Observation of *in vivo* DNA in Ice Embedded whole Cyanobacterial Cells by Hilbert Differential Contrast Transmission Electron Microscopy (HDC-TEM)

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HDC-TEM has opened a way to visualize the ultrastructure of ice embedded whole cells. The extraordinary advantage of this technique is that it exhibits structures close to the living state while retaining all the *in vivo* molecular constituents undisturbed. We attempted to identify *in vivo* DNA by incorporation of BrdU, which conferred electron density to newly synthesized DNA in ice embedded cyanobacterial cells. Localization of Br in the electron dense area in the identical cell was investigated by electron spectroscopic imaging (ESI). Br was also appeared to be associated with polyphosphate bodies, which would indicate a close relationship between newly synthesized DNA and polyphosphate bodies. While ESI indicates the DNA localization, high resolution HDC-TEM reveals the fine fibrous structures *in situ*. The combination of ESI with HDC-TEM will be extremely useful to study the *in vivo* dynamics of DNA synthesis, and its structural and conformational changes close to the living state at high resolution.

Keywords: phase contrast electron microscope, cyanobacteria, *in vivo* DNA, rapid freezing, electron spectroscopic imaging

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1. Introduction

The transmission electron microscope has provided a great deal of information about cellular ultrastructure because of its high resolving power, which is 2 orders of magnitude higher than that of the light microscope. However, lately fluorescent or laser microscopes have become more widely used for cell biological research because of their capability to locate and follow the dynamic change of functional molecules *in vivo* using various fluorescent probes. We have examined the ultrastructure of ice embedded whole cyanobacterial cells with the Hilbert differential contrast transmission electron microscope (HDC-TEM) [1, 2], one of the phase contrast electron microscopes developed recently [3]. The HDC-TEM image can display topographic features by means of a half plane π-phase plate inserted in the back focal plane of the objective lens, achieving an effect similar to the differential interference contrast in light microscopy [4]. The cells observed by HDC-TEM are characterized by smooth cell walls and membranes. Various particles, filamentous and membranous structures fill the cellular space. The aim of this study is to develop a method to identify DNA in these high resolution topographic images of ice embedded whole cells observed by HDC-TEM.

2. HDC-TEM and Conventional TEM Observation

Fig. 1a shows the HDC-TEM image of an ice embedded whole cyanobacterial (*Synechococcus* sp. Strain PCC 7942) cell. Surrounded by smooth cell walls, the thylakoid membranes, carboxysomes, and polyphosphate bodies (arrow) can be identified. The identification was confirmed by comparison with conventional TEM images of ultrathin sections of chemically fixed and resin embedded cells (Fig. 1b). Large electron dense spherical polyphosphate bodies are prominent structures in HDC-TEM images (arrow in Fig. 1a). These structures cannot be observed by conventional TEM because of the difficulty in preserving them through the specimen preparation process and usually appear simply as empty holes in ultrathin sections (arrow in Fig. 1b). The polyphosphate bodies seem to shrink considerably during chemical fixation and dehydration, leaving spaces around them, and are thus, in fact, often eliminated during ultrathin sectioning.

In TEM observation of ultrathin sectioned *Synechococcus* sp. Strain PCC 7942, fibrous structures believed to be DNA are consistently observed radiating from the holes (Fig. 1b, arrowhead) where polyphosphate bodies had been situated. In order to confirm the localization of DNA, ultrathin sections of conventionally prepared specimens were treated with osmium ammine-SO_2 (Fig. 1c),

