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Article

Serial-section scanning electron microscopy

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1. Introduction

In recent years, techniques involving three-dimensional (3D) reconstruction of consecutive scanning electron microscopy (SEM) images of resin-embedded specimens have been the subject of increased attention for the structural analysis of biological specimens. Such techniques include FIB/SEM,[1,2] which is a block-face observation method using a focused ion beam (FIB), and serial block-face SEM (SBF/SEM),[3] in which an ultramicrotome is installed inside an SEM system. There have also been reports on 3D reconstruction of SEM images of serial ultrathin sections mounted on a rigid substrate such as glass.[4-7] This is commonly referred to as serial-section SEM (or array tomography), and it offers a number of advantages over other imaging methods. First, the complex grid manipulation required for serial-section transmission electron microscopy (TEM) is not required, allowing serial ultrathin sections to be collected with ease. In addition, wide areas of the samples can be observed at one time, and any given section can be re-observed any number of times if necessary. In this report, we provide an introduction to serial-section SEM and discuss its application to the 3D structural analysis of the Golgi apparatus.

2. Preparation of samples for serial-section SEM

Serial-section SEM is a technique in which serial ultrathin sections are mounted on a rigid substrate, and images of these sections are obtained by SEM (Fig. 1). Here, we describe the various steps of serial-section SEM for the Golgi apparatus. [8), 9)]

1. Trimming of resin

First, a resin block is trimmed into a trapezoidal shape using a razor blade. It is important that the upper and lower edges of the trapezoid be trimmed in parallel to ensure that the serial sections are produced with a ribbon-shaped configuration. The dimensions of the trapezoid should be trimmed within an upper edge length of 0.7 mm, a lower edge length of 1 mm, and a side height of 0.5 mm.

2. Preparation and arrangement of serial-section ribbons

Ultrathin serial sections (100 nm thick) are cut using an ultramicrotome with a diamond knife. The length of the serial-section ribbons is chosen to ensure that they can be picked up using an iron ring with an inner diameter of 7 mm. In our case, a ribbon containing 10-15 serial sections is cut using the diamond knife, and then carefully separated from the knife using an eyelash. The process of ribbon creation and separation is repeated to produce four aligned ribbons, resulting in a total of 40-50 sections (Fig. 2A).

3. Collection of sections

The serial-section ribbons are picked up using the iron ring (Fig. 2B).

4. Transfer of sections

The ribbons are transferred from the ring to a glass slide.

