. After blocking with 2% w/v BSA in PBS (PBS-BSA) for 20 minutes, cells were incubated with anti-FtsZ polyclonal rabbit antibodies (Agrisera) diluted 1 to 200 in PBS-BSA overnight at +4°C. Then cells were washed 5 times with 0.01% Tween-20 in PBS, incubated with secondary goat anti-rabbit antibodies diluted in PBS-BSA for 1 hour at room temperature and washed another 5 times with 0.01% Tween-20 in PBS. In the case of DNA and FtsZ localization microscopy Alexa 555 conjugated secondary antibodies from Bios were used in 1 to 100 dilution and cells were then stained with DNA dye TOTO-3 (250 nM in PBS) for 10 minutes. In case of FtsZ and cell surface (cell wall and cytoplasmic membrane stained by wheat germ agglutinin(WGA) [10]) localization microscopy Alexa 647 conjugated F(ab’)2 fragments of goat anti-rabbit antibodies (LifeTechnologies) were used in 1 to 1000 dilution to visualize FtsZ and cells were additionally incubated with 5 µg/ml WGA conjugated with Alexa 488 dye for 10 minutes and 100 nM propidium iodide (LifeTechnologies) for another 10 minutes to visualize DNA. The dye combinations were chosen according to the compatibility of the dye spectra and conditions required to perform localization microscopy using these dyes. After that cells were post-fixed with 2.6% formaldehyde for 10 minutes.

Figure 2. Experimental setup. CD – microscope condenser, CL – cylindrical lens, CS – coverslip, DM – dichroic mirror, EM-CCD – microscope camera, FC – fluorescence filter cube, IL – transmitted light lamp, IRF 1 – infrared filter, passing 830 nm, IRF 2 – infrared filter, blocking 830 nm, L- lens, M – mirror, ML – mercury lamp for fluorescence excitation, O – microscope objective, PC - polarizing cube, PS – piezo stage, QD – quadrant detector, S – sample, Sh – shutter, TL – microscope tube lens.

Super-resolution images were obtained using custom experimental set-up (Figure 2) based on Axioimager.Z1 (Carl Zeiss) upright microscope, equipped with custom laser excitation block and feedback sample position stabilization system based on microsphere position detection in 3D using a quadrant detector on which the image of the bead is created by a near-infrared diode laser [11, 12]. DNA-FtsZ super-resolution images were obtained in the buffer consisting of 10% glucose, 0.5 mg/ml glucose oxidase (Sigma), 40 µg/ml catalase (Sigma), 100 mM 2-mercaptoethylamine, 2 mM cyclooctatetraen in PBS. In the case of FtsZ, DNA and cell surface imaging GOC oxygen scavenging system was replaced with PCA-PCD oxygen scavenging system (25% glycerin, 100mM TRIS-HCl pH 7.4, 100 mM 2-mercaptoethalmine, 5 mM protocatechuic acid, 60 nM protocatechuac 3,4-
dioxygenase and 2 mM cyclooctatetraene) [13]. Super-resolution images were reconstructed using ImageJ [14, 15] plug-in QuickPALM [16].

3. Results and discussion.

Dual-color localization microscopy of FtsZ and DNA in *E. coli* (Figure 3) allowed relative distribution of FtsZ and DNA to be analyzed in fine details. These images showed that FtsZ distribution in dividing cells mirrors that of DNA throughout the cell (Figure 4), with DNA localizing primarily in two bands halfway from the cell center to the poles, while FtsZ forms single- and multi-band structures at the cell center and also concentrates at DNA-free regions near the cell poles.

**Figure 3. DNA and FtsZ super-resolution images.** Columns: 1 - transmitted light, 2 – diffraction-limited (DL) images of the FtsZ structures, 3 - super-resolution (SR) images of the FtsZ structures, 4 – diffraction-limited images of the DNA (nucleoid), 5 – super-resolution images of the DNA (nucleoid), 6 – composite super-resolution image, DNA pseudo colored red and FtsZ pseudo colored green. Scale bar corresponds to 1 µm.

**Figure 4. FtsZ and DNA are distributed inversely in the E. coli cells.** Characteristic distributions of DNA (pseudo colored red) and FtsZ (pseudo colored green) along the cell.

Simultaneous cell surface and FtsZ localization microscopy combined with diffraction-limited DNA visualization (Figure 5) allows us to reveal the spatial arrangement of FtsZ structures relative to the nucleoid and the place of the septum formation. These images also clearly show that FtsZ forms not only the Z-ring, but also a variety of spiral-like and other off-center structures running along the cell axis (Figure 3, 5), which was not observed with the use of fluorescent protein fusions. This
emphasizes the need to compare data obtained by different staining methods to create an accurate model of FtsZ functioning.

**Figure 5. Cell surface and FtsZ super-resolution and diffraction-limited DNA images.** Columns: 1 – transmitted light, 2 – super-resolution (SR) images of the FtsZ structures, 3 – diffraction-limited (DL) images of the DNA (nucleoid), 4 – super-resolution images of the cell surface stained with WGA, 5 – composite images; DNA pseudo colored blue, FtsZ - green and cell surface - red. Scale bar corresponds to 1 µm.

4. **Conclusions.**
Multicolor super-resolution localization microscopy combined with immunofluorescent labeling proves to be a powerful method for the study of bacteria division machinery. Immunolabeling allows the visualization of FtsZ structures outside the Z-ring, which was not achieved with fluorescent protein fusion super-resolution microscopy. Simultaneous DNA and FtsZ super-resolution images enable deeper study of nucleoid occlusion system, whereas cell surface super-resolution imaging allows to visualize sub-membrane localization of the FtsZ structures in the cells and the place of the septum. Developed methods can be applied to the in-depth study of the FtsZ assembly regulation and its rearrangements during stress response. Three-dimensional localization microscopy should allow whole-cell visualization of FtsZ structures.

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