Investigation of sample preparation methods for EUV imaging of fixed bio-cells

T. Ejima, F. Ishida, Y. Neichi, M. Yanagihara
IMRAM, Tohoku University: 2-1-1, Katahira, Aoba-ku, Sendai, 980-8577 JAPAN
iejima@tagen.tohoku.ac.jp

Abstract. Recent development of normal-incident reflection multilayer optics allows us to observe with both high spatial resolution and large field of view in EUV wavelength region. Some bio-cells and/or tissues need both high spatial resolution and large field of view in an observation tool even in a fixed and stained foam of a sample. Both fixation and stain methods are investigated and compared for EUV imaging of bio-cells. As the results, the preparation method needs no heavy-metal staining used usually in TEM observation.

1. Introduction

Normal-incident optics coating with a reflection multilayer in EUV wavelength region ($\lambda = 3 \sim 50$ nm) have convex and concave mirrors, therefore aberration of the optics can be corrected by combination of the mirrors. Normal-incident multilayer optics show high spatial resolution because of the short wavelength in EUV region. Therefore the optics coexist large field of view with high spatial resolution, and are being applied to exposure or mask-inspection tools of EUV lithography [1].

When the EUV multilayer optics is applied to an observation tool for bio-cells, it will be possible to observe a small organelle less than several tens of nm in a large tissue more than several hundreds of $\mu$m. In a case of brain, a neuron connects to the other through a synapse, and connections of neurons integrated form neural circuits. Expression mechanism of brain functions is believed to be caused by the neural circuits. To study the neural circuits in a brain, all connection points of neurons will be clarified through observations of whole brain, and the circuits are reconstructed by the clarified connection points (“connectome” plan [2]). In the tool for the observation, both high spatial resolution (less than several tens of nm) and large field of view (as large as possible) will be needed.

An exposure tool for EUV lithography [3] or a transmission-type EUV microscope [4] using a wavelength 13.5 nm are one of the microscopes that coexist high spatial resolution with large field of view. Because the wavelength 13.5 nm is far from the absorption edges of component atoms of bio-cells, then it was considered as being unsuitable wavelength to observe bio-cells. But differences of the absorption cross sections are large between the component atoms, therefore contrast needed in observations of bio-cells will be obtained in wavelength 13.5 nm [5]. The majority of component atoms is oxygen included in water. And the absorption cross section of oxygen atoms is large, and then the oxygen atoms in bio-samples should be decreased for the observation [5].

Easy way to decrease the oxygen atoms is that water in bio-cells is removed, and that the dry block of bio-cells is sliced as thin as possible. Transmittances of sections fabricated finally will be high and contrasts of the section images will be kept by carbon and nitrogen atoms. On the other hand, preparation method for bio-cell observation is unknown in EUV wavelength region including the
wavelength 13.5 nm, therefore we investigate fixation and staining methods and fabricate a conventional evaluation tool for fixation and staining methods [6].

2. Preparation conditions for EUV observation of bio-cells

2.1. Suitable thickness for EUV observation

To decrease the large absorption of bio-cells, suitable thickness of sections was obtained changing both thickness and stain method of bio-cells. We chose cerebral cortex as the bio-cells. After the perfusion and fixation of cerebral cortex by solutions of 2% paraformaldehyde, 2.5% glutaraldehyde, and 1% osmium tetraoxide, a block of the sample was stained by Reynolds method [7]. The block sample was dehydrated by ethanol, embedded by epoxy resin, and then sliced 200 nm and 500 nm thick sections by an ultramicrotome (here after "block stain" section). In addition, some sections of each thickness were stained again by Reynolds method (here after "electric stain" section).

EUV images of the sections were taken at wavelength 13.5 nm by using an EUV microscope TXM [4]. Five EUV images were taken with 20 shots of YAG pulse (duration time was 20 nsec, repetition rate, 10Hz) at the same position. And then the images were averaged, and normalized by an averaged image of the light source. Transmittance images obtained finally have enough contrasts to distinguish organelles in the images.

Nucleuses and blood vessels were selected as the organelles and the resin, respectively. Using the measured transmittances of nucleuses, $T_n$, and of blood vessels, $T_{bv}$, contrast of nucleus to blood vessel $C = (T_n - T_{bv})/(T_n + T_{bv})$ was calculated in each section. Absorption coefficients of them were estimated from both the transmittances and thicknesses of the sections. Finally, dispersion curves of transmittances and contrasts were calculated from the absorption coefficients. Obtained results are represented in Figure 1. The result shows that the contrasts increase as transmittance decreases. At thickness region from 300 nm to 350 nm, the contrasts are balanced with the transmittances.

2.2. Wavelength dependence of the contrast in nucleuses

Thickness of sections is fixed to be 400 nm thick from the result in section 2.1, organelles for comparison are reduced to nucleus only, and then preparation methods are compared. The preparation methods are listed in Table 1. Details of the preparation methods are shown in references of the table. Measurements were carried out using SCOM, we have fabricated [6], at beamline BL11D, Photon Factory, KEK, Japan under the conditions that the wavelength resolution $\lambda/\Delta\lambda$ was 500, the exposure time, 40 ~ 360 sec. Transmission images were normalized as in section 2.1, and the images at the wavelength 13.5 nm are represented in Figure 2.

Nucleuses of the sample D are hard to be recognized, therefore no result was obtained. Transmittance values of sections prepared by the other methods decrease as the increase of wavelength.
because of the increasing values of absorption cross sections of C, N, and O atoms. The values of transmittances are different depending mainly on the preparation method except for the absorption edges of stain materials.