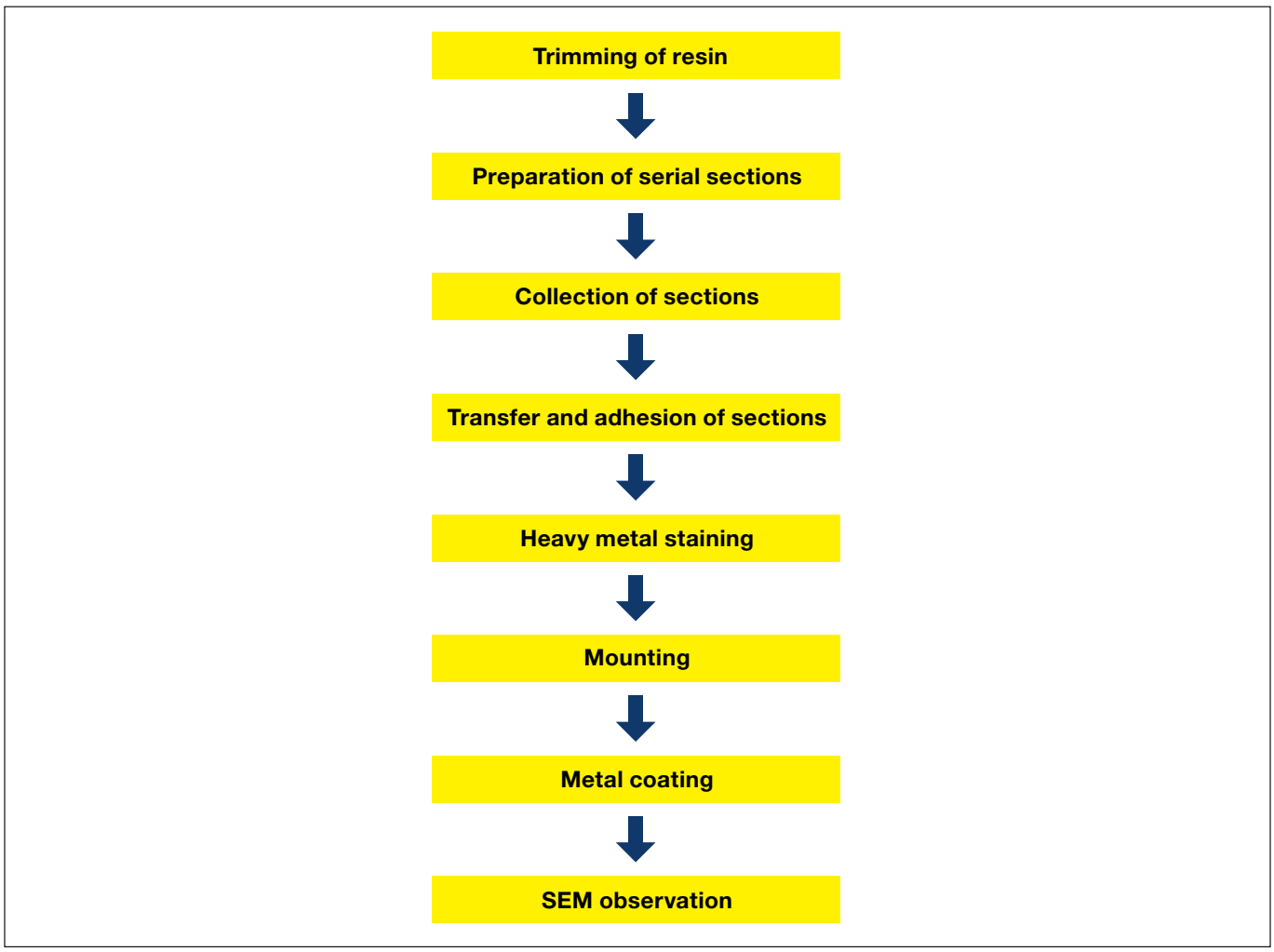

Fig.1 Flowchart showing sample preparation procedure for serial-section SEM.

5. Adhesion of sections

The glass slide with the sections is placed on a hot plate, which has been preheated at 60°C for 30 min (Fig. 2C). The required number of serial sections for 3D reconstruction is obtained by repeating steps 2–5.

6. Heavy metal staining

In contrast to FIB/SEM and SBF/SEM, serial-section SEM allows the sections to be immobilized on the glass slide. For this reason, the sections can be stained with heavy metals. Here, the sections are stained with a 1% aqueous solution of uranyl acetate for 10 min, rinsed with distilled water, and then stained with Reynolds lead solution for 5 min (Fig. 2D).

7. Mounting

To mount the glass slide with the sections on the aluminum SEM sample stub, a diamond pen is used to scribe a line on a glass slide (region with dimensions of approximately 1 cm × 1 cm), and the slide is then carefully broken by hand. Carbon tape is then used to mount the slide on the SEM sample stub (Fig. 2E).

8. Metal coating

If the glass slides with the sections are subjected to SEM, this may result in the occurrence of artifacts due to charging. To avoid this problem, the serial sections are coated with platinum/palladium with a thickness of several nanometers (Fig. 2F).

9. SEM Observation

Finally, the sections are observed by SEM system (Hitachi SU3500) equipped with a thermionic electron gun and a highly sensitive semi-annular semiconductor backscattered electron (BSE) detector at an accelerating voltage of 7 kV.

