An electron microscopic study of microorganisms: from influenza virus to deep-sea microorganisms

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Abstract: Three topics from electron microscopic studies of microorganisms carried out in my laboratory in recent ten years are described. 1) Influenza A virus was observed in water by an ice-embedding method using phase contrast electron microscopy developed in Japan. Virions appeared as spherical or elongated particles consisting of spikes, an envelope, and a core with high contrast. 2) A new term the "structome" was introduced and defined as "the quantitative and three-dimensional structural information of a whole cell at electron microscopic level." We performed structome analyses of Saccharomyces cerevisiae by using freeze-substitution and serial ultrathin sectioning electron microscopy. We found that there were one to four mitochondria and about 195,000 ribosomes in a cell. 3) In the deep-sea off the coast of Japan, we discovered a unique microorganism appearing to have cellular features intermediate between prokaryotes and eukaryotes. The organism, named as the Myojin parakaryote, was two orders of magnitude larger than a typical bacterium and had a large "nucleoid", surrounded by a single layered "nucleoid membrane", and bacteria-like "endosymbionts", but it lacked mitochondria. This organism exemplifies a potential evolutionary path between prokaryotes and eukaryotes, and the presence of the organism supports the endosymbiotic theory for the origin of mitochondria and the karyogenetic hypothesis for the origin of the nucleus. These studies show that the electron microscopy is a powerful tool for studying a wide range of problems of microorganisms.

1. Electron microscopic observation of influenza virus in water

The ultrastructure of frozen-hydrated influenza A virus was examined by Zernike phase contrast electron microscopy. Using this new technique, the virions were clearly observed with high contrast and appeared as spherical or elongated particles consisting of peripheral spikes, an envelope, and a core. Not only lipid bilayers but also individual glycoprotein spikes on viral envelopes were clearly resolved. About 450 glycoprotein spikes were present in an average-sized spherical virion. Eight ribonucleoprotein complexes, that is, a central one surrounded by seven others, were distinguished in one viral particle. Thus, Zernike phase-contrast electron microscopy is a powerful tool for resolving the ultrastructure of viruses in natural and hydrated state, because it enables high-contrast images of ice-embedded particles.

Introduction

Viruses are usually observed by negative staining method. In this method, virus suspension was placed on a support film, stained with liquid metal such as uranyl acetate, blotted with filter paper to remove excess liquid, dried in air, and observed in transmission electron microscope. But such images have many artifacts, and do not show the true structure. In order to see the real structure of the influenza virus, the virus must be observed in water. An ice-embedding method makes such observations possible. A virus suspension was placed on a microgrid that was made hydrophilic by glow discharge beforehand. The grid was blotted with filter paper to remove excess liquid and snap-frozen by dipping in liquid ethane kept in liquid nitrogen. By using this procedure, a thin film of frozen viral suspension in vitreous state was formed1,2.
Negative staining image of influenza virus

Fig. 1-1 shows an image of influenza viruses obtained by this method. Viruses appeared as round particles about 100 nm in diameter, with spikes.

Ice-embedding image of influenza virus

The grid with frozen viral suspension was transferred into a JEM-3100FFC transmission electron microscope equipped with a field emission gun, a cryostage, a Zernike phase plate, and an omega filter (JEOL Ltd., Tokyo, Japan). Micrographs were taken at a direct magnification of 40,000 in focus, at a 300 kV acceleration voltage, using a minimum-dose system, at −269°C. Novel structures of the virus as obtained from the phase contrast images are shown in Fig. 1-2. They appeared as spherical or elongated particles consisting of many glycoprotein spikes, a viral envelope, and an inner core. The images were clear and natural with high contrast.

Glycoprotein spikes on the virions were clearly visualized by the new method (Fig. 1-2b). The density of glycoprotein spikes along the virion surface was easily measured to be 10.2 ± 0.7 spikes/100 nm (average ± standard deviation). Thus, about 100 spikes were present in 10,000 nm² of the viral surface, and about 450 glycoprotein spikes were present on the surface of 120-nm-diameter virions, whose surface area (4πr²) was 45,216 nm². This number well matched the previous mass analysis estimates of 400-500 spikes per virion. In a few instances, eight ribonucleoprotein complexes, one at the center surrounded by seven others, were distinguished in one viral particle (Fig. 1-2c). The diameter of each ribonucleoprotein complex was 10.7 ± 2.5 nm (n = 8).

Comparison of influenza virus imaged by negative staining and ice-embedding methods

Spherical virions measured 121 ± 19 nm in diameter and consisted of 65 % of the population by ice-embedding method, whereas 39 % of the population was spherical by negative staining method (Table 1-1). The diameter of the elongated virions was 102 ± 7 nm, whereas it was 107 ± 11 nm by negative staining method. The length of the spikes was 14.2 ± 1.2 nm by ice-embedding method, whereas it was 10.1 ± 1.2 nm by the negative staining method. The differences described above were statistically significant, and show that distortion of the virus morphology had occurred in negative staining method.

Future studies

The Zernike phase contrast microscopy of ice-embedded viruses makes it possible to obtain high-contrast images of intact particles free of contrast transfer function artifacts. This greatly improves the...
Table 1-1 Comparison of morphology, size, and length of the spikes of influenza A virions by different methods.
(Modified from Yamaguchi et al., Kenbikyo 43, 115-120, 2008 14)

| Method               | Morphology | Number measured | Proportion | Diameter (d) and length (l): | Average ± S.D. (nm) (number measured) |
|----------------------|------------|-----------------|------------|------------------------------|---------------------------------------|
|                      | Morphology |                 |            |                              |                                        |
| Negative staining     | Spherical  | 47              | 39 %       | 127 ± 23 (d) *2              | 10.1 ± 1.2 (42) *5                     |
| Elongated            | 73         | 61 %            | 107 ± 11 (d) *3 | 161 ± 56 (l) *4               |
| Total                | 120        | 100 %           |            |                              |                                        |
| Ice- embedding       | Spherical  | 59              | 65 %       | 121 ± 19 (d) *2              | 14.2 ± 1.2 (42) *5                     |
| Elongated            | 32         | 35 %            | 102 ± 7 (d) *3 | 155 ± 19 (l) *4               |
| Total                | 91         | 100 %           |            |                              |                                        |

*1t< 52.000, P<0.001; *2t< 1.440, P >0.05; *3t< 2.800, P <0.01; *4t< 0.815, P >0.05; *5t< 15.709, P<0.001
The proportion of spherical virions*1, the diameter of elongated virions*3, and the length of the spikes*5, were significantly differ by the method employed. Distortion of virion morphology may have occurred in negative staining method. Measurements obtained by the ice-embedding method give true values.

quality and accuracy of the analysis of viruses. In order to solve the issue of the superposition and overlapping of images in transmission electron microscopy, the use of tomography should be considered. The tomography of various biological species has been performed, and the 3D structures of the specimens have been resolved at high resolution. If tomography could be combined with phase contrast cryo-electron microscopy, it would be a powerful tool for resolving the ultrastructure of viruses.