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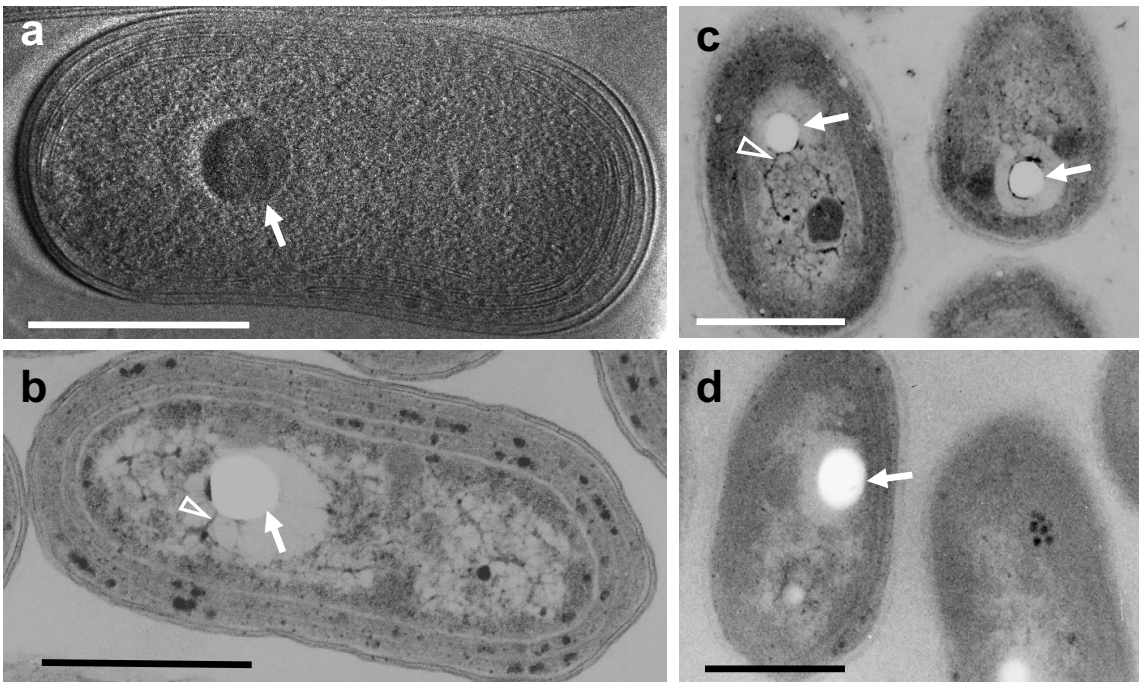

Fig. 1 (a) A 300 kV HDC TEM image of an ice-embedded whole cyanobacterial cell. The polyphosphate body (arrow) is prominent among detailed ultrastructure visualized with high contrast.
(b) A 100 kV conventional TEM image of a chemically fixed, plastic embedded, sectioned cell stained with uranyl acetate and lead citrate. The polyphosphate body is lost during the process leaving an empty hole on the section (arrow). DNA fibers (arrowhead) are radiating from the hole.
(c) A 100 kV conventional TEM image of chemically fixed, resin embedded, sectioned and osmium ammine-SO2 stained cyanobacterial cells. DNA fibers are stained intensely. An intimate association between stained DNA fibers (arrowhead) and the remnant of a polyphosphate body (arrows) is observed.
(d) A 100 kV conventional TEM image of chemically fixed, resin embedded, sectioned cells prior to osmium ammine-SO2 staining. No staining of DNA is discernable in the vicinity of empty hole (arrow). Scale bars, 500 nm.

which is known to be DNA specific [5]. Aggregated fibrous structure dispersed in cells stained intensely. The peripheral portion of the polyphosphate bodies (Fig. 1c, arrows) and filaments radiating from them (Fig. 1c, arrowhead) also stained intensely with osmium ammine-SO_2, indicating the presence of DNA. The electron dense staining of DNA was not observed on ultrathin sections before osmium ammine-SO_2 staining (Fig. 1d).

