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High Resolution Scanning Electron Microscopic Cytology - Specimen Preparation and Intracellular Structures Observed by Scanning Electron Microscopy

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HIGH RESOLUTION SCANNING ELECTRON MICROSCOPIC CYTOLOGY - SPECIMEN PREPARATION AND INTRACELLULAR STRUCTURES OBSERVED BY SCANNING ELECTRON MICROSCOPY

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Abstract

With the recent developments of the specimen preparation techniques, intracellular organization has been observed three-dimensionally by scanning electron microscopy (SEM). A suitable preparation method is the most important factor for observing intracellular structures at high resolution. Since intracellular structures are usually hidden in the cytoplasmic matrix, SEM observation of them is impossible merely by cracking the fixed cell. To remove the fixed cytoplasm, an osmic maceration technique is the most effective method which is applicable to almost all kinds of specimen preparation. In our laboratory, we developed some methods for observing intracellular structures. Those are the O-D-O method, the A-O-D-O method, O-D-W method, freeze-polishing method, and so on. Since each specimen preparation method has merits and demerits, it is necessary to select the suitable method for each purpose. To observe the intracellular membranous structures such as endoplasmic reticulum and mitochondria, the A-O-D-O method is recommended. An exfoliating method by surface tension is effective to observe submembranous structures. The freeze-polishing method is applied for observing intracellular structures of thin materials such as mesothelial cells or cultured cells. By these methods, some new findings on the three-dimensional architecture of the intracellular organelles were obtained. On the surface of sarcoplasmic reticulum ribosomes were sometimes attached forming spiral polysomes. In Golgi apparatus, the cisternae were composed of compiled cisternae which showed a whorl-like appearance seen from above. Although the newly revealed findings must be investigated further in the near future, it is obvious that three-dimensional cytology by high resolution SEM is emerging.

KEY WORDS: High resolution SEM, specimen preparation methods, intracellular structures, osmic maceration technique, sarcoplasmic reticulum, polysome, Golgi apparatus

Introduction

In the last three decades, cytologists established transmission electron microscopic (TEM) cytology mainly by the ultrathin sectioning methods. However, intracellular organelles are of three-dimensional constitution in the cell, so they are not readily appreciated in thin sectioned two-dimensional TEM images. Although scanning electron microscopy (SEM) is useful to observe materials in three-dimensions, it has been considered unsuitable for observing intracellular fine structures because of the low resolving power. In recent years, some techniques have been reported to achieve high resolution in SEM, and recent developments of specimen preparation techniques enabled us to observe intracellular organization three-dimensionally at high resolution. Hence, a new high resolution SEM cytology is emerging.

As to the specimen preparation techniques, several effective methods for revealing intracellular structures have been developed in our laboratory. As intracellular organelles are usually hidden in the cytoplasmic matrix, SEM observation of them is impossible merely by cracking the fixed cell. There are two ways to remove the excess cytoplasmic matrix; one is the use of a hypotonic solution, and another is a maceration technique using a dilute osmic solution, originally developed in the O-D-O method (Tanaka and Naguro, 1981). Although the maceration technique has been routinely used in our laboratory, we have developed some revised methods for each purpose (Table 1). An aldehyde fixative can be perfused by the A-O-D-O method (Tanaka and Mitsushima, 1984), so better preservation of ultrafine structures is expected. By this method, all of the intracellular membranous structures such as endoplasmic reticulum and mitochondria were shown three-dimensionally. To observe submembranous structures, an exfoliating method by surface tension is used (Inoué et al., 1984b). This method has been developed to peel off plasma membrane using surface tension produced between ethanol and distilled water. The freeze-polishing method (Inoué and Osatake, 1984) is effective to obtain a polished plane parallel to the specimen surface, and is applied for observing intracellular structures of thin materials such as mesothelial cells or cultured

Table 1.
Specimen preparation methods for observing intracellular structures developed in our laboratory.

Methods	Developed by	Initial fixation	To reveal internal structures	Removal of excess cytoplasmic matrix
O-D-O method	Tanaka and Naguro (1981)	1% OsO_4	DMSO freeze cracking	0.1% OsO_4
A-O-D-O method	Tanaka and Mitsushima(1984)	0.5% GA + 0.5% FA	DMSO freeze cracking	0.1% OsO_4
hypotonic method	Inoué (1982)	1% OsO_4 after hypotonic treatment	DMSO freeze cracking	(-)
O-D-W method	Inoué (1983)	1% OsO_4	DMSO freeze cracking	rinsing with D.W.
exfoliating method	Inoué et al. (1984b)	1% OsO_4	surface tension	(-)
freeze-polishing method	Inoué and Osatake (1984)	1% OsO_4 or 0.5% GA + 0.5% FA	DMSO freeze polishing	0.1% OsO_4
freeze-substitution method	Osatake et al. (1985)	rapid freezing	DMSO freeze cracking	0.1% OsO_4

cells. When combined the osmic maceration technique with the rapid freezing, freeze-substitution method (Osatake et al., 1985), we can observe more reliable structures than those obtained by chemical fixation.

This paper intends to describe the specimen preparation methods developed in our laboratory for observing intracellular structures, and to introduce the interesting SEM findings as SEM cytology.

General specimen preparation procedure

Firstly, general specimen preparation procedures in our laboratory for observing intracellular structures will be mentioned. Figure 1 shows the outline. In the O-D-O method, the removed tissues are fixed by immersion in 1% osmium tetroxide buffered with 1/15 M phosphate buffer (pH 7.2). In the A-O-D-O method, a mixture of 0.5% glutaraldehyde (GA) and 0.5% formaldehyde (FA) is perfused from the aorta or the heart. In the original description of the A-O-D-O method, the fixatives are buffered with cacodylate buffer for cytochemical studies, but we usually use 1/15 M phosphate buffer for observing intracellular structures alone. After the small pieces of the fixed tissues are rinsed with the buffer, they are immersed in 25% and 50% dimethyl sulfoxide (DMSO) solution for 30 min each. The specimens are then frozen on a metal plate previously chilled with liquid nitrogen. The frozen specimens were then split with a razor blade and a hammer. We use a freeze-cracking apparatus (TF-1, EIKO Engineering Co. Ltd., Japan) for this purpose. The split pieces are immediately placed in 50% DMSO solution and thawed at room temperature. After rinsing in the buffer completely, they are osmificated again for 1 h in 1% osmium tetroxide. They were then transferred into 0.1% osmium tetroxide buffered with 1/15 M phosphate buffer, and left standing for 3-5 days at 20°C (we call this step the osmic maceration procedure). This maceration procedure is the most important step to remove excess cytoplasmic matrix. After that, specimens are conductive-stained with 2% tannic acid for 2-12 h and then 1% osmium tetroxide for 1 h. The specimens are dehydrated through a graded alcohol series. After treatment with isoamyl acetate, they are dried in a critical point dryer (HCP-2, Hitachi Koki Co. Ltd., Japan) with solid CO_2 (Tanaka and Iino, 1974). Dried specimens are coated lightly (about 3 nm) with platinum in an ion coater with a rotating stage (VX-10R, EIKO Engineering Co. Ltd., Japan). The coated specimens are observed with a field emission SEM (HFS-2ST, Hitachi Co. Ltd., Japan) or a SEM equipped with a LaB_6 gun (ISI DS-130, Akashi Seisakusho Ltd., Japan), both operated at 25 kV.

