Video Article

Visualization of DNA Compaction in Cyanobacteria by High-voltage Cryo-electron Tomography

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Abstract

This protocol describes how to visualize the transient DNA compaction in cyanobacteria. DNA compaction is a dramatic cytoplasmic event recently found to occur in some cyanobacteria before cell division. However, due to the large cell size and the transient character, it is difficult to investigate the structure in detail. To overcome the difficulties, first, DNA compaction is reproducibly produced in the cyanobacterium *Synechococcus elongatus* PCC 7942 by synchronous culture using 12 h each light/dark cycle. Second, DNA compaction is monitored by fluorescence microscopy and captured by rapid freezing. Third, the detailed structure of DNA compacted cells is visualized in three dimensions (3D) by high voltage cryo-electron tomography. This set of methods is widely applicable to investigate transient structures in bacteria, *e.g.* cell division, chromosome segregation, phage infection *etc*., which are monitored by fluorescence microscopy and directly visualized by cryo-electron tomography at appropriate time points.

Video Link

The video component of this article can be found at https://www.jove.com/video/57197/

Introduction

DNA compaction is a dramatic cytoplasmic event that has been identified in some cyanobacteria. When *Synechococcus elongatus* was cultured under 12 h each light/dark cycle, DNA appeared condensed at the end of the light period, which was clearly different from its appearance at the other time points[1]. It has been suggested that this process is controlled by a circadian clock based on the Kai proteins[2]. Seki *et al.* have reported that the DNA stained with Hoechst 33342 was compacted in *S. elongatus* cells toward the end of the light period and showed a wavy rod-shape under a fluorescence microscope. The compacted DNA then separated into two at the center of the rod as the cell divided, and finally returned to a normal uniform distribution in each daughter cell[3]. However, its transient nature and large size for electron microscopy impeded structural analysis. Murata *et al.* combined several methods, including synchronous culture, fluorescence microscopy, rapid freezing, and high voltage electron cryo-tomography (cryo-HVET), and succeeded in identifying the structure of transient DNA compaction, including the kinetics of polyphosphate bodies (PPBs)[4]. The manuscript provides visual explanation of such a difficult material in detail by combining the experimental procedures.

S. elongatus has a capsule shape, a length of 2 to 5 µm, a width of about 0.5 µm, and the perfect DNA compaction appears in living cells only for a very short time. Therefore, the structural changes occurring in the cyanobacterial DNA compaction were unknown in detail. In order to investigate these structures by electron microscopy, it is necessary to overcome two main technical problems. One is the observation of such a thick specimen of the whole bacterium at near native conditions, and the other is the rapid fixation of a dynamic structure. As for the first problem, the inelastic mean free path (iMFP) of electrons depends on the accelerating voltage of the electron microscope[5]. In a transmission electron microscope (TEM) of 300 kV, it is less than 350 nm. For example, when an ice-embedded cyanobacterium (specimen thickness ≈ 600 nm) is observed in 200kV TEM (iMFP ≈ 250 nm), the structures inside cell are difficult to observe. By contrast, 1 MV TEM (iMFP ≈ 500 nm) can give and image of the cytoplasmic structure throughout the cell (**Figure 1**). In this protocol, as one part of the solution, a high voltage electron microscope (HVEM) at an accelerating voltage of 1 MV was employed. However, facilities that implement HVEM are limited worldwide. Possible alternative solutions are also discussed in the Discussion section. The second problem was solved by cryo-electron microscopy (cryo-EM). This is a powerful tool for visualizing dynamic structures at near native conditions, where the specimen is rapidly frozen in liquid ethane using a rapid freezing device, and the frozen moment is directly observed with a minimum of modifications[6]. Combining with tomography, a snapshot of three-dimensional (3D) structures can be reconstructed from the tilt series[7]. In this experiment, DNA compaction was reproduced in *S. elongatus* using synchronous culture under 12 h each light/dark cycle, and the timing of the freezing of the specimen was determined by monitoring under a fluorescence microscope.

The approaches described here are widely applicable to the study of dynamic structures in bacterial cells, *e.g.* cell division, chromosome segregation and phage infection, and have a potential to open new avenues in microbiological research.