2. The Structome of Saccharomyces cerevisiae determined by freeze-substitution and serial ultrathin sectioning electron microscopy

The cell structure has been studied by light and electron microscopy for centuries, and it is assumed that the whole structure is clarified by now. However, little quantitative and three-dimensional analysis of cell structure has been undertaken, and the number of ribosomes or the number and cellular distribution of endoplasmic reticula in a yeast cell are not known, for example. I have coined a new word, "structome", by combining "structure" and "–ome", and defined it as the "quantitative three-dimensional structural information of a whole cell at electron microscopic level." In the present study, we performed structome analysis of Saccharomyces cerevisiae, one of the most widely researched biological materials, by using freeze-substitution and serial ultrathin sectioning electron microscopy. Our analysis revealed that there were one to four mitochondria and about 195,000 ribosomes in a cell and 13-28 endoplasmic reticula/Golgi apparatus, which do not form networks in the cytoplasm in G1 phase. The nucleus occupied 10.1 % of the cell volume, the cell wall occupied 17.7 %, the vacuole occupied 4.0 %, the cytoplasm occupied 66.2 % and the mitochondria occupied only 1.6 % in G1 phase. These data would form a base to consider the function of cells.

Introduction

The number of cells that makes up the human body is known to be about 6 x 10^13 cells, while the human brain is known to consist of about 1.5 x 10^10 cells. But, it is not known, for example, how many ribosomes there are in a single yeast cell, nor the volume occupied by endoplasmic reticula occupy and how they localize within a cell. The word "structome" was coined by combining "structure" and "–ome," and defined as the "quantitative three-dimensional structural information of a whole cell at the electron microscopic level" 8,9.

Three-dimensional analysis of cell components such as mitochondria has been performed using serial ultrathin sectioning electron microscopy since 1969 10. A report that mitochondria, which appeared as multiple entities, were in fact connected to each other as a single unit was a big surprise to cell biologists 11. Although there have been studies using three-dimensional analysis of specific components by electron microscopy, there were no 3D analysis on all cell components. In 2003, we performed structome analysis of

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Exophiala dermatitidis yeast cells for the first time\textsuperscript{12,13}, and found that 1) there are about 195,000 ribosomes, 17-52 mitochondria, 5-10 endoplasmic reticula in a cell; 2) mitochondria occupied 9.9 \%, nucleus 7.3 \%, cell wall 21.8 \%, cytosol 47.5 \%, and only 0.2 \% endoplasmic reticula of the cell volume; 3) there is no giant mitochondrial in this species\textsuperscript{12}.

Saccharomyces cerevisiae was used for the first genome analysis in eukaryotes and is one of the most common organisms studied in cell biology worldwide. Since genetic, biochemical, and morphological information on this material has accumulated, structural information on the whole cell in this species at the electron microscopic level, i.e., the structome, would provide useful additional information. Here we report the structome of Saccharomyces cerevisiae determined by freeze-substitution and serial ultrathin sectioning electron microscopy.

**Structome analysis of Saccharomyces cerevisiae**

Saccharomyces cerevisiae, strain S288c, cells were cultured in 20 ml of YPD liquid medium (1\% yeast extract, 2\% polypeptone, and 2\% glucose) in a 100-ml Erlenmeyer flask at 30°C with reciprocal shaking at 120 rpm. Exponentially growing cells (OD\textsubscript{660} was about 1.0) were used for structome analysis. Cells were collected by centrifugation, sandwiched between two copper disks and snap frozen by plunging into melting propane cooled with liquid nitrogen. They were freeze-substitution and serial ultrathin sectioning electron-transparent, 86 ± 14 nm thick, and made of β-glucan and chitin (Fig. 2-1a).

The cell wall was 120 ± 14 nm thick and consisted of two layers (Fig. 2-1a).\textsuperscript{9} The outer cell wall layer was 34 ± 3 nm thick and composed of fibrous materials made of mannoproteins. The inner layer was electron-transparent, 86 ± 14 nm thick, and made of β-glucan and chitin (Fig. 2-1a).

1. The cell

S. cerevisiae G1 phase cells were nearly spherical ellipsoids, 3.24 ± 0.42 µm (average ± standard deviation) in the minor axis and 3.85 ± 0.48 µm in the major axis (Table 2-3). The volume was 15.2 ± 4.0 µm\textsuperscript{3} (Table 2-2).

2. The cell wall

The cell wall of S. cerevisiae was 120 ± 14 nm thick and consisted of two layers (Fig. 2-1a).\textsuperscript{9} The outer cell wall layer was 34 ± 3 nm thick and composed of fibrous materials made of mannoproteins. The inner layer was electron-transparent, 86 ± 14 nm thick, and made of β-glucan and chitin (Fig. 2-1a).

3. The nucleus, nuclear envelope, nuclear pore, and nucleolus

The nucleus was enclosed by a double layered nuclear envelope (Fig. 2-1i). The nucleus in interphase cell was spherical, 1.63 ± 0.20 µm in diameter (Table 2-3), and 1.5 ± 0.4 µm\textsuperscript{3} in volume, occupying 10.1 ± 0.8 \% of the cell volume (Table 2-2, Fig. 2-4). The nuclear pore was 93.7 ± 11.8 nm in diameter (Table 2-3) and is known to function in the transport of substances between the nucleoplasm and cytoplasm.\textsuperscript{10} There was only one nucleolus in a nucleus (Table 2-1). The nucleolus was composed of densely packed granular materials (Fig. 2-1i), 0.31 ± 0.17 µm\textsuperscript{3} in volume and occupied 18.9 ± 7.4 \% of the nuclear volume (Table 2-2).

4. The mitochondria

The mitochondria appeared oval or elongated in ultrathin sections (Fig. 2-1c) and were found to be string-shaped and sometimes branched in 3D reconstruction images (Fig. 2-2). The mitochondria had a cross sectional diameter of 232.9 ± 34.0 nm (Table 2-3) and length of up to 6.2 µm (Fig. 2-2c). The mitochondria in a cell had a total volume of 0.23 ± 0.07 µm\textsuperscript{3} and occupied 1.6 ± 0.5 \% of the cell volume (Table 2-2, Fig. 2-4). There was one giant mitochondrion\textsuperscript{17} in most cells. The outer membrane of the mitochondrion was clearly observed, but cristae were not very clear in our culture condition (Fig. 2-1c).

5. The endoplasmic reticulum and Golgi apparatus

In S. cerevisiae, Golgi apparatus rarely shows a stacked structure and exists primarily as individual cisternae throughout the cytoplasm\textsuperscript{18} (see Fig. 2-1a and g). Therefore, we analyzed the endoplasmic reticulum and Golgi apparatus as one organelle (ER/Golgi). There were 19.8 ± 6.3 ER/Golgi in a cell (Table 2-1), with a
total volume of 0.09 ± 0.05 µm$^3$, occupying only 0.6 ± 0.3 % of the cell volume (Table 2-2). Membranes of the ER/Golgi were 14.2 ± 1.8 nm thick (Table 2-4). ERs of animal cells often form networks in a whole cell, but the ER/Golgi in Saccharomyces exists mostly like islet and does not form network (Fig. 2-2).