Notes on specimen preparation

Fixation

In specimen preparation for electron microscopy, 2-3% glutaraldehyde is usually used for the initial fixation. Though this fixative is effective for TEM studies as well as for surface observations by SEM, it is not suitable for observing intracellular structures by SEM. This is because both cytoplasmic matrix and intracellular membranous organelles are fixed firmly, so that we cannot identify membranous structures in the cell.

In the O-D-O method, 1% osmium tetroxide is used for the initial fixation. However, the fixative is usually used by immersion, and the rate of osmic infiltration to the tissues is very slow. For this reason, it is difficult to expect a good fixation especially in the nervous tis-

1. Fixation: 1% OsO_4 (O-D-O method)
   0.5% GA + 0.5% FA (A-O-D-O method)
2. Freeze cracking: 25%, 50% DMSO
3. Osmic maceration: 0.1% OsO_4
4. Conductive staining: 1% OsO_4 & 2% tannic acid
5. Dehydration
6. Critical Point drying
7. Metal Coating: ion-sputter coating (Pt)
8. Observation

Fig. 1 The outline of the specimen preparation methods for observing intracellular structures by SEM.

sues. On the contrary, in the A-O-D-O method, a mixture of 0.5% GA and 0.5% FA can be perfused through vessels, better preservation of fine structures can be expected than the O-D-O method. The mixed fixative of 0.5% GA and 0.5% FA may be too dilute to fix the cell sufficiently, but this fixative is widely used for cytochemical studies and we have not encountered significant artifacts due to the fixation. One more merit of the A-O-D-O method is its applicability to cytochemical studies. Using backscattered electron imaging, Tanaka and Mitsushima (1984) demonstrated AcPase activity of hepatocytes stained with the Gomori's lead phosphate method. Since in both the O-D-O method and the A-O-D-O method, cytoplasmic matrix is slightly fixed, the maceration technique to remove the matrix is necessary after cracking the tissues.

Freeze cracking

The freeze cracking method is the most effective way to reveal intracellular structures. Many kinds of cryofracture or freeze cracking methods for SEM have been developed. By these methods, frozen specimens previously treated with a cryoprotectant are fractured on a metal plate chilled with liquid nitrogen using a razor blade and a hammer. As cryoprotectants, glycerol (Nemanic, 1972), ethanol (Humphreys et al., 1974), dimethylsulfoxide (Tokunaga et al., 1974) are used. In our laboratory, we use a freeze cracking method using DMSO, a slightly modified method of Tokunaga et al. (1974). The use of organic solvents such as ethanol and isoamyl acetate as a cryoprotectant is not recommended for observing intracellular structures because cytoplasmic matrix has been fixed firmly with the solvent.

Removal of excess cytoplasmic matrix

The osmic maceration technique is the most effective method to remove the excess cytoplasmic matrix. The development of this technique enabled us to observe the three-dimensional images of intramembranous structures such as endoplasmic reticulum, mitochondria and Golgi apparatus. However, as pointed out by Tanaka and Mitsushima (1984), it is not yet clear why the matrix is successfully removed, while the membranous structures are well preserved. In rare cases fixed with osmium tetroxide alone, intracellular structures can be satisfactorily revealed without the maceration procedure. The extraction effect of osmium tetroxide is well known (Hayat, 1981). It is recognized that tissues fixed with osmium tetroxide lose proteins during both fixation and dehydration. Dallam (1957) showed mitochondria in rat liver tissue lost - 22% of their proteins during the fixation in osmium tetroxide and a further 12% during dehydration. The mechanism of loss of proteins reported is summarized by Hayat (1981). It is true that proteins are lost during osmic fixation and the following dehydration. As to the effect of a dilute osmic solution which is used as a maceration procedure, it is well known in light microscopic technique that dilute solutions of some metal salts such as osmium tetroxide, chromium trioxide and potassium dichromate are effective in macerating the cytoplasm. Although the biphasic effects (gelation and then extraction) of osmium tetroxide on tissue constituents is also well known (Hayat, 1981), it is uncertain whether the dilute osmic solution used for the maceration technique has these biphasic effects or not. Actually, however, it is true the fixed cytoplasmic matrix is progressively extracted with the dilute osmic solution. The progressive extraction was clearly shown in the study of intracellular organization of cultured cells (Inoué et al., 1984a). Time needed for the proper extraction depends on the kinds of tissues and fixation method, so we must divide the specimens into groups and macerate them for different periods. It has been said the maceration entirely dissolves the filamentous structures within the cell, so that both O-D-O method and A-O-D-O method are unsuitable for observing cytoskeletal elements (Tanaka and Mitsushima, 1984). However, we found that the cytoskeletal elements of cultured cells can be retained by using a proper buffered solution and by reducing the maceration period (Inoué et al., 1984a). In this specimen preparation, cytoskeletal elements such as microtubules and actin filaments can be disclosed under SEM (details are discussed in cytoskeleton).

To remove the excess cytoplasmic matrix, a hypotonic treatment prior to fixation is also effective. Inoué (1982) demonstrated the continuity of endoplasmic reticulum of rat spermatids prepared by the hypotonic method prior to the fixation with 1% osmium tetroxide. He used 1/15 M phosphate buffer for the hypotonic solution, and treated the specimens for 1-2 min immediately after the removal of the tissues. Although long-time immersion of the specimen in the hypotonic solution caused cell rupture, some cells undergo some degree of swelling and others little when the hypotonic treatment has been suitably performed. Slight swelling is effective for ascertaining the continuity of the endoplasmic reticulum.

Another method to remove the cytoplasmic matrix is the use of distilled water as a rinsing solution. During the course of the study of rinsing solutions for SEM, Inoué (1983) found that rinsing with distilled water removed the excess cytoplasmic matrix revealing intracellular structures under SEM. According to this method, specimens fixed with 1% osmium tetroxide are prepared by rinsing with distilled water in all rinsing steps, and the osmic maceration procedure is omitted. In this preparation, muscular fibers

such as myosin and actin were shown (Fig. 2), while the O-D-O method cannot demonstrate these fibers. In addition, by this method, cells and membranes were sometimes dissociated by the hypotonic shock, so the submembranous structures or the surface of the basement membrane was successfully demonstrated.