Protocol

1. Synchronous Culture of Cyanobacteria

1. Culture *S. elongatus* PCC 7942 on sterilized BG 11 plate (in a 9 cm sterile plastic petri dish) containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate[8].
2. Place the plates in a growth chamber at 23 °C with a light intensity of 50 μE/m^2/s and subject to 12 h light/12 h dark cycles.
3. Transfer the cells onto fresh BG11 agar plates once a week.
   NOTE: The cultures on the agar will appear as green bands of actively proliferating cells after one week under this culture condition.
4. Take green clumps of cells with a flame sterilized wire loop and streak the cells onto a fresh BG11 agar plate. Do this on a clean bench.

2. Monitoring by Fluorescence Microscopy

1. Use cells cultured on the agar plate for 6 days to observe DNA compaction. Collect cells from the plate at the end of the light period by pouring 1 mL of 0.2 M sucrose solution over the cells. Repeat pouring the solution onto the cells so that most of the cells are collected. Transfer the suspended-cell solution into a micro tube for DNA staining.
2. Add DNA staining dye (*e.g.* Hoechst 33342) solution to 500 μL of the suspended cell solution in a micro tube to a final concentration of 1 μg/mL. Then, keep the tube in the dark for 10 min.
3. Centrifuge for 1 min at 2,000 x *g* to sediment the cells. Discard the supernatant and add 10 μL of 0.2 M sucrose solution to obtain a dense cell suspension.
4. Transfer 1 μL of the solution containing stained cells to a slide glass, put a cover slip and observe with a fluorescence microscope equipped with a UV filter using an objective lens with a magnification of 100X and immersion oil.
5. Confirm that the DNA compaction is observed at this point in most cells, then prepare the sample for the next freezing step.

3. Sample Freezing for Cryo-HVET

1. Set up a plunge-freezing device. Fill the tank with liquid nitrogen and start cooling the cryo-chamber after connecting the tank and the chamber with a Teflon tube.
2. Fill liquid ethane into a small cupper pot inside the chamber after cooling down the chamber to liquid nitrogen temperature. Wear glasses during operation, because liquid ethane is explosive.
3. Glow discharge the carbon-side of a holey carbon-coated EM grid (holey grid) for 30 s at 50 mA using a plasma ion bombarder.
4. Apply 1 μL of BSA gold tracer (15 nm) to the holey grid as a fiducial marker.
5. Apply a 2.5 μL aliquot of cells in the DNA compaction stage to the holey grid. Blot off the excess solution with a filter paper. Plunge the grid into liquid ethane using a plunger in the plunge freezing device immediately.
6. Store the frozen grids in liquid nitrogen storage until they are examined.

4. Cryo-HVET

1. Set up the HVEM at a high voltage of 1 MV.
2. Mount the frozen grid in a cryo-specimen holder for HVEM precooled to -150 °C with liquid nitrogen inside the cryo-workstation, and load it into the HVEM. Be careful to avoid contamination by ice.
3. Select an imaging area at a lower magnification of 1,000X. Adjust the eucentric z-axis height.
4. Tilt the specimen stage to -60° and remove the backlash of the tilting rotation.
5. Adjust the focus near the target location at a magnification of 10,000X. Set an under focus of 6 to 10 μm by deviation from the focused image. For imaging, set the dose to 2 $e^-/Å^{-2}$ or less on the specimen beforehand.
6. Measure the electron dose by the current density on the image screen in HVEM. Take an image on an electron film or by digital camera at the same magnification as in the focusing process.
7. Collect tilt images manually by the same procedure as in (4.5) from -60° to +60° at a tilt angle increment of 2° to 4°.
   NOTE: In many modern electron microscopes, the acquisition of the tilt series is automated by the combination of a digital camera. In that case, follow the instruction manual. For negative films, develop the films for 12 min at 20°C in a developer tank using a full-strength developer and fix in a fixer for 10 min. Digitize the films at a resolution of 4,000 dpi (0.635 nm/pixel on the image) using a flatbed scanner. In the case of a digital camera, subject the collected images directly to the next image processing step. If necessary, reduce the image size by a median filter using two to four binning (size reducing factor) and appropriate software (*e.g.* ImageJ).