6. Vacuoles, autophagosomes, and multivesicular bodies

There were 1.8 ± 1.7 vacuoles in a cell (Table 2-1). Vacuoles were usually spherical (Fig. 2-1o) with a size of (0.87 ± 0.46) µm x (0.93 ± 0.49) µm (Table 2-3). The total vacuolar volume was 0.68 ± 0.49 µm$^3$, and occupied 4.0 ± 2.5 % of the cell volume (Table 2-2, Fig. 2-4). Vacuolar membrane was 19.4 ± 2.7 nm thick and consisted of three leaflets with the inner leaflet and the outer leaflet having similar thickness (Table 2-4).

Autophagosomes (Fig. 2-1d) were membrane-bound ellipsoid structures in the cytoplasm and had a size of (263.0 ± 67.4) nm x (316.0 ± 76.7) nm (Table 2-3). They often contained ribosome particles that might be in the process of digestion (Fig. 2-1d). Their membranes consisted of three leaflets (Fig. 2-1d) and had a thickness of 16.8 ± 2.3 nm (Table 2-4). The autophagosome is the precursor of the autophagic body in the vacuole.

![Fig. 2-1](https://example.com/fig2-1.png) High magnification images of various cell components in S. cerevisiae. a The cell wall (CW) and plasma membrane (PM). O, outer cell wall layer; I, inner cell wall layer. ER, endoplasmic reticulum. b Invagination (Inv). c Mitochondrion (M) with outer membrane (OM). d Autophagosome (A). e Multivesicular body (MVB). f Peroxisome (P). g Endoplasmic reticulum/Golgi apparatus (ER/Golgi). h Virus–like particles (VLP). i Nucleus (N), Nuclear envelope (NE), Nuclear pore (NP), and Nucleolus (Nu). j Ribosomes (R). k Filasome (F). l Small vesicles (Ves). m Spindle pole body (SPB), Microtubule (Mt). n Microfilaments (Mf). o Vacuole (V) with vacuolar membrane (VM). Bar = 100 nm. (Reprinted from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011)
### Table 2-1  Numbers of organelles and cell components in *S. cerevisiae* cells.
(Modified from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011(*)

| Organelles/components | G1 phase (Average ± S.D. of 3 cells) | Early G1 phase (Average ± S.D. of 3 cells) | Total (Average ± S.D. of 6 cells) |
|-----------------------|--------------------------------------|--------------------------------------|-----------------------------------|
| Nucleus               | 1 ± 0                                | 1 ± 0                                | 1 ± 0                             |
| Nucleolus             | 1 ± 0                                | 1 ± 0                                | 1 ± 0                             |
| Spindle pole body     | 1 ± 0                                | 1 ± 0                                | 1 ± 0                             |
| Mitochondria          | 23 ± 1.2                             | 23 ± 1.5                             | 23 ± 1.2                          |
| ERs/Golgi apparatus   | 21.7 ± 7.8                           | 18.0 ± 5.3                           | 19.8 ± 6.3                        |
| Vacuoles              | 2.0 ± 1.7                            | 1.7 ± 0.6                            | 1.8 ± 1.7                         |
| Autophagosomes        | 0.3 ± 0.6                            | 1.7 ± 0.6                            | 1.0 ± 0.9                         |
| Multivesicular bodies | 1.7 ± 2.9                            | 0.0 ± 0.0                            | 0.8 ± 2.0                         |
| Ribosomes             | 217000 ± 48000                       | 175000 ± 62000                       | 195000 ± 55000                    |
| Ribosomes/µm³         | 19600 ± 600                          | 19400 ± 1500                         | 19500 ± 1000                      |
| Small vesicles        | 9.7 ± 6.4                            | 5.3 ± 4.9                            | 7.5 ± 5.6                         |
| Filasomes             | 29.3 ± 9.5                           | 20.7 ± 2.3                           | 25.0 ± 7.8                        |
| Virus-like particles  | 82.7 ± 38.1                          | 58.0 ± 15.1                          | 70.3 ± 29.2                       |
| Peroxisomes           | 0.0 ± 0.0                            | 0.0 ± 0.0                            | 0.0 ± 0.0                         |
| Invaginations         | 31.7 ± 12.6                          | 22.7 ± 15.6                          | 27.2 ± 13.6                       |

### Table 2-2  Volumetric measurement of *S. cerevisiae* cells and cell components
(Measured in µm³, numbers between parentheses show proportions against the whole cell)
(Modified from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011(*)

| Cell/organelles | G1 phase (Average ± S.D. of 3 cells) | Early G1 phase (Average ± S.D. of 3 cells) | Total (Average ± S.D. of 6 cells) |
|-----------------|--------------------------------------|--------------------------------------|-----------------------------------|
| Cell            | 17.1 ± 3.1 (100 ± 0 %)               | 13.3 ± 4.3 (100 ± 0 %)               | 15.2 ± 4.0 (100 ± 0 %)            |
| Cell wall       | 2.9 ± 0.6 (17 ± 2 %)                 | 2.4 ± 0.6 (18 ± 3 %)                 | 2.6 ± 0.6 (18 ± 3 %)              |
| Nucleus         | 1.8 ± 0.4 (10.5 ± 1.0 %)              | 1.3 ± 0.4 (9.7 ± 0.3 %)               | 1.5 ± 0.4 (10.1 ± 0.8 %)           |
| Nucleolus       | 0.40 ± 0.10 (22.1 ± 1.5 %)*          | 0.22 ± 0.19 (15.7 ± 10.1 %)*         | 0.31 ± 0.17 (18.9 ± 7.4 %)*        |
| Mitochondria    | 0.28 ± 0.03 (1.7 ± 0.4 %)             | 0.17 ± 0.04 (1.4 ± 0.6 %)             | 0.23 ± 0.07 (1.6 ± 0.5 %)          |
| ERs/Golgi apparatus | 0.12 ± 0.04 (0.7 ± 0.3 %)          | 0.06 ± 0.02 (0.5 ± 0.1 %)             | 0.09 ± 0.05 (0.6 ± 0.3 %)          |
| Vacuoles        | 0.99 ± 0.28 (5.8 ± 1.0 %)             | 0.36 ± 0.45 (2.2 ± 2.4 %)             | 0.68 ± 0.49 (4.0 ± 2.5 %)          |
| Autophagosomes  | 0.004 ± 0.007 (0.03 ± 0.05 %)         | 0.006 ± 0.008 (0.04 ± 0.04 %)         | 0.006 ± 0.008 (0.03 ± 0.04 %)      |
| Multivesicular bodies | 0.008 ± 0.014 (0.04 ± 0.06 %)       | 0 ± 0 (0 ± 0 %)                     | 0.003 ± 0.008 (0.02 ± 0.04 %)      |
| Cytosol         | 11.0 ± 2.2 (64.3 ± 0.6 %)             | 9.0 ± 3.0 (68.0 ± 3.6 %)             | 10.0 ± 2.6 (66.2 ± 3.1 %)          |