Other specimen preparation methods

Exfoliating method using surface tension (Inoué et al., 1984b). In general, it is difficult to observe intracellular structures beneath the plasma membrane. This method is intended to demonstrate submembranous intracellular structures by peeling off the plasma membrane using surface tension. After specimens were fixed with 1% osmium tetroxide, they were dehydrated in a graded series of ethanol up to the concentration of 70-100%, they were thrown into distilled water. Then the specimens moved about on the surface of the distilled water for some time and eventually sank down into the water. At this step, some parts of the cells and membranes were separated by the surface tension produced between the water and the ethanol. After conductive staining, specimens were dehydrated and critical point dried. In this method, the osmic maceration procedure is omitted to demonstrate intracellular fibrous structures. In intestinal absorptive epithelial cells prepared by this method, some parts of the cells and microvillous borders were separated by the surface tension, revealing intracellular structures such as fine networks of the intestinal terminal web. The underside of the microvillous border was also revealed, where numerous openings and some protrusions were shown, which correspond to the basal portion of the microvilli and the pinocytotic vesicles, respectively (Fig. 3).

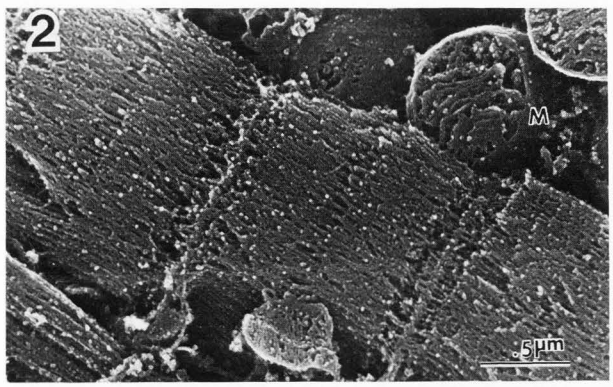
Freeze-polishing method(Inoué and Osatake, 1984) This method has been developed for observing intracellular structures of thin cells such as mesothelial cells or mono-layered cultured cells. In general, it is difficult to split such kinds of cells parallel to the specimen surface using the freeze cracking method. By this method, however, intracellular structures could be revealed by polishing or abrading the frozen samples previously treated with DMSO. For the polishing, we used a polishing film (Imperial Lapping Film, 3M Co. Ltd., Minnesota, U.S.A.), of which the surface is covered with fine particles of aluminum oxide. Specimens were fixed with 1% osmium tetroxide or a mixture of 0.5% GA and 0.5% FA, and treated with DMSO. The polishing film was mounted on a metal plate with adhesive tape, chilled with liquid nitrogen, and placed on the cooled aluminum plate in a freeze cracking apparatus (TF-1, EIKO Engineering Co. Ltd., Japan). Then the frozen sample was held with a precooled forceps, and the desired surface was polished on a series of successively finer films (grain sizes: 3, 1, and 0.3 μm). After thawing in 50% DMSO at room temperature, specimens were placed in 0.1% OsO_4 for the osmic maceration, conductive stained, dehydrated, and critical point dried. Using this method, we could demonstrate intracellular structures of mesothelial cells, intestinal smooth muscle cells, and cultured cells. In the mesothelial cells, a part of the surface cell membrane was found to be turned over, and the cytoplasmic side of the membrane was seen. Here, many pinocytotic vesicles were attached on the membrane. Where the cells were polished by this method, intracellular structures were shown three-dimensionally. The endoplasmic reticula formed a network in the cell, some of them surrounding the mitochondria (Fig. 4). By this method, pinocytotic vesicles on the basal cell membrane were also shown. In the small intestine, various layers were exposed by the polishing. They were the peritoneal layer, the outer muscular layer, the myenteric nerve plexus of Auerbach, and the inner muscular layer. In the smooth muscle cells, the relationship between the sarcoplasmic reticulum and surface vesicles was clearly demonstrated three-dimensionally (Fig.5).

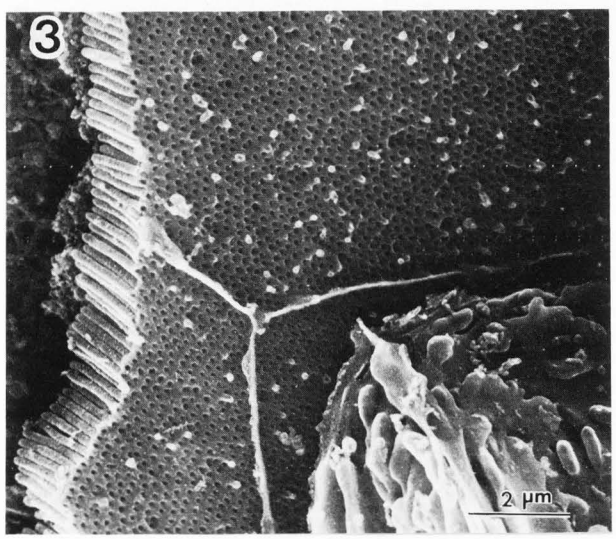
Rapid freezing, freeze substitution method (Osatake et al., 1985) Rapid freeze-fixation is effective in preserving fine structures, because it can be performed within seconds after removal of tissues, arresting the transient physiological process in cells. We devised a combined technique of the rapid freezing, freeze substitution-fixation method and the osmic maceration method. Small pieces of tissues were frozen by a metal contact method using a rapid freezing apparatus (RF-2, EIKO Engineering Co. Ltd., Japan). The frozen specimens were then transferred into 2% osmium tetroxide in acetone at -80°C and kept at -80°C for 24 h. After substitution of ice on the specimen at low temperature, the solution was gradually warmed to room temperature. After washing in acetone, the specimens were rehydrated through a descending acetone series, and immersed in 25% and 50% DMSO. They were split by the freeze cracking method, and macerated with 0.1% osmium tetroxide for 3 days or more at 20°C. After conductive staining, they were dehydrated and critical point dried. This method is effective to study the true structures of intracellular structures in three dimensions. Though the deeper layer of the tissue is destroyed by ice crystal formation, a good preservation of intracellular structures was obtained in the superficial layer of the tissue. Some differences in the intracellular morphology was noted when

Fig. 2 The longitudinally cracked surface of a rat cardiac muscle cell prepared by the O-D-W method. M: mitochondria

Fig. 4 Intracellular structures of the mesothelial cells lining the outer surface of a rat small intestine. This specimen was prepared by the freeze-polishing method. The smooth endoplasmic reticulum forms a network structure, and mitochondria (M) is partially surrounded with the endoplasmic reticulum.