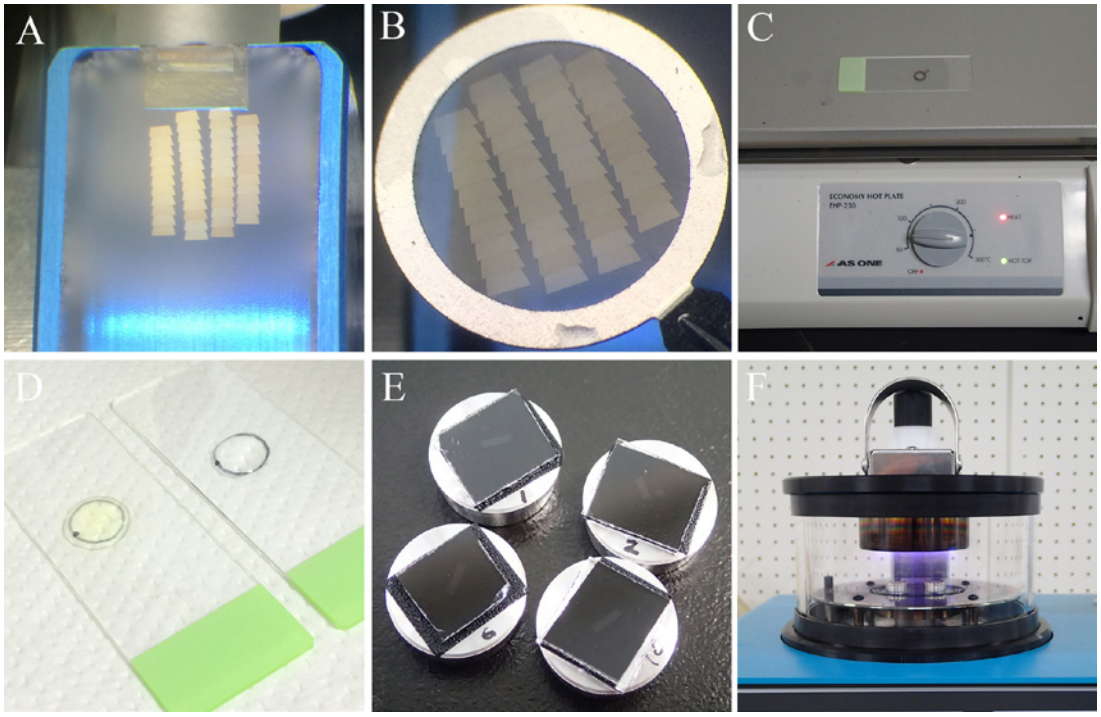

Fig.2 Serial-section SEM.
A. Four aligned ribbons of serial sections. Each ribbon consists of roughly 15 sections, so the number of samples that can be acquired at once is approximately 50.
B: Collecting sections using the iron ring.
C: Adhesion of sections. The slide is heated for approximately 30 min on a hot plate (60 °C).
D: Staining with uranium and lead. It is easy to apply heavy metal staining to sections mounted on glass slides.
E: Mounting. After being cut to a predetermined size using a diamond pen to make cracks, the slide is mounted on the aluminum SEM sample stub.
F: Metal coating. To prevent artifacts due to charging of the specimen, a platinum/palladium coating is applied.

3. BSE images of ultrathin sections

Figure 3A shows a BSE image of an ultrathin section of an osmium-stained Golgi apparatus, and Fig. 3B shows a black-white inversion of the same image. The black-white inversion of the BSE image exhibits contrast that is similar to that in TEM images. In Fig. 3B, osmium deposits are visible in the cisterna on the cis side of the Golgi apparatus, due to a higher BSE intensity. Similarly, by double staining with uranium and lead, it is possible to observe cellular organelles such as the endoplasmic reticulum, mitochondria, nuclei and secretory granules.