*Proportions against the nucleus

### Table 2-3  Diameter (minor axis) and major axis of *S. cerevisiae* cell and cell components (average ± S.D.)
(Modified from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011(*)

| Cell/organelle/component | Diameter (minor axis) | Major axis          | Number measured |
|--------------------------|-----------------------|---------------------|-----------------|
| Cell                     | 3.24 ± 0.42 µm        | 3.85 ± 0.48 µm      | 36              |
| Nucleus                  | 1.63 ± 0.20 µm        | 36                 |
| Vacuole                  | 0.87 ± 0.46 µm        | 0.93 ± 0.49 µm      | 47              |
| Nuclear pores            | 93.7 ± 11.8 nm        | 20                 |
| Spindle pole body        | 117.2 ± 18.8 nm       | 8                  |
| Mitochondria             | 232.9 ± 34.0 nm       | 20                 |
| Autophagosomes           | 263.0 ± 67.4 nm       | 25                 |
| Multivesicular bodies    | 185.9 ± 38.9 nm       | 206.3 ± 43.3 nm     | 16              |
| Microvesicles in...      | 39.3 ± 4.5 nm         | 14                 |
| Ribosomes                | 20.2 ± 1.2 nm         | 50                 |
| Small vesicles           | 70.2 ± 6.3 nm         | 20                 |
| Filasomes                | 175.2 ± 33.2 nm       | 33                 |
| Virus-like particles     | 51.7 ± 6.2 nm         | 100                |
| Microtubules             | 25.1 ± 0.5 nm         | 24                 |
| Microfilaments           | 7.3 ± 0.8 nm          | 7                  |
Table 2-4  Membrane thickness of organelles in *S. cerevisiae* (Average ± S.D.) (Modified from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011)

| Organelle                      | Thickness (nm) | Number measured |
|-------------------------------|----------------|-----------------|
| Plasma membrane               | 15.6 ± 2.1     | 20              |
| Outer leaflet                 | 4.6 ± 1.0      | 20              |
| Middle leaflet                | 3.8 ± 0.6      | 20              |
| Inner leaflet                 | 7.3 ± 1.9      | 20              |
| Vacuole                       | 19.4 ± 2.7     | 20              |
| Outer leaflet                 | 7.8 ± 2.2      | 20              |
| Middle leaflet                | 4.1 ± 0.8      | 20              |
| Inner leaflet                 | 7.6 ± 1.7      | 20              |
| Autophagosome                 | 16.8 ± 2.3     | 11              |
| Multivesicular body           | 16.9 ± 2.6     | 8               |
| Nuclear outer envelope        | 12.6 ± 1.5     | 20              |
| Nuclear inner envelope        | 13.3 ± 1.6     | 20              |
| ER/Golgi apparatus            | 14.2 ± 1.8     | 20              |
| Mitochondrial outer membrane  | 14.1 ± 2.0     | 20              |

Fig. 2-2  Three-D reconstructions of *S. cerevisiae* Cell 1–6. Cell 1–3 are in G1 phase, and Cell 4–6 are in early G1 phase. Red, nucleus; Green, mitochondria; Blue, vacuoles; Purple, endoplasmic reticula/Golgi apparatus; Yellow points, small vesicles; Red points, virus-like particles; * in Cell 4–6, position where mother cell attaches. (Reprinted from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011)

Fig. 2-3  Proportions of the cytosol (C), cell wall (CW), nucleus (N), mitochondria (M), vacuoles (V), and other cell components (O) in Cells 1–6. Schematic representation of Table 2-2. Values are expressed as percent to the cell volume. “Others” include endoplasmic reticulum/Golgi apparatus, autophagosomes, and multivesicular bodies. The volume of cytosol includes the volume of ribosome particles, Small vesicles, and virus-like particles. (Modified from Yamaguchi et al., J Electron Microsc 60, 337-351, 2011)

Fig. 2-4  Average proportions of the cytosol (C), cell wall (CW), nucleus (N), mitochondria (M), vacuoles (V), and other cell components (O) in *S. cerevisiae*.

Fig. 2-5  Average proportions of the cytosol (C), cell wall (CW), nucleus (N), mitochondria (M), vacuoles (V), and other cell components (O) in *E. dermatitidis*. (Modified from Biswas et al., J Electron Microsc, 52, 133-143, 2003).
Multivesicular bodies were spherical membrane-bound organelles containing microvesicles (Fig. 2-1e). Up to five multivesicular bodies were present in a cell, having a size of (185.9 ± 38.9) nm × (206.3 ± 43.3) nm (Table 2-3). The membrane of the multivesicular body was 16.9 ± 2.6 nm thick (Table 2-4) and consisted of three leaflets (Fig. 2-1e). Multivesicular bodies are considered to be part of the lysosomal system since they contain acid phosphatase.

Small vesicles and filasomes
Small vesicles are vesicles having diameters of 70 nm with clear circumference and were often found in the cytoplasm near cell walls (Fig. 2-1i). There were 7.5 ± 5.6 in a cell (Table 2-1) and they showed glucanase activity.

Filasomes were spherical, 175.2 ± 33.2 nm in diameter, and consists of central small particle and outer filamentous material (Fig. 2-1k, Table 2-3). There were 25.0 ± 7.8 in a cell (Table 2-1), and often observed near cell wall.

The spindle pole body, microtubules, and microfilaments
Fig. 2-1m shows the spindle pole bodies (SPBs) of interphase cells. The SPB was located within a nuclear envelope and consisted of disc elements with cytoplasmic and nuclear microtubules. The disc elements measured 117.2 ± 18.8 nm in diameter (Table 2-3).

Microtubules (Fig. 2-1m) had a diameter of about 25.1 ± 0.5 nm (Table 2-3). Most were associated with the SPB and few were found in the cytoplasm. Microfilaments (Fig. 2-1n) were 7.3 ± 0.8 nm in diameter (Table 2-3).

Peroxisomes and virus-like particles
Peroxisomes were observed as electron-dense spherical organelles enclosed by a membrane (Fig. 2-1f). They sometimes contained more electron-dense crystal-like structures (Fig. 2-1f). There were few peroxisomes in the present culture condition and no peroxisomes were seen in the cells 1-6.

The plasma membrane and membrane systems
The plasma membrane was 15.6 ± 2.1 nm thick and consisted of three leaflets (Fig. 2-1a, Table 2-4). The outer leaflet was electron-dense, the middle leaflet was electron-transparent, and the inner leaflet was electron-dense. They measured 4.6 ± 1.0 nm, 3.8 ± 0.6 nm, and 7.3 ± 1.9 nm, respectively (Table 2-4). The plasma membrane was invaginated into cytoplasm in certain places (Fig. 2-1b). There were 27.2 ± 13.6 invaginations in each cell (Table 2-1).

Membranes of S. cerevisiae can be classified into two groups according to their thickness. The first group had a thickness of 16–19 nm (Table 2-4) and included the plasma membrane (Fig. 2-1a), vacuolar membrane (Fig. 2-1o), membranes of autophagosomes (Fig. 2-1d), and multivesicular body (Fig. 2-1e). The second group had a thickness of 13–14 nm (Table 2-4) and included the outer and inner nuclear envelope (Fig. 2-1m), the ER/Golgi membranes (Fig. 2-1a and g), and the mitochondrial outer membrane (Fig. 2-1c).