5. Tomographic Reconstruction

1. Make a stack image file from the individual tilt images using the command "tif2mrc" or "newstack" in IMOD software[9].
2. Start eTomo GUI software in IMOD and set image parameters: pixel size, fiducial diameter, image rotation, *etc.* Then create scripts.
3. Execute individual programs according to the software listed in tables of materials, where the tilt series is aligned using fiducial markers (with a mean residual error less than 0.5). Finally, reconstruct a 3D tomogram using the SIRT algorithm in IMOD.
4. Extract a region of interest (ROI) from the tomogram and denoise using a denoise filter: an anisotropic diffusion filter in IMOD, a bilateral filter in EMAN[10], or a mathematical morphology filter[11], *etc.*, with appropriate parameters to enhance the contrast.

6. Segmentation of the Feature of Interest

NOTE: The procedure described below is specific to the software used (see **Materials Table**) but other software packages can be used instead. Refer to their user guide.

1. In the 3D Viewer window, open the tomogram file on Amira software and generate an OrthoSlice.
2. In the Segmentation Editor window, create a segmentation file by selecting a new "label field".
3. Manually trace the border of the feature of interest (FOI). Follow the FOI through all tomogram slices. For the second FOI, create a new "material" and repeat the same operation.
4. Generate a surface rendering by selecting the "SurfaceGen" menu. To visualize the segmented volume, select the "SurfaceView" menu. To move, rotate, and zoom in the 3D volume, use the tools in the 3D Viewer window.
5. For automatic segmentation, use the Magic Wand Tool. Click on an object, and adjust the sliders in Display and Masking to cover the range of values so that the object is fully selected by its features.

Representative Results

In a precise synchronous culture under 12 h each light/dark cycle, DNA labeled with Hoechst shows a normal uniform distribution in the dark condition (**Figure 2a**). However, it progressively compacts within the cell during the light period, and appears as a wavy rod-like structure (**Figure 2b**) at the end of the light period. Finally, the rod divides at the center (arrows in **Figure 2c**) and its two parts are distributed into the daughter cells (**Figure 2d**). After cell division, the compacted DNA disappears immediately, and the DNA returns to a normal uniform distribution.

When an aliquot of cells containing the cells in the final stage of DNA compaction were immediately transferred onto a holey grid and rapid frozen in liquid ethane, and the frozen grid was observed by 1 MV cryo-HVEM, the internal structures of cyanobacteria including DNA, thylakoid membrane layers, cell walls, and PPBs, appeared as in a snapshot at the moment of freezing (**Figure 3a**). Many cells showed distinct DNA compaction in the cells (white arrows in **Figure 3a**), and could be easily distinguished from normal cells (white arrowheads in **Figure 3b**). Some exhibited a constriction at the center of the cells as expected before cell division (yellow arrow in **Figure 3a**).

In 3D tomograms, major organelles of the cell could be segmented; cell wall, thylakoid membrane layers, DNA, and PPBs could be distinguished (**Figure 4**). In particular, the compacted DNA was separated by a distinct gap in the cytoplasm where the DNA was surrounded by low density material and the thylakoid membrane layers were distorted along the wavy rod of compacted DNA. Dynamic behavior of PPBs was newly observed: in DNA compacted cells, many small PPBs were seen to adhere to DNA, whereas they are large and fewer in normal cells. In addition, most of the PPBs appeared as pairs, and some DNA appeared to be in a process of separation from the PPBs. This suggested that PPBs themselves are divided into two by DNA duplication and function as suppliers of phosphate for DNA synthesis.