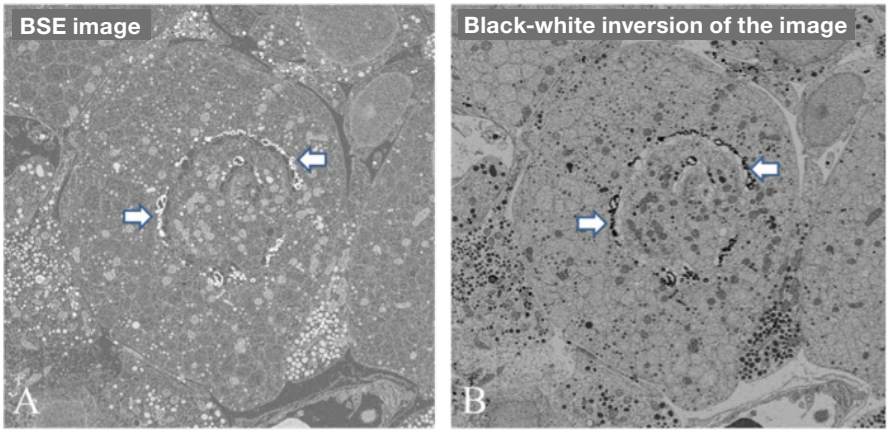

Fig.3 Ultrathin section of osmium-stained Golgi apparatus.
A: BSE image.
B: Black-white inversion of Fig. A. By performing a black-white inversion of the BSE image of the section, we obtain an image similar to that which would be obtained by TEM. Osmium deposits are visible in the cis-Golgi cisterns (arrows).

4. Methods for 3D reconstruction

After serial sections have been observed by SEM, 3D reconstruction of the target structures must be performed. Here, we briefly introduce the 3D reconstruction method, which is schematically illustrated in Fig. 4.

1. Selection of observation region

Considering an example in which 300 serial sections have been prepared, observations should begin with sections near the center of the series, i.e., around the 150th section. Information from this section can be used to determine the region that should be subjected to 3D reconstruction.

2. Capturing serial sections

After determining the region of interest, observations are performed on successive sections until target structures such as the Golgi apparatus, nucleus and cytoplasm disappear from this region.

3. Adjusting alignment

The serial section images obtained by SEM are imported into a 3D computer program and automatic alignment is performed. After visual inspection, fine adjustments to the alignment may be carried out manually.

4. Segmentation

After alignment, the images are imported into an image-processing computer program, where the Golgi apparatus, nuclei, and cytoplasm are segmented by tracing their boundaries.

5. Stacking and 3D reconstruction

After segmentation of the target structures in the serial images, the images are re-imported into the 3D software, and 3D reconstructed surface-rendered images are made.