Comparison between structomes of G1 phase and early G1 phase cells
The sizes of cells, and the volumes of the cell walls, nucleus, nucleolus, mitochondria, ER/Golgi, vacuoles, and cytosols tended to be smaller in early G1 phase cells than in late G1 phase cells (Table 2-2), although the values were not statistically significant. This is understandable since the daughter cells are usually smaller than the mother cells. The ratios of the vacuolar volume were noticeably smaller in early G1 phase cells than in late G1 phase cells. These results show that structome dynamics have an interesting relationship to the cell cycle; this aspect of the study should be further pursued in the future.
Analysis of distances between ER/Golgi and mitochondria

Three-D reconstruction of cells enabled us to measure distances between the ER/Golgi and mitochondria in each cell in three dimensions (Table 2-5). Distances between the ER/Golgi and mitochondria in Cell 1 (G1 phase) were 775 ± 634 nm, and those in Cell 5 (early G1 phase) were 231 ± 253 nm. This difference was found to be statistically significant. It can be assumed that a higher energy supply would be necessary in early G1 phase cells than in G1 phase cells, resulting in closer association between the ER/Golgi and mitochondria in the former. It is interesting that similar results were reported in acinar cells of pancreas in mouse.

Generation of autophagosomes, multivesicular bodies, and peroxisomes

Autophagosomes were not found in two out of six cells examined, multivesicular bodies were not found in five cells, and no peroxisomes were found in all six cells examined (Table 2-1). The presence of cells that have none of these organelles means that these organelles may not always be necessary for the cell to live and that they could be generated de novo in the cytoplasm when necessary.

Comparison with another yeast structome

We have previously reported the structome of Exophiala dermatitidis, an ascomycetous black yeast, in G1 phase, making it possible to compare structomes between S. cerevisiae and E. dermatitidis.

There were no lipid bodies, glycogen granules, nor storage materials in S. cerevisiae, whereas these components were present in E. dermatitidis. On the other hand, there were small vesicles, virus-like particles, and peroxisomes in S. cerevisiae, whereas these components were not present in E. dermatitidis. There were fewer mitochondria in S. cerevisiae than in E. dermatitidis (2.3 ± 1.2 vs. 25.6 ± 14.8). Also, the volume of mitochondria in S. cerevisiae was much less than that of E. dermatitidis (1.6 ± 0.5 % vs. 9.9 ± 1.7 %). The proportion of cytosol in S. cerevisiae was greater than in E. dermatitidis (66.2 ± 3.1 % vs. 47.5 ± 3.5 %). It will require more information on structomes of other cells to understand what these differences between S. cerevisiae and E. dermatitidis mean.

Application of structome analysis for finding unknown microorganisms

An example that structome analysis can lead to the discovery of an unknown microorganism will be shown here. In 2010, we collected samples in a deep sea off the coast of Japan and observed microorganisms by electron microscopy. We found a yeast-like microorganism that had a cell wall and bacteria-like endosymbionts, but had no true nucleus nor mitochondria. By structome analysis it became evident that this microorganism was not a true eukaryote nor a true prokaryote. We named this microorganism “Myojin parakaryote” and scientific name “Parakaryon myojinensis”. Details of this study will be described in chapter 3.

Structome of prokaryote

In writing a paper on Myojin parakaryote, we looked for a paper that described the volume of Escherichia coli. The paper described the volume of E. coli, but reported little further structural information. Since E. coli is one of the most studied organisms, we considered that the structome of E. coli would give important information for other studies. We are now conducting structome analysis of E. coli by rapid freeze-freeze substitution and serial ultrathin sectioning. Further, we finished structome analysis of the pathogenic prokaryote, Mycobacterium tuberculosis, and found some interesting features.

Other means for structome analysis than serial ultrathin sectioning

Recently, serial slice scanning electron microscopy are getting popular for 3D reconstruction of animal tissues. Although these new approaches are very useful, the resolution of the images is inferior to transmission electron microscopy of ultrathin sections. Improvement of resolution of images obtained using these methods should come about in the future.

Concluding remarks

The genome is the whole genetic information of an organism; the proteome is the whole protein information of a cell or organism. Similarly, the structome is the whole structural information of a cell at the electron microscopic level, an important concept for understanding cell function. The concept of structome includes information on individual protein positions within the cell which, we hope, will become possible in the future.

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3. Deep-sea microorganisms and the origin of the eukaryotic cell

There are only two kinds of organisms on the Earth: prokaryotes and eukaryotes. Eukaryotes are thought to have developed from prokaryotic predecessors; however, the large differences in their cellular structures result in equally large questions of how the process might have occurred. In 2012, in the deep-sea off the coast of Japan, we discovered a unique microorganism appearing to have cellular features intermediate between prokaryotes and eukaryotes. The organism, named as the Myojin parakaryote, was two orders of magnitude larger than a typical bacterium and had a large "nucleoid", consisting of naked DNA fibers, surrounded by a single layered "nucleoid membrane", and bacteria-like "endosymbionts", but it lacked mitochondria. This organism exemplifies a potential evolutionary path between prokaryotes and eukaryotes, and the presence of this organism supports the endosymbiotic theory for the origin of mitochondria and the karyogenetic hypothesis for the origin of the nucleus. In this chapter, we describe how the Myojin parakaryote was discovered, the features of this organism, the significance of the discovery, and perspectives on future research.

Introduction

There are only two known ways of being a living cell: the prokaryotic and the eukaryotic. Prokaryotes include the Bacteria and Archaea. Prokaryotic cells are generally only a few micrometers in size, have simple cellular structures including cytoplasm with a fibrous nucleoid, ribosomes, a plasma membrane, and a cell wall. Eukaryotic cells are much more complex and include both single- and multi-cellular organisms; e.g., animals, plants, fungi, and protists. Both groups, particularly the eukaryotes, have wide cellular size ranges; however eukaryotic cells typically have nearly 10000 times the volume of prokaryotic cells. Eukaryotic cells also have a nucleus enclosed by a double membrane and show complex membranous cellular structures: endoplasmic reticula, Golgi apparatuses, peroxisomes, lysosomes, endosomes, and various sizes and types of vacuoles. Additionally, eukaryotic cells have either one or both of two distinct types of organelles that contain their own DNA: mitochondria and chloroplasts. Eukaryotic cells also have various types of cytoskeletal structures: centrioles, microtubules and microfilaments (Fig. 3-1) [26,27].

Eukaryotes are thought to have evolved from prokaryotes [26]; however, until recently, there were no known examples of intermediate forms between the vastly different prokaryotic and eukaryotic levels of organization. In fact, the differences in cellular structure between prokaryotes and eukaryotes are so seemingly insurmountable that the problem of how eukaryotes could have evolved from prokaryotes is one of the greatest puzzles in biology. One way to address this question is to find an organism with intermediate cellular structure between those of prokaryotes and eukaryotes [20]. This organism was described using freeze-substitution electron microscopy and structome analysis (see Chapter 2). The organism was named the 'Myojin parakaryote' with the scientific name of Parakaryon myojinensis ("next to (eu)

In 2012, in the deep-sea off the coast of Japan, we found a unique microorganism that appears to have an intermediate cellular structure between those of prokaryotes and eukaryotes [20]. This organism was described using freeze-substitution electron microscopy and structome analysis (see Chapter 2). The organism was named the ‘Myojin parakaryote’ with the scientific name of Parakaryon myojinensis (‘next to (eu)

![Fig. 3-1 Schematic diagrams of a prokaryotic cell (colon bacillus, a) and a eukaryotic cell (rat pancreas, b). (Reprinted from Yamaguchi et al., Kenbikyo 48, 124-127, 2013 [27].)'](image-url)
karyote from Myojin”) after the discovery location and its intermediate morphology between the prokaryotes and the eukaryotes20. In this chapter, I describe how the Myojin parakaryote was discovered, the features of this organism, the significance of the discovery, and perspectives on future research.

