Visualization of the intracellular structures of bacteria using expansion microscopy

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Abstract. Expansion microscopy (ExM) is a powerful tool which allows to visualize intracellular structures with increased resolution due to physical widening of the sample. ExM resolution is comparable to that of some super-resolution microscopy techniques, but it does not require the use of complex and expensive optical equipment, thereby providing improvement in resolution even if relatively simple microscopes are used. In this work we successfully utilized ExM to visualize structures formed by FtsZ protein and DNA in Escherichia coli cells. The results of the work demonstrate that structures formed by both FtsZ and DNA are highly sensitive to the sample preparation procedure which should be optimized in each case of the ExM use.

1. Introduction

Light microscopy is one of the most important tools in biology and medicine [1]. Many discoveries have become possible only due to improvement in the resolution of microscopes. However, the diffraction limit imposes fundamental restriction on the resolution of light microscopy (about 0.2 μm) and complicates the study of small biological objects, for example, the localization of proteins in the cell [2]. One of the ways to overcome the diffraction limit is the utilization of super-resolution microscopy methods. These methods allow to achieve a resolution which is far beyond the diffraction limit - about ten nanometers [3]. They may be divided into 2 groups - deterministic and stochastic methods. Deterministic methods are based on the known information about the spatial distribution of the exciting and emitted light [4]. They can significantly increase the resolution, but require the use of complex and expensive optical equipment. Stochastic methods are based on the fundamental possibility of separating the emission of single fluorophores in time, using their chemical nature, and thus localizing individual fluorescent molecules with significantly enhanced accuracy [4]. For these methods, it is necessary to use special photoactivatable or photo-switchable fluorescent proteins or synthetic dyes, as well as complex optical equipment, which is not always possible. Nevertheless, it was the development of such methods of super-resolution microscopy that made it possible for the first time to visualize intracellular structures more accurately. For example, we can point at the deciphering of the Z-ring organization in the bacterial cell, which appears to have discontinuous structure, and consists of many clusters of different dimensions randomly overlapping with each other [5, 6].
In this work, we used an alternative method that allowed us to significantly increase the resolution without overcoming the diffraction limit - expansion microscopy (ExM) [7]. This method does not require the use of complex optical equipment and is based on the physical expansion of samples by about 4 times in each dimension. During ExM experiments samples are mounted into a polyelectrolyte hydrogel, which expands when placed in deionized water [8]. ExM allowed us to obtain detailed fluorescent images of the Z-ring and DNA (nucleoids) in *Escherichia coli* cells with a resolution significantly higher than conventional light microscopy methods allow.

2. Materials and methods
In current study, BW27783 strain of *Escherichia coli* was used, in which FtsZ (bacterial cytoskeleton protein) is fused with the green fluorescent protein mNeonGreen [9]. This fusion allowed us to visualize various structures in the bacterial cell using fluorescence microscopy. Cells were cultivated in lysogeny broth (LB) medium at 37 °C up to the middle of exponential growth phase and then fixed by adding directly into medium formaldehyde and glutaraldehyde in the sodium phosphate buffer (pH = 7.5, 32 mM) to the final concentration of 2.6%, and 0.04%, respectively. Alternatively, cells were washed twice in phosphate-buffered saline (PBS) and fixed in PBS containing 4% formaldehyde and 0.04% glutaraldehyde. After fixation, cells were washed with PBS, then with 100 mM glycine in PBS to quench the fixation. Permeabilization was carried out using 1% Triton X-100 in PBS. After permeabilization, samples were rinsed three times with PBS. Then, the samples were treated overnight with 0.1 mg / ml Acryloyl-1-X SE in PBS, due to which reactive groups were added to the biomolecules, and then these groups were included in the hydrogel polymers during its polymerization. To form a gel, the sample was placed in a demountable microscopic chamber and incubated with a monomer solution which recipe is described in paper [8] with addition of tetramethylethylenediamine 10% (v / v) and ammonium persulfate 10% (w / v) for several hours at 37 °C. After polymerization, the gel was removed from the chamber and subjected to proteolytic cleavage by autoclaving for 1 hour at 121 °C or adding a solution containing proteinase K at a concentration of 8 U / ml. Then the gel was placed into water, in which, due to the interaction of charged groups, the gel expanded with the cells. TOTO-3 DNA-specific dye was also added to water to final concentration of 100 nM. The acquisition was carried out on a Nikon Ti-E fluorescence microscope with sCMOS Andor Zyla 4.2 camera. To detect dyes fluorescence the following filter sets were used: YFP HQ (YFP-2427B (Semrock)) for mNeonGreen, and Cy5 (Cy5-4040C (Semrock)) for TOTO-3. Data processing was carried out using the ImageJ software [10].