Fig. 5 (a) Intracellular structures of the smooth muscle cells from a rat small intestine revealed by the freeze-polishing method. Surface vesicles show the linear arrangement along the long axis of the cell. Mitochondria is situated on the linear aggregates of the vesicles. M: mitochondria. (b) A higher magnified view of the sarcoplasmic reticulum (SR). A spiral structure of polysomes is attached on its surface (arrowhead).





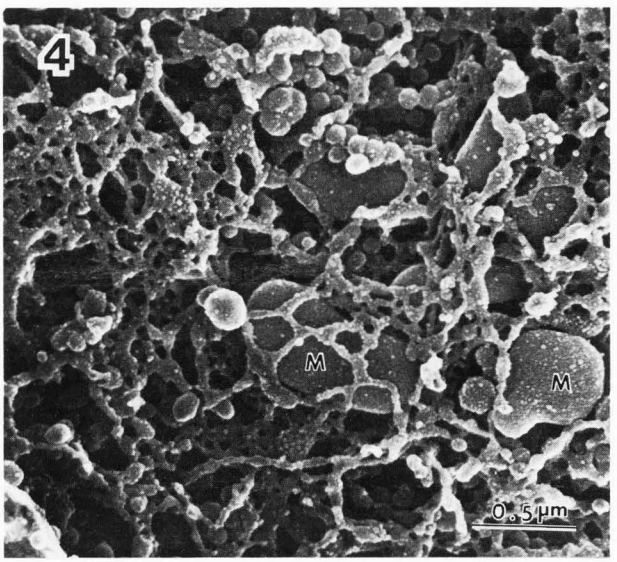
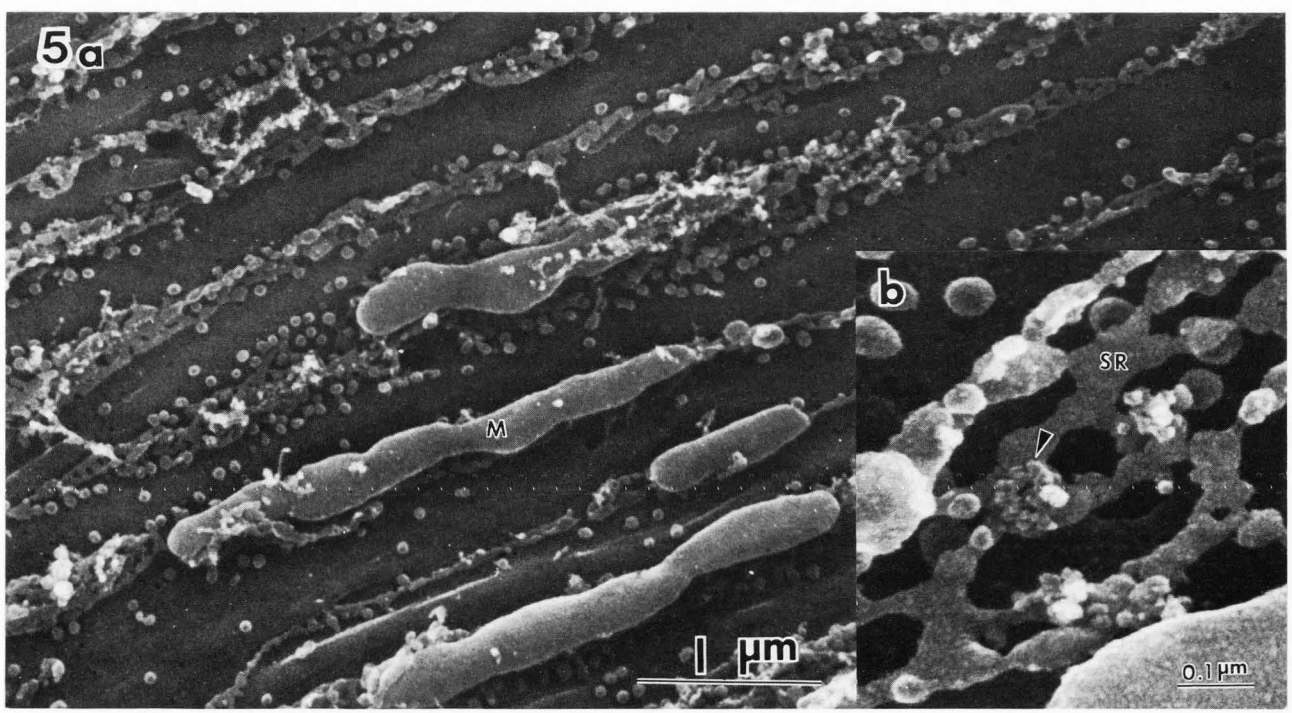


Fig. 3 Cytoplasmic side of the microvillous border of a rat intestinal cell seen from below. Many openings and some protrusions are seen. They are pinocytotic vesicles and the basal portion of the microvilli, respectively. This specimen was prepared by the rapid freezing, freeze substitution method and the exfoliating method using surface tension. At the rehydration step, some parts of the intestinal epithelia have been dissociated as well as by the exfoliating method using surface tension, revealing the underside of the microvillous border.



compared with the samples fixed with chemical fixatives (details described later).

SEM cytology of intracellular structures

Cytoplasmic side of plasma membrane

SEM is useful to observe surface structures such as microvilli and microplica, and many studies have been performed. However, the cytoplasmic side of the plasma membrane has not sufficiently been studied. As specialized structures of the inner side of the membrane, pinocytotic vesicles and the opening of microvilli and so on are cited. Using a glycerin and hypotonic treatment, Inoué (1981) demonstrated pinocytotic vesicles of mesothelial cells. The apical surface membrane of an intestinal absorptive epithelium is characterized by many microvilli and a few pinocytotic vesicles. Vial and Porter (1975) first demonstrated the underside of the microvillous border by SEM using a mechanical dissociation technique after treatment with boric acid. We also showed the underside structure at high resolution by an exfoliating technique using surface tension or a rapid freezing, freeze substitution method (Fig. 3). In this micrograph, numerous openings and some protrusions are seen, which correspond to the basal portion of microvilli and pinocytotic vesicles, respectively. The distribution of pinocytotic vesicles and microvilli can be clearly seen in this micrograph. Surface vesicles in the smooth muscle cells is another case of the specialization of the plasma membrane. Although the distribution has been demonstrated by TEM study (Devine et al., 1972), SEM is useful for obtaining a bird's-eye view of the distribution (Fig. 5).