Sample collection

In May 2010, we left Yokosuka harbor on the research vessel Natsushima heading to the Myojin Knoll (32°08.0'N, 139°51.0'E), which is located about 100 km south of Hachijo Island off the coast of Japan. Samples were collected from hydrothermal vents at a depth of 1240 m using a remotely operated vehicle, Hyper-Dolphin (Fig. 3-2). There was a dense population of larger creatures near the hydrothermal vents (Fig. 3-3). We collected small invertebrates, such as Polychaetes, and their associated microorganisms. Most of the collected creatures were alive when they were lifted onto the deck of the ship. The animals were photographed directly or under a stereomicroscope to record whole morphological features and aid in species identification (Fig. 3-4a). The specimens were fixed with 2.5% glutaraldehyde in sea water, kept on ice, and transported to the laboratory in Chiba University.

Electron microscopic observation of microorganisms by conventional chemical fixation

The glutaraldehyde-fixed samples were post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were cut to a thickness of 70 nm, stained with uranyl acetate and lead citrate, covered with Super Support Film (Nissin EM, Tokyo, Japan), and observed in a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Many microorganisms were found associated with the chaetae of scale worms (Polynoidae) (Fig.3-4b). Although the ultrastructure of microorganisms cannot be observed by light microscopy, they were clearly observed by electron microscopy (Fig.3-5). Fig.3-5a shows spherical microorganisms with cell wall-like structures inside the cells. They measure about 2.5 µm in diameter and are postulated to divide by binary fission because there were 4 individuals side by side. If the electron-lucent gull-shaped structures are indeed internal cell walls, these bacteria are the first observed with such a unique morphology. Cell walls of known bacterial cells are always external to the cell membrane, thus this configuration must have divergent and unknown advantages. This inner cell wall structure might be an example of the diverse and seemingly bizarre adaptations that are found in extreme environments, such as the deep sea, but are currently unknown on the surface of the Earth32.
Fig. 3-5a shows another example of the poorly-studied microbe community in the deep sea. These spherical microorganisms measure about 1.0 µm in diameter. Like the organisms in Fig. 3-5a, they may divide by binary fission since there are 4 individuals side by side. These microbes have unique comma-shaped nuclei-like structure and lack mitochondria. Fig. 3-5b shows yet another example of the types of interesting life forms we are finding in the deep sea. In this case, the microorganism is lens-shaped with a gull-shaped cell wall-like structure inside the cell very similar to that seen in Fig. 3-5a32.

Through the initial observations shown in Fig. 3-5, we noticed that it is necessary to observe deep-sea microorganisms in three-dimensions to truly understand the whole cell structure. We also noticed that the ultrastructure of microorganisms is heavily damaged by conventional chemical fixation and thus there was a need to develop methods for observing intact morphology at high resolutions. (Therefore, the above descriptions, which are based only on one ultrathin section of chemically fixed cells, must be considered preliminary until we examine the 3D ultrastructure of the cells with the more precise methods elucidated below)32.

Development of better specimen preparation methods

We knew that rapid-freeze freeze-substitution fixation would give a significantly better preservation of the ultrastructure of deep-sea specimens because we previously had good results from freeze-substitution of glutaraldehyde-fixed yeast cells31.

The chaetae with associated microorganisms were cut from glutaraldehyde-fixed scale worms, rinsed with phosphate buffer (pH 7.2), and sandwiched between two copper discs. They were snap-frozen by being plunged into melting propane kept in liquid nitrogen. The specimens were freeze-substituted in acetone containing 2% osmium tetroxide at –80°C for 2 to 6 days and embedded in epoxy resin34 (We refer to this new method as CF/FS (chemical fixation/freeze-substitution) method, as opposed the conventional chemical fixation method (CF method))32. Ultrathin sections were stained with uranyl acetate and lead citrate, covered with Super Support Film, and observed in a JEM-1400 electron microscope the same as with the conventional method.

Fig. 3-6 clearly shows improvement of cell structure preservation of deep-sea microorganisms by this new method. Fig. 3-6a is bacteria-like microorganisms, 0.3 to 0.5 µm diameter, prepared using CF method. They appear to consist of a cell wall/outer cell membrane and cytoplasm. Parts of the cytoplasm in the center are often devoid of electron-dense components and seem to have had the contents artificially extracted. Fig. 3-6d shows microorganisms similar to those in Fig. 3-6a, but prepared using CF/FS method. The cytoplasm appears to contain electron-dense components and an electron-lucent vacuole, which is spherical in shape34.

Fig. 3-6b shows a 2.2 to 2.5 µm diameter microorganism prepared using CF method. It appears to consist of an outer membrane and cytoplasm with several cellular compartments that are surrounded by membranes but appear to be devoid of electron-dense...
materials inside. Fig. 3-6e shows a microorganism similar to the one in Fig. 3-6b, but prepared using CF/FS method. The outer membrane has a very smooth and circular circumference consisting of an outer electron-dense leaflet, a middle electron-lucent leaflet, and an inner electron-dense leaflet34).

Fig. 3-6c shows an apical part of a filamentous multicellular microorganism with cell walls prepared using CF method. The cytoplasm of each cell contains several components, some of which are electron-dense while others are electron-lucent. Fig. 3-6f shows an apical part of a similar filamentous microorganism prepared using CF/FS method. The cytoplasm of each cell is filled with similar components in both specimens, but the morphology of each component appears clearly only in the CF/FS method specimen34).

As these examples show, by applying rapid-freeze freeze-substitution after glutaraldehyde fixation (CF/FS method), it is possible to observe the ultrastructure of deep-sea microorganisms at high-resolutions with minimal disturbance of their natural morphologies34).

Discovery of a “Myojin parakaryote”

We prepared 420 specimen blocks by this new CF/FS method, made serial ultrathin sections for all specimens, stained with uranyl acetate and lead citrate, and observed them with an electron microscope. After one year of sectioning and observation, we found a yeast-like microorganism several microns in size with a cell wall (Fig. 3-7)20,32,35,36.