3. Results and discussion
3.1. The influence of proteolytic cleavage conditions on the expansion efficiency of the sample
In the literature there is a large number of protocols for the implementation of ExM, however, in each case, separate optimization of sample preparation is required. All the protocols we tested allowed us to obtain a physical expansion of *E. coli* cells to a greater or lesser extent (see Figure 1). To assess the degree of increase in sample size after expansion, the average cell length was measured before and after expansion. The results demonstrate that, in the case of *E. coli* cells, the degree of expansion is highly dependent on proteolytic cleavage conditions. Either autoclaving or proteinase K was used for digestion in current work. When treating the sample with proteinase K, the cell length during subsequent expansion increased on average 3-4 times from 5 to 16-19 microns, while when autoclaving it was only 1.5-2.5 times to 7-13 microns, respectively. Probably, this may be due to the fact that autoclaving provides a mild proteolysis, in which there is no sufficient destruction of the bonds between the components of the cell, which prevents its expansion. Thus, for efficient expansion of *E. coli* cells, a protocol involving cleavage with proteinase K is more preferable.
Figure 1. Micrographs of *E. coli* cells before and after the expansion procedure (only Proteinase K digestion was used). Cells were visualized in the fluorescent channel of the mNeonGreen protein. The left column shows the cells before expansion (A, C), the right - after expansion (B, D). Note that different cells are shown on left and right panels. Scale bar - 5 micrometers.

3.2. The influence of sample preparation conditions on the preservation of the Z-ring structure

On the example of the Z-ring we were able to compare how various conditions of sample preparation during the ExM implementation affect the preservation of the bacterial cytoskeleton. For fixation, formaldehyde was used at a final concentration of 2.6% or paraformaldehyde 4% (w / v), for digestion - proteinase K cleavage or autoclaving. It was possible to observe the Z-ring in some cells only with the formaldehyde fixation and the proteinase K cleavage (see Figure 2). When using other combinations of fixation and cleavage, the structures were not preserved, and after expansion in the cells, either a uniform fluorescence signal or its uneven foci that did not correspond to the localization of FtsZ in the cells before expansion could be observed. The use of lysozyme at a concentration of 2 mg / ml overnight at 37 °C instead of treatment with Triton X-100 (to digest the cell wall) was also tested, and there were no differences in the results.
Figure 2. Fluorescent images of structures formed by the FtsZ protein obtained by ExM. The FtsZ-mNeonGreen fusion protein was marked with false green (mNeonGreen fluorescence channel), DNA was marked with false magenta (TOTO-3 dye). Scale bar - 5 micrometers.

3.3. DNA expansion is uneven
It was also found that during expansion microscopy, the expansion of DNA occurs unevenly, which differs significantly from the corresponding distribution before expansion (see Figure 3). Perhaps this phenomenon affects the preservation of other intracellular structures, for instance, those that are bound to the chromosome. Probably, in addition to partial proteolysis using proteinase K, partial DNA cleavage (for example, by use of restriction endonucleases) is required before the sample expansion, which should lead to a more adequate distribution of DNA throughout the cell.

Figure 3. Images of the DNA structures in E. coli before and after expansion. Before expansion (A), the DNA is separated into 2 nucleoids corresponding to future daughter cells. After expansion (B), the DNA forms uneven structures in the cells, for instance, some strands or bundles which are not present on images before expansion. Scale bar - 5 micrometers.
4. Conclusion
The results obtained indicate that expansion microscopy allows visualization of FtsZ structures in \textit{E. coli} cells with a significantly higher resolution than in case of conventional fluorescence microscopy. We estimated spatial resolution of ExM using resolution of light microscopy and average expansion coefficient of the cells, and thus it was about 60 nanometers. Due to this, it is possible to observe and analyze the localization of protein structures in the cell using this method. Conditions were optimized that ensure effective expansion and preservation of the structure of the Z-ring, however, additional optimization of the protocol is required in order to ensure the preservation of the DNA structure in the cell. In the future, we plan to use this method to analyze the structures of bacteria such as mycoplasmas, whose characteristic size is comparable to the diffraction limit and thus complicates their investigation.

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