3. Labeling *in vivo* DNA by BrdU Incorporation

Since HDC-TEM allows not only observation of intact ultrastructure, but also identification of its constituents in their *in vivo* states, we attempted to visualize DNA by incorporation of BrdU (bromodeoxyuridine) into newly synthesized DNA, a technique widely used to investigate DNA synthesis *in vivo* in cell biology research. However, instead of labeling BrdU using fluorescence probes which is a routinely used procedure, we first tried to visualize Br directly by its electron density. Rapidly growing *Synechococcus* sp. PCC 7942 cells were cultured in BrdU containing liquid media for 24 h, and the collected cells were rinsed thoroughly, then frozen rapidly in liquid ethane. The samples were observed with a JEOL JEM-3100FFC electron microscope equipped with a helium stage and omega type energy filter at 300 kV accelerating voltage.

HDC-TEM images of the BrdU treated cells exhibited electron dense areas (Fig. 2a, arrowhead) other than polyphosphate bodies. Electron dense strands extending from the polyphosphate bodies (Fig. 2a, arrows) are visible in this area. An almost identical electron dense area was observed when the sample was observed without phase plate (Fig. 2b) however, ultrastructural details were lost. To confirm the localization of Br in the electron dense areas, electron spectroscopic imaging (ESI) was conducted subsequently after HDC-TEM observation on the same cells. Mapping images of Br (69 eV) and P (132 eV) were obtained by Gatan Digital Micrograph. Three window Br maps were acquired using an energy filter slit width of 10 eV, positioning the slit at 79 eV for post edge image and 63 and 55 eV for pre-edges. Three window P maps were obtained using an energy filter slit width of 20 eV, positioning the slit at 152 eV for the post edge image and 120 and 100 eV for pre-edge images. Since confirmation by electron energy loss spectroscopy (EELS) is necessary

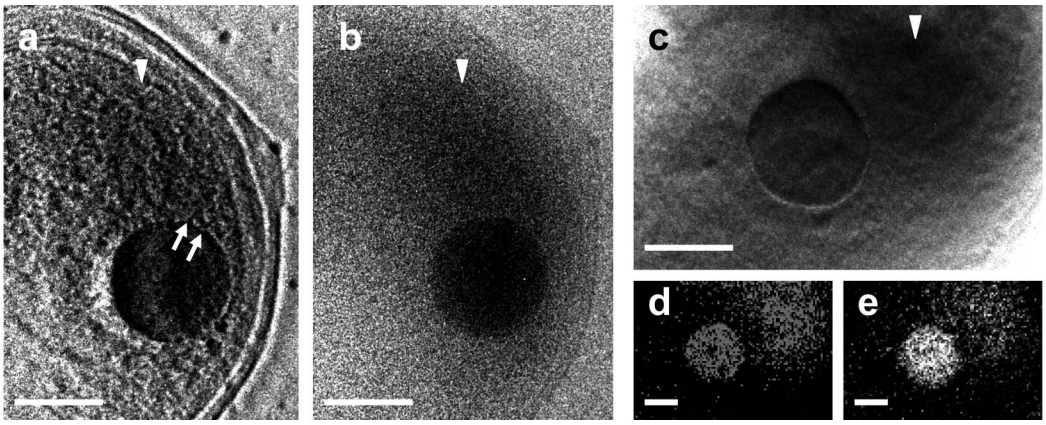

Fig. 2 300 kV TEM images of an ice-embedded whole cell cultured in BrdU containing medium for 24 h. (a) HDC-TEM, (b) conventional TEM, (c) HDC-TEM, (d) ESI of "Br", identical area of (c), (e) ESI of "P", identical area of (c). Electron dense areas (arrowheads) due to BrdU incorporation occur in the vicinity of polyphosphate bodies. Both "Br" and "P" were detected in polyphosphate bodies and electron dense areas. Scale bars, 100 nm.

to assign elements, localization of Br and P by ESI in this study is not conclusive and the putative signals are denoted by names of the elements in quotation marks. Fig. 2d and e illustrate the results of ESI for Br and P respectively, performed on the whole ice embedded cyanobacterial cell following HDC-TEM observation (Fig. 2c). An intense "Br" signal (Fig. 2d) was detected at the electron dense area (Fig. 2c, arrowhead). The "Br" signal was also clearly detected in polyphosphate bodies. ESI of P (Fig. 2e) showed that the "P" signal was most intense in the polyphosphate bodies and thus indicated the presence of phosphorous in them.