However, this microorganism was found to lack a nucleus enclosed by a double membrane and mitochondria (Fig. 3-7). Instead, it had “endosymbionts” with bacteria-like morphology consisting of ribosomes and fibrous nucleoids but no cell wall (Fig. 3-8a, b). The “nucleoid” of the host cell had a highly irregular shape and occupied most of the host cytoplasm (Fig. 3-7). It consisted of fibrous material (DNA) and ribosomes (Fig. 3-8g). Interestingly, the “nucleoid” was different from both the true nucleoids of prokaryotes and the true nuclei of eukaryotes in that it was enclosed by single-layer membrane, which we refer to as the “nucleoid membrane” (Fig. 3-7 and 3-8g). The nucleoid membrane was not a closed membrane system but was interrupted by gaps (Fig. 3-8i) through which the nucleoid region was connected to the cytoplasm, and was notably different from the nuclear envelopes of eukaryotic cells that are made of closed double membranes20.

The cell wall consisted of one layer and had a thickness of 80-120 nm (Fig. 3-8g). The plasma membrane appeared to be a typical three leaflet structure of electron-dense, electron-transparent, and electron-dense material (Fig. 3-8h) and had a thickness of 19.4 ± 3.9 nm. The cell lacked mitochondria, chloroplasts, a nucleolus, plastids, Golgi apparatuses, peroxisomes, centrioles, spindle pole bodies, and microtubules20.

P. myojinensis was 10 µm in length and 3 µm in diameter. By 3D reconstruction from the 67 complete serial sections and structome analysis, we found that the four putative endosymbionts apparent in the sectioned image in Fig. 3-7 (labeled E) were actually different parts of the one large spiral endosymbiont (E1) (Fig. 3-9a and d) and there were a total of three endosymbionts in the cell. The other two endosymbionts (E2 and E3; Fig. 3-9d) were both rod-shaped and small, together being only around one tenth the volume of the large endosymbiont20.

Fig. 3-6 Development of better specimen preparation method for deep-sea microorganisms. a, b, c Ultrathin sections with conventional chemical fixation. Note that membranes are not smooth and cytoplasmic structures appear to be distorted and extracted. d, e, f Ultrathin sections prepared using freeze-substitution after glutaraldehyde fixation. Note that membranes are very smooth, cytoplasm is filled with electron-dense components and vacuoles and the cell (e) are nearly spherical, showing natural forms and high-resolution images. C, cytoplasm; CW, cell wall; D, electron-dense components; L, electron-lucent components; M, membrane; OM, outer membrane; V, vacuole. (Reprinted from Yamaguchi et al., J Electron Microsc, 60, 283-287, 201134).
Fig. 3-7 An ultrathin section of Parakaryon myojinensis. Note the large irregular ‘nucleoid’ (N) with single layer ‘nucleoid membrane’ (NM), the presence of endosymbionts (E), and the absence of mitochondria. Also labeled are the cell wall (CW) and plasma membrane (PM). (Reprinted from Yamaguchi et al., J Electron Microsc. 61, 423-431, 2012).

Fig. 3-8 The cellular components of P. myojinensis under high magnification. a and b The largest endosymbiont, Endosymbiont 1 (E1), showing the endosymbiont nucleoid (EN) with DNA fibers (F), ribosomes (R), and the endosymbiont cell membrane (EM), as well as the cytomembranes (CM) and the phagosome space (PS) of the host. c The second largest endosymbiont (E2). d The smallest endosymbiont (E3). e A vacuole (V). f Small granular electron-transparent materials, which might be storage materials (S). g High magnification of the host ‘nucleoid’ region (N) showing DNA fibers (F), the ‘nucleoid membrane’ (NM), ribosomes (R), cell wall (CW), and plasma membrane (PM). h High magnification of the plasma membrane (PM). i The nucleoid (N) enclosed by the nucleoid membrane (NM) with a gap (G). (Reprinted from Yamaguchi et al., J Electron Microsc. 61, 423-431, 2012).
Fig. 3-9 The three dimensional reconstruction of *P. myojinensis*. a The whole cell. b The nucleoid. c The cytomembrane system of the host cell. d The endosymbionts. e The distribution of vacuoles in the host cell. f The distribution of the small granulated electron-transparent materials in the host cell. g Trace image of Fig. 3-8i. (Reprinted from Yamaguchi et al., J Electron Microsc, 61, 423-431, 2012)

The “nucleoid” of the host occupied 40.8 % of the cell volume (Fig. 3-10) and was surrounded by a complicated cytomembrane system (Fig. 3-9c), which occupied 1.7 times the area of the plasma membrane. There were about 100 small vacuoles in the cell (Fig. 3-9e), which occupied 1.4% of the cell volume (Fig. 3-10). The cell also contained small granulated electron-transparent materials that occupied 0.6% of the cell volume (Fig. 3-10) (Fig. 3-9f), and are typically considered to be storage materials. The cytosol, including the plasma membrane, cytomembranes, and ribosomes, occupied 22.4 % of the cell volume (Fig. 3-10).

**Features of Parakaryon myojinensis**

*P. myojinensis* is more than 100 times larger than *E. coli*, three times larger than *S. cerevisiae*, and 1.5 times larger than *E. dermatitidis*. The size of prokaryotes is typically confined to a few micrometers because their metabolism is dependent on the diffusion of molecules. Because *P. myojinensis* exceeds the normal size for prokaryotes, it likely has some kind of transport system within the cell; for example, cytoskeleton molecules like actin. We do not know what kind of intracellular transport system this organism has and further study is necessary, but the size and complexity of the “nucleoid” and the cytomembrane system are suggestive of their potential involvement.

The “nucleoid” of *P. myojinensis* occupies more than 40% of the cell volume, whereas the nuclei of the yeasts occupy only 7-11% of the cell volumes. The endosymbionts in *P. myojinensis* occupy 4.9 % of the cell volume, a percentage that is in between the mitochondrial volumes in *S. cerevisiae* (1.6 %) and *E. dermatitidis* (9.9 %). The presence of a giant endosymbiont is reminiscent of the presence of a giant mitochondrion in *S. cerevisiae*. The endosymbionts themselves of *P. myojinensis* might fuse or divide during the cell cycle as do the mitochondria of yeast cells. Further study is needed to clarify the nature of the symbiosis between the host and the endosymbionts. Table 3-1 summarizes the features of *P. myojinensis*.

**Possible origins of the *P. myojinensis***

*P. myojinensis* seems to be a stable species that originated through an endosymbiotic event in the past involving a larger prokaryote and smaller bacteria as
Table 3-1  Features of Parakaryon myojinensis (Reprinted from Yamaguchi et al., J Electron Microsc, 61, 423-431, 2012 [20]).

|   |               |
|---|---------------|
| 1 | Cell size     | Much larger than ordinary prokaryotes; more than 100 times larger than E. coli; three times larger than S. cerevisiae |
| 2 | Nucleoid      | Consists of prokaryote type DNA fibers and no nucleolus structure; very large and consists of more than 40% of the cell volume |
| 3 | Nucleoid membrane | Single membrane surrounding the nucleoid; pierced with gaps |
| 4 | Endosymbionts | Similar ultrastructure to modern eubacteria consisting DNA fibers and ribosomes; lack cell walls but enclosed by cell membranes |
| 5 | Other organelles | Cell wall, plasma membrane, complex cytomembrane systems, many vacuoles, small granular electron-transparent materials; none of the following: mitochondria, chloroplasts, plastids, Golgi apparatus, peroxisomes, centrioles, spindle pole body, microtubules |

It is fervently hoped that genetic and biochemical work will be possible in the future when another specimen of P. myojinensis is found to enable estimates of its phylogenetic position and the timing of its endosymbiotic event.