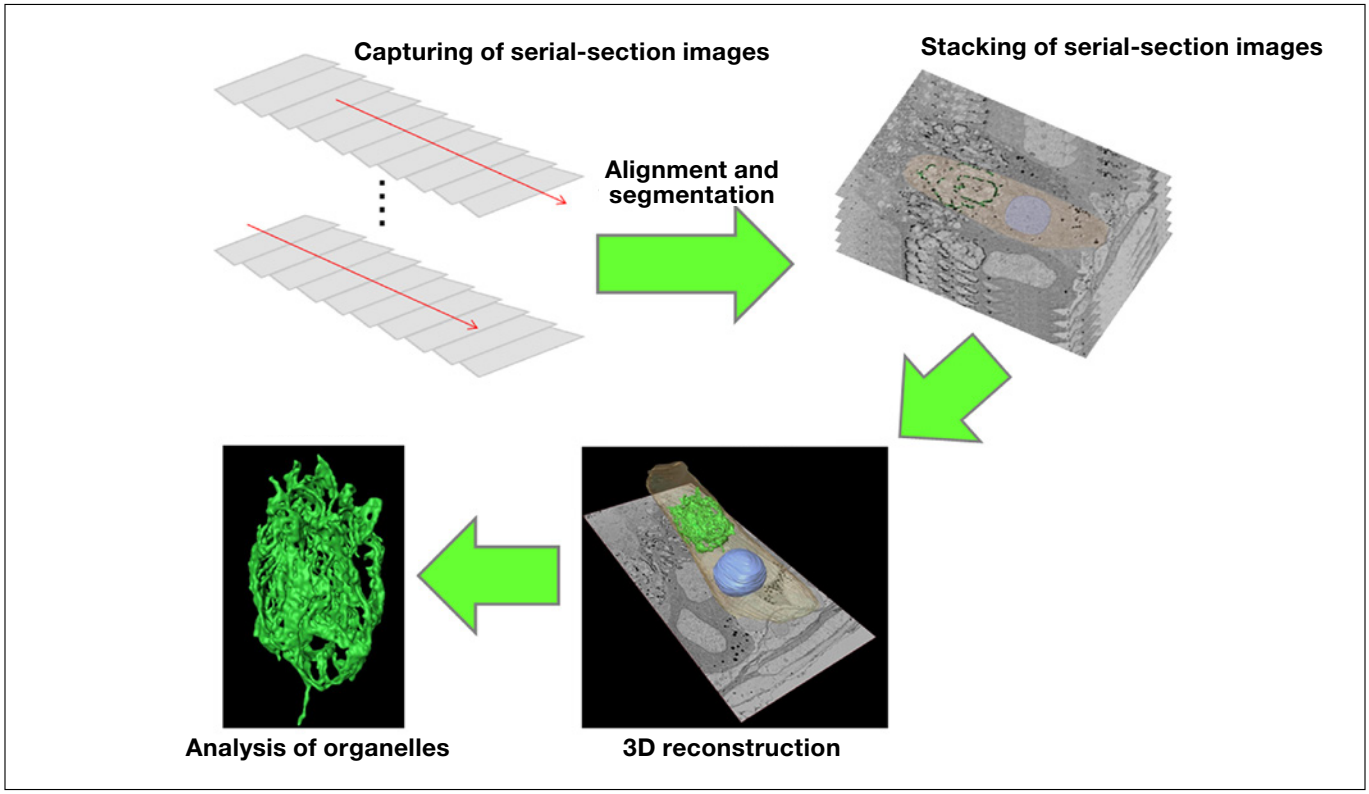

Fig.4 Procedure for 3D reconstruction of serial-section images.

5. Application of serial-section SEM

Figure 5A shows 3D reconstructed images of the nucleus, the cytoplasm, and the Golgi apparatus in an epithelial principal cell in a rat epididymis. In the cell, 150 ultrathin serial sections were used to produce a 3D reconstructed image of the Golgi apparatus. The Golgi apparatus is located in a large region of cytoplasm between the juxtanuclear region and the lumen. Fig. 5B shows closer view of the 3D reconstruction of the Golgi apparatus in Fig. 5A, and its spatial complexity is clearly evident. Although the continuity of the Golgi cisterna cannot be determined based on observations of a single ultrathin section, it is very clear in the 3D reconstructed image. By combining serial-section SEM and 3D reconstruction techniques, it is therefore possible to analyze the overall shape of the Golgi apparatus with the spatial resolution of electron microscopy.

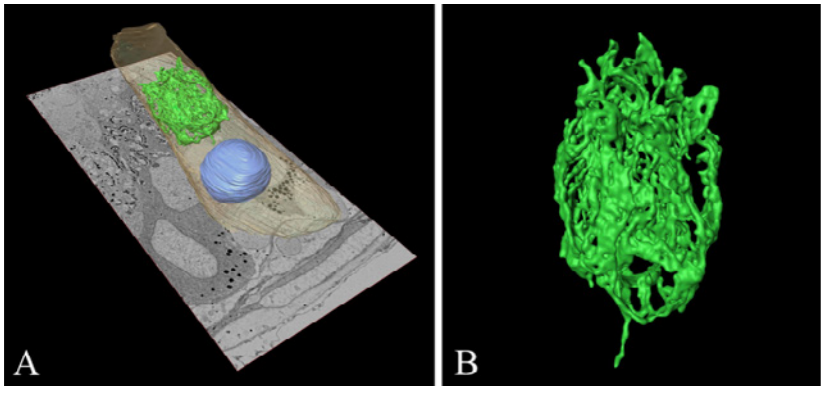

Fig.5 3D reconstructed images of the Golgi apparatus in an epithelial principal cell in a rat epididymis.
A: Golgi apparatus (green), nucleus (blue), and cytoplasm (brown) in the cell.
B: Closer view of the Golgi apparatus showing complex cisterna that resemble the petals of a tulip.

6. Advantages of serial-section SEM

Serial-section SEM offers a number of advantages over other techniques. Here, we discuss some of the merits of this method.

1. Easy collection of serial sections

In serial-section TEM, serial sections in a ribbon must be detached and transferred to a single slot grid. This grid manipulation process is complicated and requires training and practice. If carried out by an inexperienced operator, there is a high risk of damaging or losing sample sections. In contrast, in serial-section SEM, the sample sections are adhered to a rigid substrate such as a glass microscope slide, so that such grid manipulation is unnecessary. Collecting ribbons of serial ultrathin sections using the iron ring is particularly easy and causes little damage to the individual sections.