Endoplasmic reticulum

According to the TEM findings, the endoplasmic reticulum is divided into two types; rough endoplasmic reticulum and smooth endoplasmic reticulum. There are several contributions to SEM cytology on the morphology of endoplasmic reticulum. Firstly, the continuity of endoplasmic reticulum in a cell has been proved under SEM. In rat spermatids of their early developing phase, Inoué (1982) showed all of the endoplasmic reticulum is continuous, forming a single continuous system. Though several researchers described a three-dimensional scheme of endoplasmic reticulum from the examination of numerous ultrathin sections, it is not a direct evidence of the continuity. Secondly, the whole architecture of the sarcoplasmic reticulum and transverse tubules of muscle fibers has been shown in three-dimensions. Ohmori(1984) observed the three-dimensional morphology of these membranous systems of rat skeletal muscle cells and heart muscle cells. According to his study, the architecture obtained by SEM corresponds well to those previously described by many researchers, but differs in some respects. Figure 6 a and b shows the sarcoplasmic reticulum of the skeletal muscle from the rat tongue. The sarcoplasmic reticulum is regularly arranged along the myofilaments which has been removed during the osmic maceration, forming a continuous network system. Triads are observed at the A-I junction, and are partially surrounded with the sarcoplasmic reticulum (Fig. 6b). When comparing the network of the sarcoplasmic reticulum in the A band with that in the I band, the latter is more closely packed than the former. This may be concerned with the contraction mechanism or Ca transport. Further studies are now being continued in our laboratory. The third contribution of SEM cytology to the architecture of endoplasmic reticulum is the demonstration of the attachment of polysomes on the surface of endoplasmic reticulum which has been considered to be a smooth one. The sarcoplasmic reticulum of smooth muscle cells has been considered to be a kind of smooth endoplasmic reticulum. In our study, however, polysomes were often attached on the surface of sarcoplasmic reticulum, and showed a typical spiral structure (Fig. 5b). In the mesothelial cells lining the intestinal outer surface, we also observed the attachment of a few polysomes on the surface of endoplasmic reticulum which had been considered to be an intracellular vacuole.

Polysomes

According to TEM studies, two types of polysomes are known; free polysomes and polysomes attached on membranous structures such as endoplasmic reticulum or the nuclear envelope. Although free polysomes seem to be floating in the cytoplasmic matrix, they are actually supported with cytoskeletal elements or fine strands of "microtrabecular lattice" (Wolosewick and Porter, 1979). In the specimen preparation described here, they cannot be observed in the cytoplasm. Because, the "microtrabecular lattice" has been entirely dissolved with the maceration, ribosomes have been washed away with the treatment. On the other hand, polysomes attached on the endoplasmic reticulum or the nuclear envelope are clearly demonstrated under high resolution SEM. Their polysomes often appear to be arranged spirally (Fig. 5b). Polysomes consist of two subunits of unequal size. It has been speculated that where polysomes are associated with the endoplasmic reticulum, ribosomes are attached to its membrane by their larger subunits. In our high resolution SEM study, however, both small and large subunits seemed to be associated with the membrane (Fig. 7). Further studies are needed to clarify how ribosomes are associated with the membrane, and this must be clarified in the near future by the stereoscopic study using ultra high resolution SEM.

Mitochondria

The shape of mitochondria is more properly appreciated by SEM than by two dimensional ultrathin sections. The external form shows various kinds of shape under SEM. Mitochondria usually show spherical, elliposidal and club-like shapes, but sometimes shows complex irregular branching shapes, which cannot be readily appreciated by TEM.

In the cracked surface of mitochondria, mitochondrial cristae can be observed. SEM is also useful to understand the three-dimensional architecture of the cristae. Masunaga (1979) studied the mitochondrial structures of the rabbit heart muscle, and classified the cristae into four types. Yamagata (1982) showed the internal structures of mitochondria in the dividing stage by SEM. According to his study, the septum of the dividing mitochondria has no cristae. Kirschner and Rusli (1976) reported that

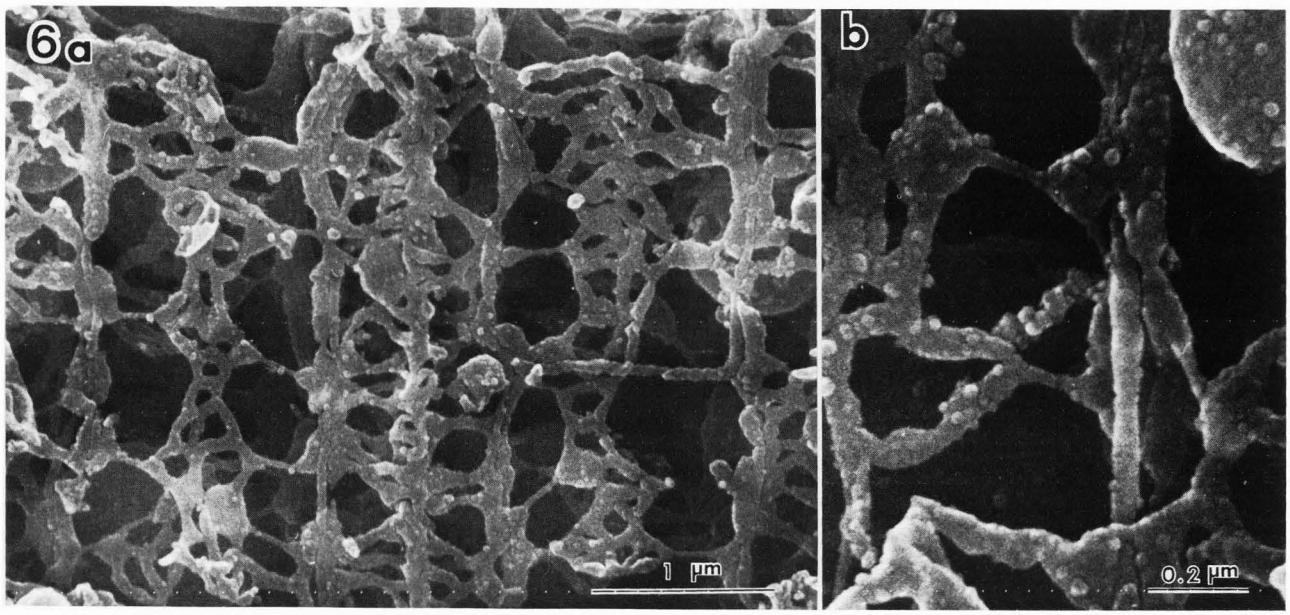


Fig. 6 (a) Sarcoplasmic reticulum of the skeletal muscle from a rat tongue, prepared by the A-O-D-O method. Since the osmic maceration had been performed for 4 days, myofilaments were completely removed. All of the sarcoplasmic reticulum is found to be continuous and the triads are observed at the A-I junctions. Note the different pattern of the network of the sarcoplasmic reticulum in each band. (b) A higher magnified view of the triad. The T-tubule is partially surrounded with the sarcoplasmic reticulum.

the inner membrane of isolated mitochondria had a granular surface and these granules might represent respiratory enzyme complex of the ATPase particles. Tanaka (1981) also demonstrated small particles on the mitochondrial tubuli of a hamster hepatocyte, and assumed that such particles corresponded to the inner membrane particles seen in the negative contrast preparations.

