Super-resolution microscopy of Mollicutes cells

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Abstract. Super-resolution microscopy is especially useful for visualization of structures in small cells which sizes are comparable to the diffraction limit, for instance, in Mollicutes species. In this work we obtained super-resolution images of FtsZ and IbpA protein structures in fixed Acholeplasma laidlawii & Mycoplasma gallisepticum cells using immunofluorescence staining.

1. Introduction
Mycoplasmas (Mollicutes class) are the smallest known organisms that can live on artificial media. Mycoplasmas are able to cause acute and chronic diseases of human, animals and plants [1]. All species of Mollicutes lack peptidoglycan layer and show various cell morphology [2]. They also demonstrate reduced genomes which lost a considerable portion of their ancestors’ non-essential genes, thus mycoplasmas are considered to be close to the concept of the “minimal cell” [3]. FtsZ protein is conserved in most mycoplasmas species. FtsZ is a well-known prokaryotic tubulin homologue being a key protein involved in division process in a majority of bacteria. In most bacteria it assembles into a ring structure, the so-called Z-ring, at the site of cytokinesis and recruits downstream proteins to form a large protein machine — the divisome, which major role is considered to be the synthesis of cell-wall [4]. The role of FtsZ in mycoplasmas is not yet clear, in particular due to the lack of cellwall. Also it is believed that Mollicutes have several other than FtsZ cytoskeleton-like proteins, that may help to maintain the shape of the cell. The study of structures formed by cytoskeleton-like proteins in mycoplasmas is complicated by small size of their cells. That’s why super-resolution microscopy techniques seem to be promising solution to study their internal organization. Single-molecule localization microscopy (SMLM) was previously successfully used to obtain images of FtsZ structures in Escherichia coli cells using immunofluorescence staining [4-6]. In current work SMLM was utilized to visualize FtsZ protein of two species – Acholeplasma laidlawii & Mycoplasma gallisepticum and small heat-shock protein IbpA of A. laidlawii with a resolution well below the diffraction limit.

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2. Materials and methods

2.1. Bacterial strains and sample preparation

The following strains of microorganisms were used in the work: *Acholeplasma laidlawii* PG8 (Collection of cell cultures of the Institute of Cytology RAS), *Mycoplasma gallisepticum* S6 (kindly provided by Gleb Fisunov, Federal Research and Clinical Centre of Physical-Chemical Medicine Federal Medical Biological Agency, Moscow, Russia). Cells were grown at 37°C in custom-made medium, based on 25 mM Tris-HCl, pH=7.4, and supplemented with ampicillin (100 µg/ml), peptone (2% w/v), NaCl (0.5 % w/v), KCl (0.13 % w/v), horse serum (10% v/v), yeast dialysate (5% v/v), glucose (1%) and phenol red indicator. After change in medium color from red to yellow, cells were harvested by centrifugation, washed in PBS and fixed by addition of formaldehyde (2%) and glutaraldehyde (0.1%) for 10 minutes at room temperature followed by 50 minutes on ice. After that the cells were 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 for 5 minutes. Next cells were blocked using 2% w/v BSA in PBS (PBS-BSA) and after that with addition of non-rabbit antibodies in order to avoid nonspecific binding of target antibodies, both steps for 0.5 hour. Then samples were incubated overnight with custom-made rabbit anti-FtsZ or anti-IbpA polyclonal antibodies at the final dilution from 1:20 to 1:100 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-made active stabilization system was used [7]. To track sample position, polystyrene microspheres (2.1 µm, Spherotech) were immobilized on the coverslip surface. The antibodies used in the experiments were preliminarily obtained by immunizing rabbits with appropriate recombinant FtsZ and IbpA proteins to obtain an immune response. After collection, the blood serum was affinity purified using FtsZ proteins cross-linked with the CNBr-Activated Sepharose 4B (GE Healthcare Life Sciences) resin according to the producer protocol.

2.2. Experimental setup and data processing

All images were obtained using custom set-up based on AxioImager.Z1 (Carl Zeiss) motorized microscope which was described previously [8]. 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 [9] in PBS-Tris buffer with pH 7.5, containing 10% w/v glucose, 10 mM 2-mercaptoethamine combined with 50 mM 2-mercaptoethanol, 2 mM cyclooctatetraene and an oxygen scavenging system (2.5 mM protocatechuic acid and 50 mM protocatechuic acid 3,4-dioxygenase) [10]. 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.

Data processing was performed using ImageJ, SMLM reconstruction was carried out using ThunderSTORM plugin for ImageJ [11].

3. Results and discussion

Using SMLM in combination with immunofluorescence staining we were able to obtain fine super-resolution images of IbpA protein in *A. laidlawii*. After 1.5 hour incubation upon conditions of cold shock IbpA protein in *A.laidlawii* cells seems to form some well-ordered structures such as filaments, arcs or rings (see Figure 1). This behavior may be due to either binding of IbpA to some structures in cell or to tendency of IbpA to multimerize. Further work is needed to explain this observation.

The same approach was used to visualize FtsZ protein in two mycoplasmas – *A.laidlawii* & *M.gallisepticum* (see Figure 2). It is clear that SMLM technique allows to visualize structures in more
details that are unreachable in the case of conventional fluorescence microscopy. Preliminary data does not yet allow us to draw specific conclusions based on the structures formed by FtsZ and IbpA proteins. We can only conclude that in some cells these proteins form structures resembling cytoskeleton elements. To interpret the patterns of protein distributions in cells obtained by the LM method, it is necessary to accumulate more experimental data, as well as data verification using other methods (for example, electron microscopy).

**Figure 1. SMLM visualization of IbpA protein in *Acholeplasma laidlawii* cells.** Left panel – cells before fixation were incubated for 1.5 hours at +4°C, right – at +37°C. DNA Fluo – conventional fluorescence image of DNA stained with YOYO-1 dye; IbpA SMLM – super-resolution image of IbpA protein. Scale bar corresponds to 1 μm.
Figure 2. SMLM visualization of FtsZ protein in *Acholeplasma laidlawii* (left) and *Mycoplasma gallisepticum* (right) cells. TL – transmitted light image, Membrane Fluo – conventional fluorescence image of membrane, FtsZ Fluo – conventional fluorescence image of FtsZ, DNA Fluo – conventional fluorescence image of DNA stained with DAPI dye; FtsZ SMLM – super-resolution image of FtsZ protein. Scale bar corresponds to 1 μm.

4. Conclusion
Using the method of single-molecule localization microscopy, we visualized FtsZ and IbpA proteins in two mycoplasma species. The data obtained show that the method of localization microscopy makes it possible to visualize intracellular structures in mycoplasmas that could not be resolved using conventional fluorescence microscopy. Further study of these proteins using SMLM and other techniques may help to better understand their function in corresponding species.

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