2. Re-observation of serial sections

In methods such as FIB/SEM and SBF/SEM, the surface of a specimen embedded in resin is removed using a FIB or a diamond knife, and the newly exposed block face is examined by SEM. Since this is a destructive process, it is not possible to perform repeated observations of the same specimen. In contrast, in serial-section SEM, serial sections on a slide can be preserved semi-permanently, allowing samples to be re-observed any number of times.

3. User-determined observation area

Serial-section SEM allows wide areas of resin-embedded specimens to be observed, because the dimensions of the trimmed resin block can be chosen to suit the purpose of the observations. The region of the resin block that is trimmed—that is, the region observed by SEM—can be arbitrarily chosen. Depending on the sample, in some cases it is best to observe a wide region, while in other cases a narrow region will suffice. In addition, the region of the specimen that is observed (z-direction) can be tailored by choosing the number of sections.

4. Reduced electron-beam damage

In serial-section TEM, sections mounted on a single slot grid are easily damaged, due to expansion or contraction under electron beam irradiation. In contrast, in serial-section SEM, the serial sections are adhered to a rigid substrate such as a glass slide, ensuring that the damage inflicted by the electron beam is minimized.

5. Heavy metal staining

Because samples mounted on glass slides can be subjected to heavy metal staining using uranium and/or lead, it is possible to perform SEM observations on samples prepared under conditions similar to those for conventional TEM samples.

6. Sections can be processed to confer electrical conductivity

When sections mounted on glass slides are observed by SEM, charging artifacts can lead to image drift, and can cause thermal damage to samples. These problems can be solved by making the sections electrically conductive using a metal or carbon coating.

7. Immunohistochemical staining (immunostaining) can be studied

With serial-section SEM, it should be possible to apply immunostaining procedures to sections on glass slides. In particular, using a hydrophilic acrylic resin such as LR white, it should even be possible to directly compare immunofluorescence images and 3D reconstructed images.

7. Disadvantages of serial-section SEM

In serial-section SEM, the resolution in the *x*- and *y*-directions, i.e., the section plane, is determined by the instrument used, whereas the resolution in the *z*-direction depends on the thickness of the sections. The *z*-direction resolution is inferior to that of FIB/SEM, because in serial-section SEM, a section thickness of 100 nm is thought to be best for creating a stable ribbon of serial sections, whereas the milling pitch can be as little as 10 nm per step in FIB/SEM. In addition, since image alignment is essential in serial-section SEM, it is important to perform accurate alignment procedures in order to obtain high-quality 3D reconstructed images.

8. Why serial-section SEM?

The osmium-maceration method, an imaging technique developed more than 30 years ago by the group of Professor Keiichi Tanaka at Tottori University, is a useful method for imaging cellular organelles by SEM.[10), 11)] Using this method, we have studied the 3D ultrastructure of cell organelles (Fig. 6A). In the osmium-maceration method, because the cracked surface of cells is observed by SEM, it is useful for analyzing the 3D ultrastructure of the cristae of mitochondria, ribosomes on the cisterna of the endoplasmic reticulum, and the cisterna of the Golgi apparatus. However, it is difficult to determine the overall shape of the Golgi apparatus and the endoplasmic reticulum, which are located in a large region of cytoplasm (Fig. 6B). Therefore, we came to feel strongly that serial-section SEM was a crucial technique for solving this problem. Because there were no nearby facilities where we could perform FIB/SEM or SBF/SEM observations, we developed an original method of applying serial-section SEM to the Golgi apparatus. By repeated trial-and-error over the course of approximately one year, we eventually succeeded in obtaining serial sections. As a result, we were able to image the overall shape of the Golgi apparatus, only the cracked surface of which could be observed by the osmium-maceration method, with the spatial resolution of electron microscopy.