In the cells cultured without BrdU, only the "P" signal, but no "Br" signal, was detected in polyphosphate bodies (data not shown). The incorporation of Br was not observed in cultures not actively growing. When cells from older cultures were used for incubation with BrdU for 24 h, no "Br" signal was detected in the DNA area, nor in polyphosphate bodies (data not shown).

4. Discussion

We attempted to identify *in vivo* DNA in ice embedded cyanobacterial cells. Incorporation of BrdU produced electron dense areas in HDC-TEM images and the apparent Br signal by ESI was localized in these areas. Br was also appeared to be localized associated with polyphosphate bodies.

4.1 ESI application to biological specimens

Electron spectroscopic imaging (ESI) is a widely used technique in material sciences to analyze constituent elements. However, not many applications of ESI to biological specimens have been reported [6–8]. Recently ESI was applied to dehydrated cells [9] and to frozen bacterial cells [10] to show the existence of phosphorous in certain distinct structures, possibly polyphosphate bodies. Although multi scattering effects due to the thickness of the ice embedded samples cannot a priori be excluded, we conclude, in view of the control experiments, that the "Br" signal observed in polyphosphate bodies in Fig. 2d is significant.

The Br localization associated with polyphosphate bodies is not likely a result of random incorporation of Br into polyphosphate bodies. Uptake of various metals such as Mg, Ba, Mn by polyphosphate bodies in cyanobacteria has been detected by X ray microanalysis of air dried whole cells of *Plectonema boryanum* [11]. However incorporated metals are all positively charged, whereas negatively charged Cl was not incorporated although the metals were applied as chloride salts [11]. We conclude that it is extremely unlikely that Br, which is also a halogen, is accumulated into polyphosphate bodies by a process unrelated to biological activity.

4.2 Polyphospate bodies and DNA

It has been known that cyanobacterial cells commonly contain distinct polyphosphate bodies. These are often found in the nuclear area and are known to be composed of polyphosphate and various metal ions [12]. The occurrence of DNA fibrils in the bodies has been suggested [13], but no experimental confirmation has so far been obtained. Currently it is believed that the main functions of the polyphosphate bodies are storage of phosphate that is essential for growth and metabolism, and the sequestration of metals [11, 14]. The existence of Br associated with the polyphosphate bodies indicates that at least part of the newly synthesized DNA co-localizes with the bodies. It is also apparent that outside of polyphosphate bodies, the major location of phosphorous utilization is DNA synthesis. These data suggest that one of the main functions of the

polyphosphate bodies in cyanobacteria is to provide phosphate as material for DNA synthesis.

Conventional TEM techniques suggest that DNA fibers are associated with polyphosphate bodies (Fig. 1c, d), although polyphosphate bodies are extremely difficult to preserve during the process from chemical fixation to ultrathin sectioning, often leaving empty holes. Another problem with conventional chemical fixation is that DNA fibers tend to aggregate. By combination of HDC-TEM and ESI, on the other hand, and by mapping the intracellular elements onto the topographical structural images, an intimate association between polyphosphate bodies and newly synthesized DNA was suggested. Further, a portion of the fibrous structures visualized by HDC-TEM radiating from polyphosphate bodies overlapped with the "Br" and "P" signals, indicating that they are DNA. By pursuing this methodology, it will be possible to visualize *in vivo* events of DNA synthesis, and its conformational change at high resolution.

4.3 Combining HDC-TEM and ESI

By a combination of HDC-TEM and ESI, DNA in ice embedded cyanobacterial cells was observed. The technique is especially promising since both structural and functional analysis can be conducted successively in the same instrument in a relatively short time. We believe that the technique has an enormous potential to develop into a new area of biological ultrastructural research in which various functional molecules can be localized in correlation with high resolution ultrastructural images of the cells close to the living state.

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