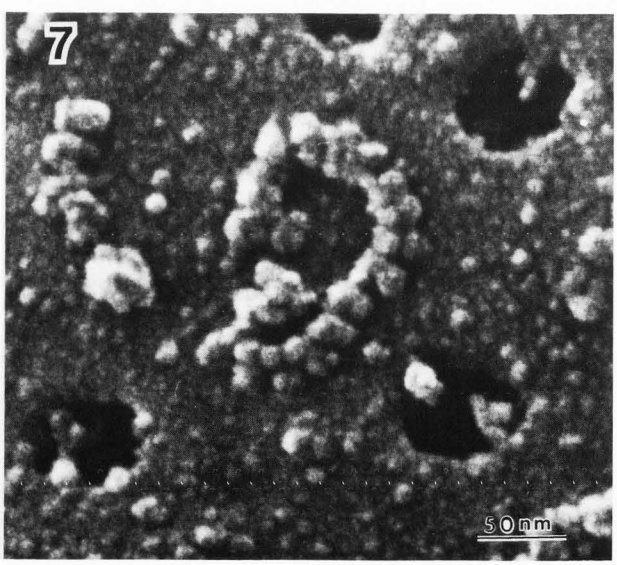


Fig. 7 A high magnified view of polysomes on the nuclear envelope of a rat hepatocyte prepared by the freeze polishing method. Two kinds of ribosomal subunits are clearly demonstrated under high resolution SEM.

Golgi apparatus

The greatest contribution of SEM cytology is the elucidation of three-dimensional architecture of Golgi apparatus. According to the TEM findings, the Golgi apparatus is constructed with three elements; stacks, vesicles and vacuoles. In the TEM micrographs, the Golgi stacks seem to be simple structures which consist of several flattened cisternae arranged in parallel. In fact, the three-dimensional schematic drawings by TEM show that the stacks are piled up. However, SEM studies proved that the Golgi stack is a greatly complicated structure, as shown in Figures 8 and 9. Through the efforts of Tanaka and his co-workers, new structural details of the Golgi apparatus have been revealed (Tanaka and Kinose, 1981; Tanaka and Fukudome, 1983). First, each Golgi stack is joined together, forming a whole Golgi complex. This continuity was also shown in the metal impregnated, mouse pancreatic acinar cells by high voltage TEM (Noda and Ogawa, 1984). Secondly, Golgi stacks are connected to rough endoplasmic reticulum with slender tubules. Thirdly, the cisternae in a stack are often observed to be continuous to each other. Tanaka and Fukudome (1983) also found a stack that appears to consist of multiple parallel arranged cisternae, but, in fact, it is made up of only one helically wound cisternum. In our laboratory, further studies are now being performed to clarify the three-dimensional architecture of

Golgi apparatus.

Myofilaments and Cytoskeleton

To observe muscle fibers by SEM, the osmic maceration technique cannot be used, because the filaments have been dissolved with the maceration. With the progress of the maceration, myofilaments are removed in the following order: actin filaments, Z disks, and myosin filaments. Though the O-D-W method preserves myofilaments, the initial fixation should be performed for a short time at low temperature (4°C) to prevent the destruction of actin filaments (Maupin-Szamier and Pollard, 1978). Figure 10 shows the higher magnified view of the rat heart muscular cells. In this micrograph, each myofilament is identified, and the ends of actin filaments are connected to the Z disk.

Intracellular fibers referred to as the cytoskeleton have been receiving increased attention by cytologists from the viewpoint of cellular motility and morphology. Three major types of cytoskeletal elements are known; microtubules, intermediate filaments and microfilaments. SEM studies of them have been carried out mainly on cultured cells using some non-ionic detergents like Triton X (Pudney and Singer, 1979; Bell, 1981). However, Triton X dissolves membranous components of the cell, preventing simultaneous observations of the cytoskeleton and membranous organelles. The osmic maceration technique has been considered unsuitable for observing the cytoskeleton, because cytoskeletal elements are removed by the procedure together with the cytoplasmic matrix. When the specimens are prepared without osmic maceration technique, cytoskeletal elements were sometimes observed. The exfoliating method using surface tension as well as the O-D-W method is effective for observing the cytoskeleton. By this method we observed terminal webs and submembranous intracellular fila-