Implications for theories of mitochondrial and nuclear development

According to the endosymbiosis theory, the ancestor of mitochondria is believed to be α-proteobacteria. There are several lines of evidence for this theory. 1) Mitochondria have their own DNA. 2) They have a double membrane structure. 3) They have 70S ribosomes that are similar to bacterial ribosomes and different from eukaryotic ribosomes. 4) They multiply within the cell by division. 5) The genome of Rickettsia prowazekii (α-proteobacteria) was found to be similar to the mitochondrial genome [37].

The relationship between P. myojinensis and its endosymbionts must be a beneficial one for it to have lasted long enough for the endosymbionts to lose their cell walls and host to gain its cell wall. It seems likely that the endosymbionts in P. myojinensis are descendants of bacteria engulfed by a larger prokaryote in the past, thus the micrographs of the present study may provide an example that shows another endosymbiotic event that lends support to the hypothesis that the highly-derived mitochondria in eukaryotes could indeed have evolved from bacteria [20].

The origin of the eukaryote nucleus is another mystery to which P. myojinensis may provide clues. There are currently several hypotheses vying for dominance [38]. The eukaryote nucleus could have evolved gradually by the development and elaboration of an inner cytomembrane system [39], but it could also have been engendered by fusion or symbiosis between multiple prokaryotes.

The ‘nucleoid’ of P. myojinensis is not a true nucleus because it does not contain chromatin (DNA associated with histone proteins), which is a hallmark of eukaryotic nuclear organization. Instead the ‘nucleoid’ contains naked DNA fibers, much like the prokaryotic nucleoid. However, true prokaryotic nucleoids are not surrounded by membranes and that of P. myojinensis is surrounded by a single layered membrane with scattered gaps. Furthermore, this membrane differs from the eukaryotic nuclear membrane, which is double-layered and complete. This nucleoid membrane could be a form of primitive nuclear membrane. If this is the case, it follows that nuclear membranes could have evolved from the cytomembranes of prokaryotes that had developed inner membrane systems [20].

There is still a question about whether a nucleus was formed before mitochondrial ancestors started the process of endosymbiosis [40]. The nuclear region of P. myojinensis is not a completely formed eukaryotic nucleus but internalized endosymbionts are already in the host cell. This suggests that a fully formed and differentiated nucleus was not necessary before eubacteria could start to be integrated within the prokaryote host cell. Thus, the formation of the eukaryotic nucleus might not have been linked in any way to the transformation of bacteria into mitochondria [40].

A model of eukaryote origins

Fig. 3-11 shows a model of eukaryote origins and a plausible evolutionary position of P. myojinensis [32] partly adapted from Whittaker (1969) [31] and Woese et al. (1990) [42]. Primitive Archea with no cell wall may have engulfed α-proteobacteria by phagocytosis and become
The ancestor of protists, fungi, animals, plants, and the parakaryote. The ancestor of plants may have engulfed cyanobacteria at a later time to become modern plants. Cell walls likely arose independently in the plant, fungi, and parakaryote lineages after the endosymbiosis process was complete\textsuperscript{32}.

At this point, the phylogenetic position of the parakaryote is a matter of pure conjecture. Further genetic studies and biochemical analysis are necessary to elucidate the nature and phylogenetic position of the parakaryote as these are impossible to perform on specimens prepared for electron microscopy. Whatever the true position of the parakaryote in the tree of life, the existence of an apparently transitional life form between prokaryote and eukaryote provides a useful model of what the ancestor to all eukaryotic life could have been like and so how the transition from prokaryote to eukaryote could have proceeded. It is likely that the cellular physiology and functioning of the parakaryote are as unique as is its morphology and by providing a third perspective could potentially give a much deeper understanding of cellular mechanisms in general\textsuperscript{32}.

The problems and potential of deep-sea microorganism studies

There are a few studies of newly discovered deep sea microorganisms that were characterized morphologically, genetically and/or biochemically after being cultured in the lab. However, culturing practices are always biased towards certain types of microorganisms with particular tolerances. Considering the fact that standard methods fail to successfully culture most microbes\textsuperscript{43}, most organisms are overlooked by these methods. Our strategy of direct observation of individual microorganisms is time- and labor-intensive but has the advantage of sampling deep-sea microorganisms without bias toward organisms able to thrive in particular culturing conditions\textsuperscript{20}.

Standard methods of identifying microorganisms through culture and genetic and biochemical characterization are some of the greatest successes of modern biology. However, the natural bias inherent to cell culture techniques severely limits the exploration of unique microorganisms from extreme environments or with unusual requirements. Rather than ignoring these microorganisms and their potentially paradigm-altering structures and adaptations, we believe that careful preparation and morphological characterization can be a useful and important first step in discovering and describing new and unique microorganisms. To do so effectively, we developed the CF/FS method to observe the natural morphology of microorganisms at high resolution by using freeze-substitution electron microscopy\textsuperscript{34}. To identify different microorganisms morphologically, we used a serial ultrathin sectioning technique to conduct structome analysis\textsuperscript{9,12}. These
method enabled us to observe and record a variety of microorganisms from the deep sea, many of which exhibit unusual morphologies. These unusual morphologies are likely mirrored by unusual biochemistry and evolutionary histories and may hold clues to important questions in evolutionary biology, such as the transition from prokaryote to eukaryote, the origins of mitochondria and nuclei, and the origins of centrioles, spindle pole bodies, flagella, and other organelles. The deep sea is an extremely stable environment in which there might be very little selective pressure for change and low levels of competition, leading to still surviving “living fossils” that may retain features long absent from more typical lineages in more “normal” environments.

**Perspectives on future research**

As discussed above, it is difficult to culture deep-sea microorganisms under laboratory conditions. Yet, it is important to obtain genetic data for each microorganism observed with an electron microscope to clarify its phylogenetic position. This presents a tremendous problem because, generally, electron microscopy specimens are fixed with chemicals, embedded in a resin, thinly sliced, stained with heavy metals, and radiated by an electron beam for observation. These harsh treatments must be avoided if the DNA is to remain intact for analysis.  

Matching up morphology with phylogenetics would be possible if the same individual cell were able to be used for both types of analysis. This could be achieved by using serial sections of the target microorganism. That is, the specimen is freeze-substituted with acetone only, embedded, thinly sliced, photographed by electron microscope, and the target is mapped. The next section is cut thick for light microscopy, the target is mapped.  

The DNA/RNA of the target microorganism is never treated by electron microscope, and the target is mapped. The DNA/RNA is sequenced. In this technique, since the DNA/RNA of the target microorganism is never treated with chemicals like osmium tetroxide and never irradiated by electron beams, it could be used for sequencing. If this technique is perfected, research on deep-sea microorganisms would advance significantly. This technique would be also useful for other research where the microorganisms cannot be cultured. As it stands now, the partnership between morphology and genetics of microbes lags far behind that of multicellular organisms, but hopefully, this will rapidly change in the near future.

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