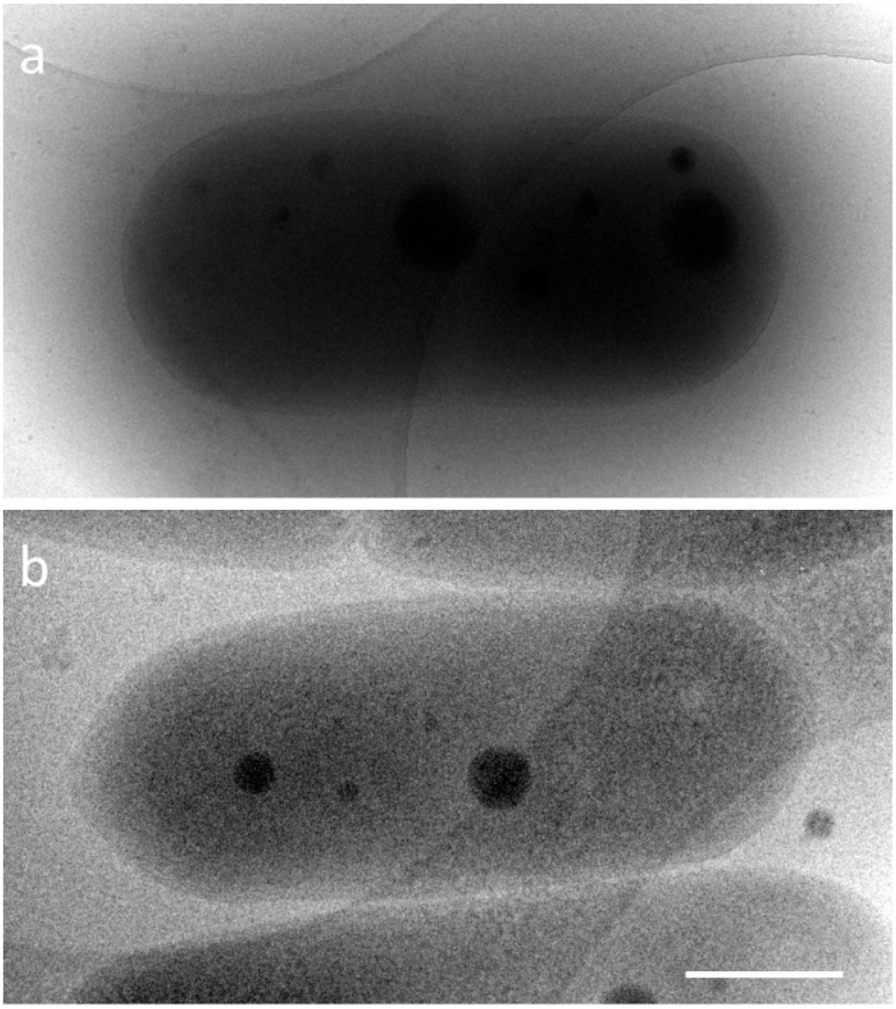

Figure 1. Cryo-EM images of ice-embedded normal cyanobacteria, *S. elongatus* PCC 7942 at different accelerating voltages. a) 200kV and **b)** 1000kV. Scale bar = 500 nm. Please click here to view a larger version of this figure.