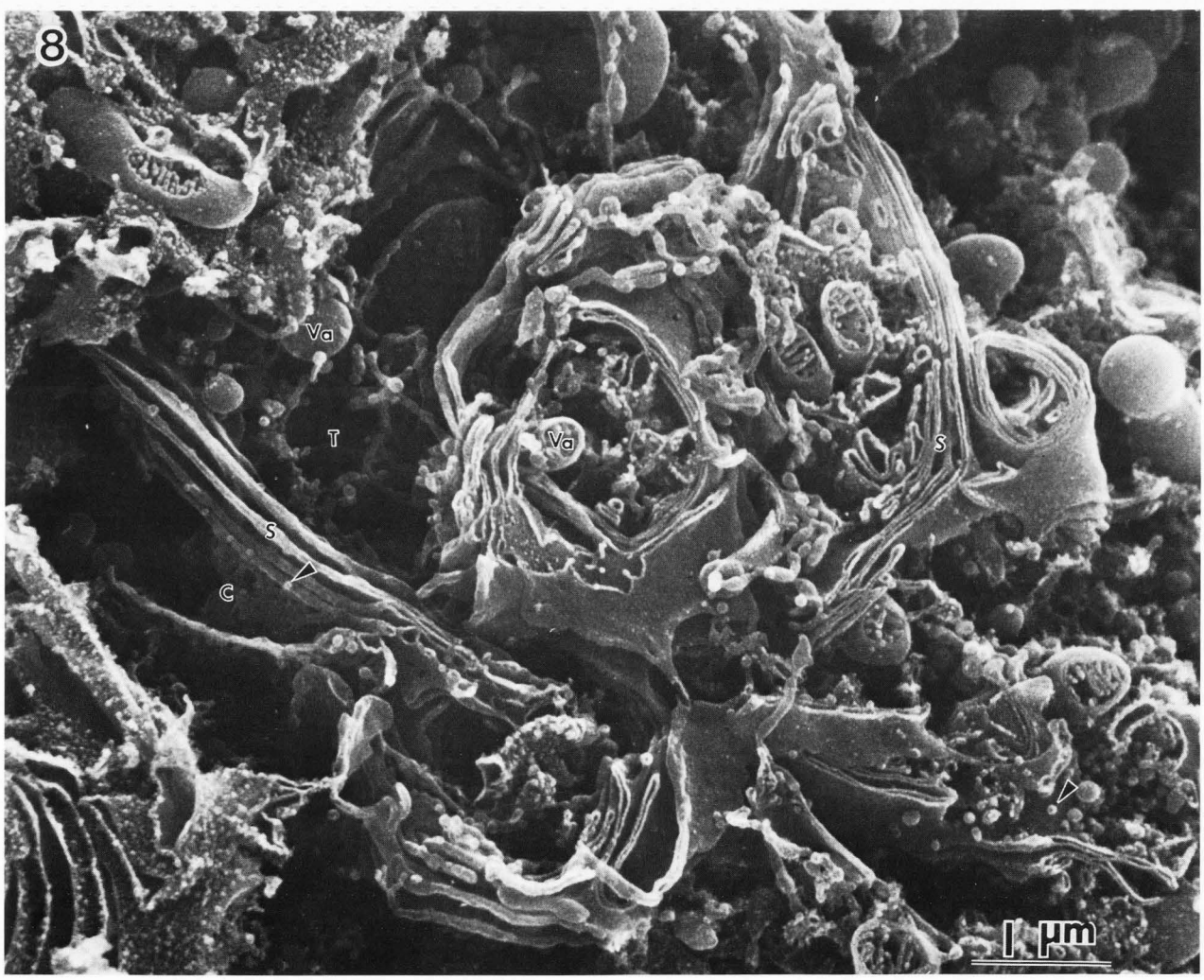


Fig. 8 Golgi apparatus of a rat lacrimal glandular cell prepared by the A-O-D-O method. Three major elements of the Golgi apparatus (stack: S, vacuole: Va, and vesicle: arrowheads) are seen three--dimensionally. A fenestrated cisterna is seen at the cis side(C), and anastomotic tubules at the trans side(T).

ments in the small intestine. Recently, we tried simultaneous observations of cytoskeleton and intracellular organelles of cultured cells using both the freeze-polishing method and the osmium maceration technique (Inoué et al., 1984a). In this study, we used a HEPES-PIPES stabilizing buffer developed by Schliwa and van Blerkom (1981) for the stabilization of microtubules, and excess cytoplasmic matrix was removed by the osmic maceration procedure for a shorter period than usual. As a result, cytoskeletal structures were most clearly seen in 48 h macerated samples, and we can identify the cytoskeletal elements together with the intercellular membranous system such as mitochondria (Fig. 11). At higher magnification, the relationship of the cytoskeletal elements was clearly shown (Fig. 12) With the progress of osmic maceration for more than 72 h, cytoskeletal elements were completely removed, while membranous structures such as endoplasmic reticulum were well retained. In this study, we found that cytoskeletal components can be observed together with membranous structures by suitable choices of both the stabilizing buffer and the maceration time.

Attempts for observing more reliable structures

When the specimens are prepared by chemical fixation, dehydration and critical point drying, artifacts such as shrinkage and deformation are inevitable. To resolve this problem, low temperature SEM is effective for observing the rapidly-frozen samples in which intracellular organelles can be frozen, preserving the in situ organization. However, the magnification of the micrographs thus far obtained are below 10,000 fold, and it is difficult to study the intracellular structures sufficiently at this magnification. In the last three years, we tried to observe intracellular structures of rapidly frozen samples at high resolution using a simple cryo SEM method which we devised (Inoué et al., 1983; Inoué and Koike, 1984). In this study, intracellular structures such as endoplasmic reticulum and mitochondria were shown three-dimensionally