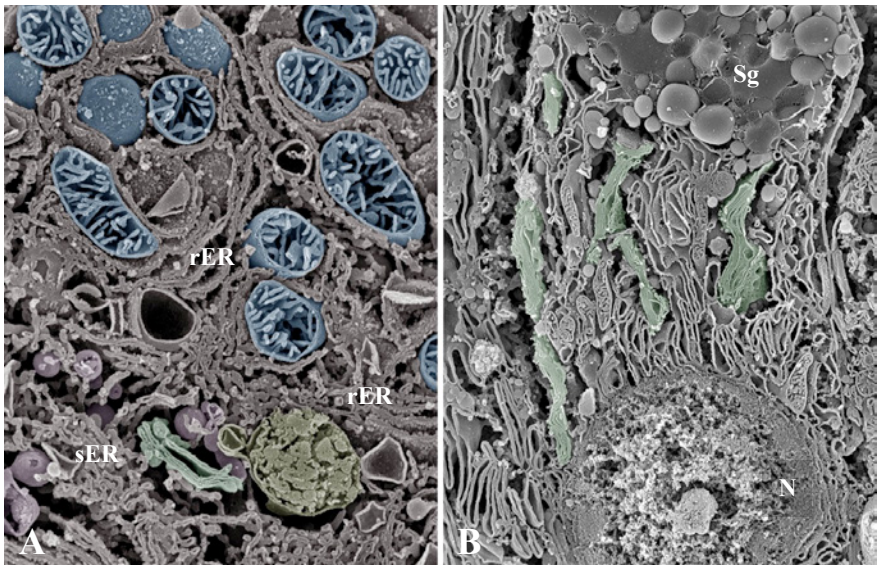

Fig.6 SEM images of osmium-macerated tissues.
A: Osmium-maceration image of hepatocyte. The cracked surfaces of the mitochondria (blue), the rough/smooth endoplasmic reticulum (rER/sER), and the Golgi apparatus (green) are observed. Cristae are also identified within the matrix space of the mitochondria.
B: Osmium-maceration image of a goblet cell in the rat jejunum. Because the cracked surfaces of cells are being observed, the continuity of the Golgi cisternae is not discernible, and it is difficult to analyze the overall structure of the Golgi apparatus (green). Sg: Secretory granules. N: Nuclei.

9. Osmium-maceration method vs. serial-section SEM

Finally, we would like to discuss our opinions regarding the osmium-maceration and serial-section SEM methods. The osmium-maceration method allows direct observation of the 3D ultrastructure of organelles such as mitochondria and the Golgi apparatus without 3D reconstruction. In contrast, in serial-section SEM, computer software is used to reconstruct serial-section images. It must therefore be kept in mind that the resulting 3D image is ultimately nothing more than a product created by a computer. On the other hand, because in the osmium-maceration method the cracked surfaces of cells are being observed, it is difficult to use this method to capture the overall shape of structures that are widely distributed throughout the cytoplasm, such as the Golgi apparatus and endoplasmic reticula. In contrast, serial-section SEM can image the overall shape of a single cell. It is therefore possible to analyze the overall structure and continuity of specific organelles. In view of these advantages and drawbacks, we believe the proper question is not "which of these two imaging techniques is better?", but rather "what do you wish to see?" In other words, it is important to choose the appropriate method based on the purpose of the study. Also, in addition to the osmium-maceration method, there are excellent sample-preparation methods that are all-too-easily overlooked, such as the KOH digestion method for observing cellular components hidden in tissues, the alkali-water maceration method for analyzing collagen fiber networks, and the vascular casting method for 3D visualization of the vascular system. In the future, it can be expected that the combination of serial-section SEM and a variety of SEM sample-preparation methods will lead to an expansion of SEM applications in the field of biology.

10. Conclusions

In this report, we have provided an introduction to serial-section SEM. This method is easier than serial-section TEM and avoids the high cost and specialized equipment characteristic of FIB/SEM and SBF/SEM, while nonetheless yielding 3D reconstructed images that are as accurate as those obtained using these other methods. Serial-section SEM is particularly effective for analyzing the overall 3D configuration of the Golgi apparatus, which has been difficult to achieve until now.

Moreover, although in recent years there has been a rapid increase in interest in SEM by many researchers due to the development of 3D SEM methods such as FIB/SEM, SBF/SEM and serial-section SEM, we must not overlook the fact that the ability to perform direct 3D observations of cells and tissues is one of the fundamental features of SEM.

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