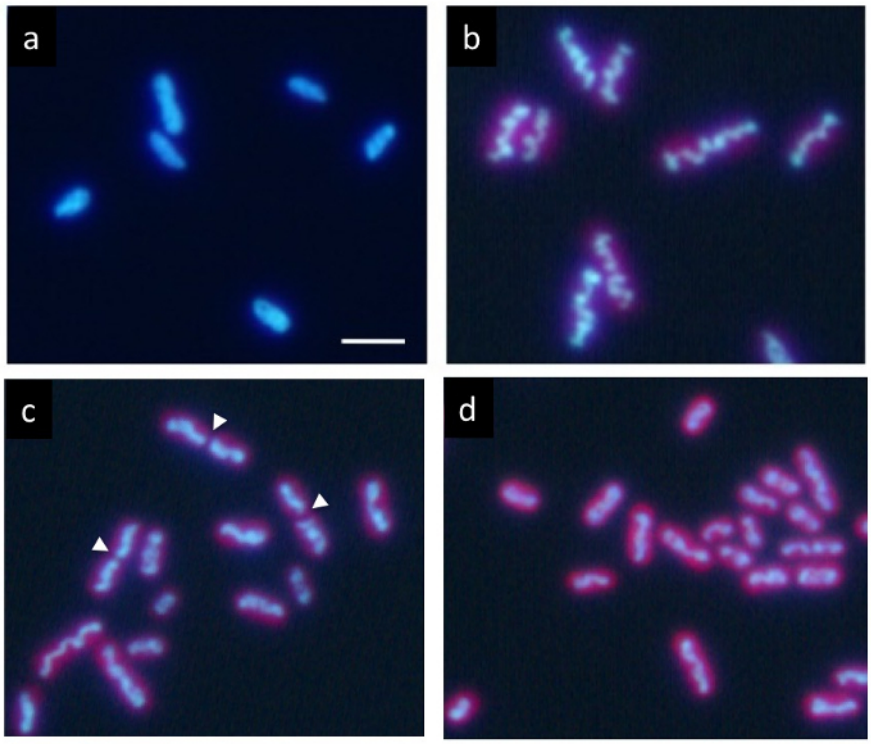

Figure 2: Fluorescence microscopy of cyanobacterium *S. elongatus* cells. After synchronous culture under 12 hour each light/dark cycles, cells were stained with Hoechst 33342. **(a)** Cells after 2 h of onset of the dark state show uniform DNA labeling. The DNA in many cells gradually condensed during the light period. It ultimately formed a thick wavy rod **(b)** typical of DNA compaction. After this stage, the compacted DNA structures quickly divided at their centers (arrowheads) during cell division **(c),** and the two fragments separated into the daughter cells **(d).** The daughter cells returned to uniform DNA labeling again in the dark period. Scale bar = 2 µm. Please click here to view a larger version of this figure.

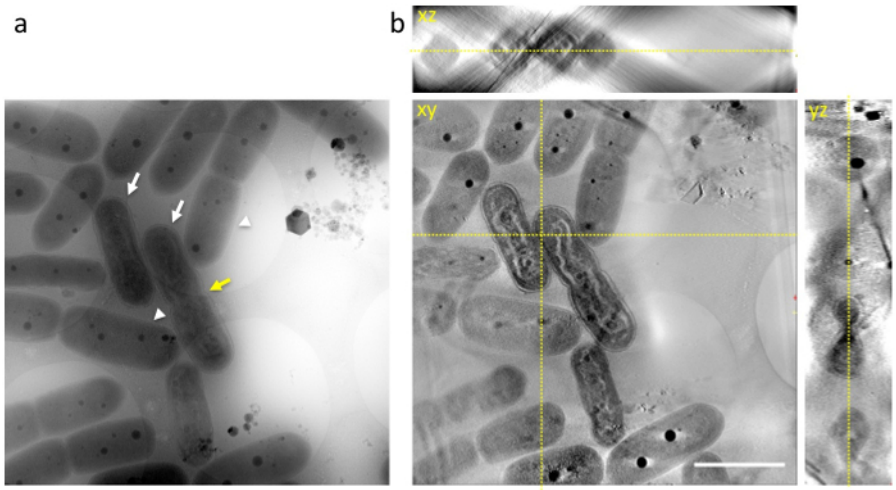

Figure 3: Cryo-HVEM image of cyanobacteria embedded in amorphous ice. (a) shows a raw image at 0° tilt. Images were taken at the end of the light period. Some cells show wavy rod-shaped DNA bodies similar to eukaryotic condensed chromosomes (white arrows). In normal cells, cyanobacteria exhibit a discernable DNA structure within the cytoplasm (white arrowheads). Some exhibit a constriction at the center of the cells as expected before cell division (yellow arrow). **(b)** xy, xz, yz-slices of a 3D tomogram. Yellow dotted lines show intersections of slices. Scale bar = 2 µm. Please click here to view a larger version of this figure.

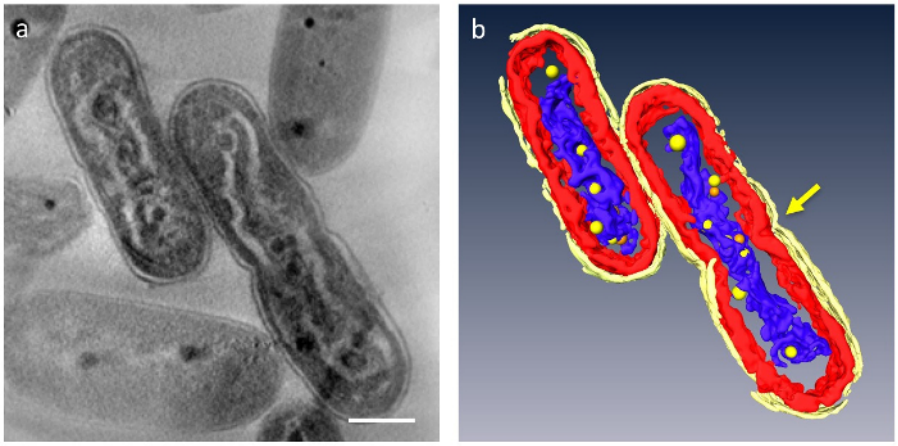

Figure 4: Ultrastructure of cells containing compacted DNA. The major components are segmented: cell wall (bright yellow), thylakoid membranes (red), and DNA (blue). **(a)** shows a z-slice of a 3D tomogram. **(b)** all segments. The constriction indicating cell separation appears in the center of the cell (arrow). PPBs are modeled as yellow or orange spheres; each orange sphere represents the counterpart of the closest yellow sphere. Scale bar = 500 nm. Please click here to view a larger version of this figure.