Contrasts of nucleuses to blood vessels (designated as N and VB, respectively, in Figure 2) obtained finally in rat hepatic cells are shown in Figure 3. Sample E that was embedded by acrylic resin without stain shows high contrast value in all wavelengths. This is due to the simpler structure of acrylic compared to other embedding and staining media. And the result of the simple structure, absorption by the embedment material itself is lower than the other materials of the samples. Then the absorption by the organelles, which have complex molecular structures, is higher than that of the acrylic resin.

3. Evaluation of preparation method by merit function

A merit function is newly introduced to evaluate the preparation methods on the basis of the experimental results. When an object forms an image on a CCD sensor and the image is recognized as the object itself, the object will have a contrast to the background around the object in the image. The contrast of the object to the background is represented by a difference of photon number between the object and the background images. Using the contrast $C$ and the average photon number $\overline{N}$ between the two, the difference of photon number will be approximated as $2C\overline{N}$. When the photon number on CCD sensor is high enough, a noise of the object image is represented by the fluctuation of the photon. Because the photon number detected on CCD sensor obeys Poisson distribution, the fluctuation of the photons is a standard deviation value $\sqrt{\overline{N}}$ of the photon number. In this situation, the signal to noise ratio $SN$ of the object is as follows:

$$SN = 2C\overline{N}/\sqrt{\overline{N}} = 2C\sqrt{\overline{N}}.$$  (1)

The average photon number $\overline{N}$ between an object image and a background will depend on: a photon intensity of irradiation light, a throughput of an imaging optics, a transmittance of an object, and exposure time for taking an image. When we assume that the parameters are constant except for the transmittance value of the object and that the photon number of the background is low enough, the average photon number is proportional to the transmittance of the object. Therefore, the average photon number $\overline{N}$ will be replaced by the transmittance value $T$, and the SN ratio is represented by

$$SN \propto C\sqrt{T}.$$  (2)

Therefore the merit function Eq. (2) is represented by the use of transmittance and contrast.

Using Eq. (2), suitable thickness for EUV observation considered in section 2.1 is evaluated again. Transmittances and contrasts calculated from both the absorption coefficients and changing thickness of the section were used to calculate the merit functions for each stained section. The results are shown in Figure 4. The value of the SN ratio increases as the increase of the thickness. The value shows the maximum at around 300 $\sim$ 700 nm thick in the block stained section, and at 200 $\sim$ 500 nm thick in the electric stained sample. The common thickness between the sections measured was 300 $\sim$ 500 nm. The result coincides with the result in section 2.1.

Finally, values of merit function for nucleus are calculated in all preparation methods of Table 1.

| Table 1. Rat hepatic cells changing the embedment and stain methods. |
|---------------------------------------------------------------|
| **Embedment** | **Stain** | **Transmittance** | **Contrast** |
|----------------|-----------|-------------------|--------------|
| Epoxy embedment | Sample A [8] | Sample B [7] | Sample C [10] | Sample D [11] |
| Acrylic embedment | × | × | × | Sample E [9] |
The results are shown in Figure 5 for comparison with each other. Values of Sample E are higher than those of the other samples in wavelength region from 7 to 15 nm. This result shows that the sample embedded by acrylic resin will be suitable in this wavelength region.

The merit function introduced here focuses on the differences of contrast and photon number, and the spatial distance is arbitrary between two objects. In the present evaluation, the organelles chosen for the evaluation were large in size and the distances between the organelles are relatively long comparing with the spatial resolution. Then a merit function including spatial information between objects will be required, when visual confirmation in the order of spatial resolution is required in preparation methods for EUV observation.

4. Summary

Instead of the usual motivation, which is an observation of "living" organelles in a "living" bio-cell at Water-window wavelength region, present results show that the microscopes in EUV wavelength region will be applied to some bio-cell samples, which need an image with coexistence of both high spatial resolution and large field of view as needed in "Connectome" plan. In addition, preparation method for EUV observation needs no heavy-metal stain used usually in TEM observation. Different information of bio-cells will be given comparing with that from TEM observation.

References

[1] for example, D. Attwood, Soft X-rays and extreme ultraviolet radiation, (Cambridge University Press, Cambridge, 2000) Chap. 4.
[2] O. Sporns, G. Tononi, R. Kotter, PLoS comp. bio. 1, (2005) e42.
[3] P. P. Naulleau, K. A. Goldberg, E. Anderson, J. P. Cain, P. Denham, K. Jackson, A.-S. Morlens, S. Rekawa, and F. Salmassi, J. Vac. Sci. Technol. B 22(6), 2962-2965.
[4] T. Ejima, F. Ishida, H. Murata, M. Toyoda, T. Harada, T. S., T. Tsuru, T. Hatano, M. Yanagihara, M. Yamamoto, and H. Mizutani, Opt. Exp. 18, (2010) 7203-7209.
[5] http://henke.lbl.gov/cgi-bin/mldata.pl
[6] T. Ejima, et al., ibid.
[7] E. S. Reynolds, J. Cell Biol., 17 (1963) 325.
[8] After sample fixation by 4% paraformaldehyde, 2.5% glutaraldehyde, and 1% osmium tetraoxide solutions, a block of sample was stained by 1% uranyl acetate solution. Then it was dehydrated by ethanol, embedded by epoxy resin, and sliced sections by an ultramicrotome.
[9] After sample fixation by osmium tetraoxide solutions, a block of sample was fixed again by 4% paraformaldehyde and 2.5% glutaraldehyde. Finally, a block of the sample was dehydrated by ethanol, and embedded by acrylic resin.
[10] P. Mayer, Mitt. Zool. Stat. Neapel., 10 (1981) 170.
[11] After sample fixation by 4% paraformaldehyde, 2.5% glutaraldehyde, and 1% osmium tetraoxide solutions, a block of sample was dehydrated by ethanol, embedded by epoxy resin.