Discussion

We have presented a sequence of protocols for visualizing transient DNA compaction in cyanobacteria. The basic concept is similar to that of correlative light and electron microscopy (CLEM)[12]. In addition, in this method, live cyanobacteria were monitored by fluorescence microscopy, rapidly frozen on EM grids, and directly visualized with high voltage cryo-electron tomography. As a first application, the detailed structure of DNA compacted bacterial cells was successfully visualized in 3D. Currently, this procedure is specific to this subject, but it will be applied more extensively, with modified methodology in some cases. Here, the advantages, the limitations, and the future possibilities of this method are discussed.

One of the advantages of this method is the 3D visualization of the whole cell. 1 MV HVEM successfully visualized the dynamic structure of the subcellular organelles in the DNA compacted cells. However, the fine structure inside normal cells could not be distinguished due to low image contrast. Increasing inelastic and multiple scattering in thick specimens blurs the image[13]. Zero-loss and most-probable-loss image filtering by an energy filter can improve image contrast by reducing inelastic scattering[14,15], but it will not work for specimens thicker than iMFP. The zero-loss and most-probable-loss peaks drastically decrease with specimen thickness. It is particularly difficult to obtain a sufficient signal-to-noise ratio for the electron sensitive ice-embedded specimens. Murata *et al.* have shown that 1MV scanning transmission microscopy (STEM) gives higher image contrast than a bright field image in plastic embedded yeast cells with 5 µm thickness, where the image contrast is primarily given by amplitude contrast[13]. However, it is expected that the effect of knock-on damage on higher accelerated electrons creates another limitation to the irradiation dose for damage-sensitive cryo-specimens[16]. The application of Volta and Zernike phase plates[17,18] for HVEM may be able to reduce knock-on damage by reducing the total dose in the future. Another limitation to using HVEM for thick specimens comes from the fact that the user facilities that provide HVEM are scarce worldwide.

Using an alternative methodology to observe thick specimens, cryo-STEM tomography at 300 kV has demonstrated high contrast images on frozen-hydrated specimens with thickness in excess of several hundred nanometers[19]. To retrieve phase contrast in cryo-STEM, electron pthychographic microscopy has also been introduced, in which the phase plate in the condenser lens transposes a phase-modulated diffraction to a pixelated 2D detector[20]. The images are retrieved by calculation from multiple diffractions. For direct and fast 3D cryo-imaging of large native frozen samples, cryo-FIB-SEM can also be used[21], where serial sectioning with a focused ion beam and block face imaging is applied for imaging fully hydrated frozen specimens. Although these technologies expand the viewing range of biological specimens, it is difficult to find the target location of the bacteria, *e.g.* labeled bacteria, because the target is completely under the ice and cannot be identified before trimming.

DNA compaction produces a distinct structure in cyanobacteria. DNA compacted cells are readily distinguished even without being stained due to a large density bias within the cells that is not present in normal cells. However, in order to visualize more local events within the cell, it is necessary to transfer the fluorescently labeled ROI into the electron microscope. For correlative light and electron microscopy (CLEM), light microscope images and electron microscope images are generally correlated using fluorescent latex beads or quantum dots on EM finder grids[12]. The labelling particles must be of high electron density in addition to fluorescence. They can accurately and reliably correlate the positions between the two images. Furthermore, by confirming the labeled area with cryo-light microscopy, complete overlap of ROI can be achieved between the two microscopes. When characterizing more detailed structural events in DNA compaction, these particles and cryo-light microscope will be an indispensable tool for a more robust and accurate correlation in the future.

This article shows how to characterize the transient structure of DNA compaction in cyanobacteria by a combination of synchronous culture, fluorescence microscopy and high voltage cryo-electron tomography. This protocol focuses on the observation of compacted DNA. By combining this method with other new technologies mentioned above, it will be possible to investigate the process of DNA compaction in further detail, and suitably modified methods are widely applicable to other dynamic structural events in bacteria.

Disclosures

The authors declare no competing financial interests.

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