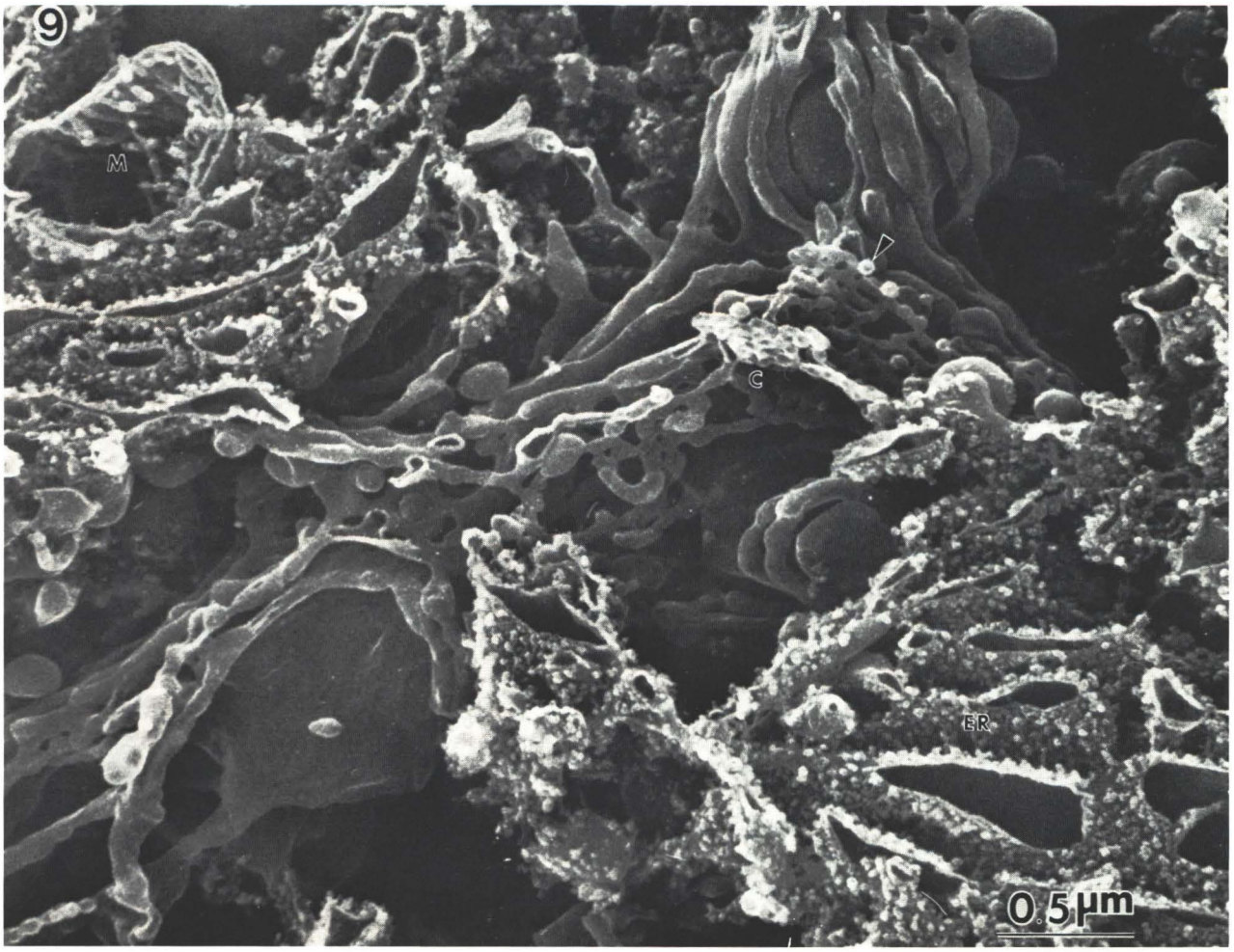


Fig. 9 A bird's eye view of the Golgi apparatus of a rat lacrimal glandular cell revealed by the A-O-D-O method. The uppermost cisterna is of cis side is fenestrated (C). The Golgi stack is not only composed of simple compiled cisternae, but also constituted by a whorl-like structure (upper right). ER: rough endoplasmic reticulum, M: mitochondria, arrowhead: Golgi vesicle.

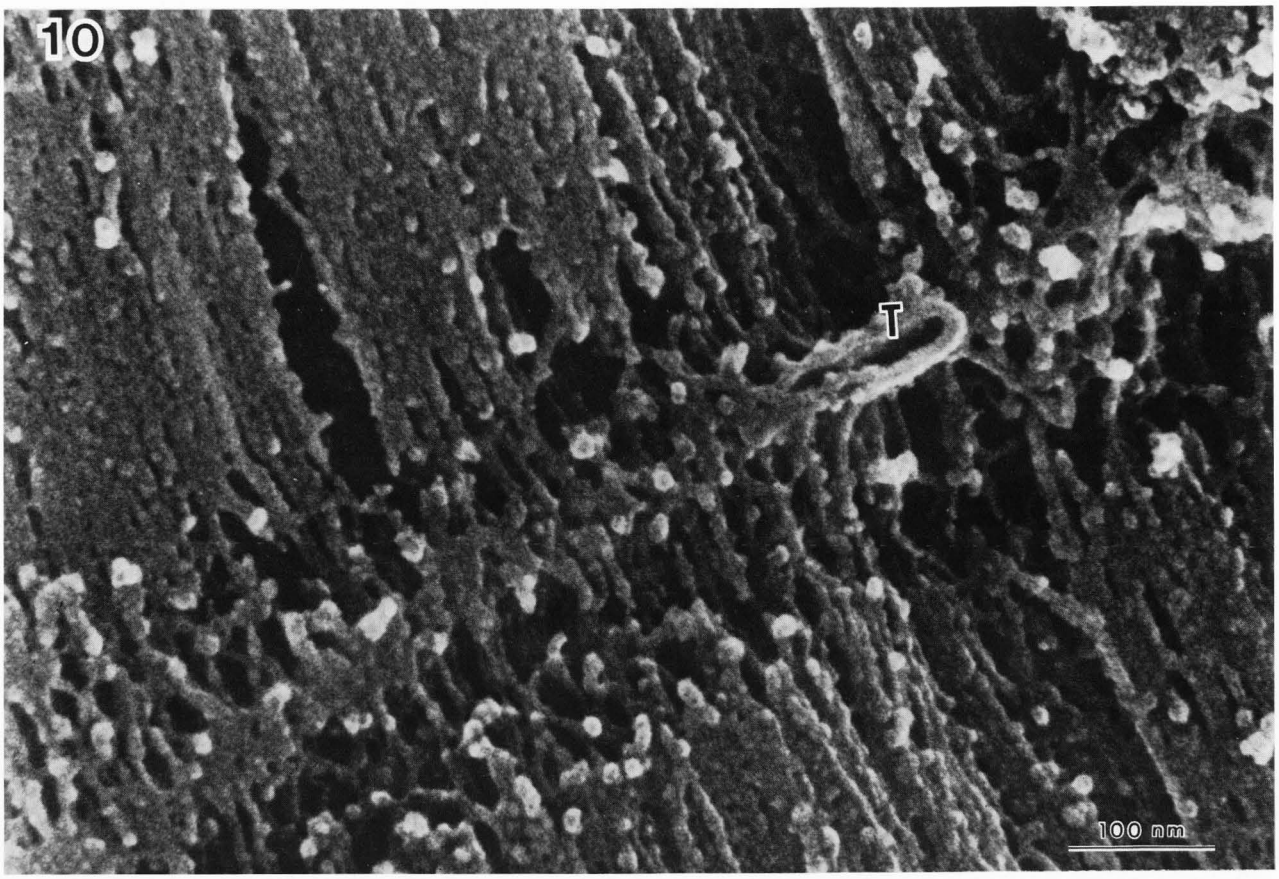


Fig. 10 A high magnified view of a rat cardiac muscle prepared by the O-D-W method. Two kinds of myofilaments (actin and myosin) are shown, and the ends of actin filaments are connected to the Z band. T: T-tubule

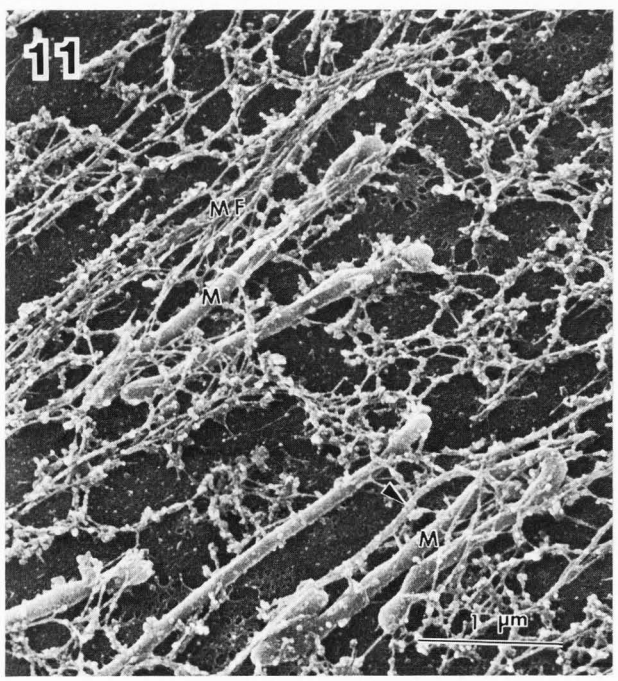
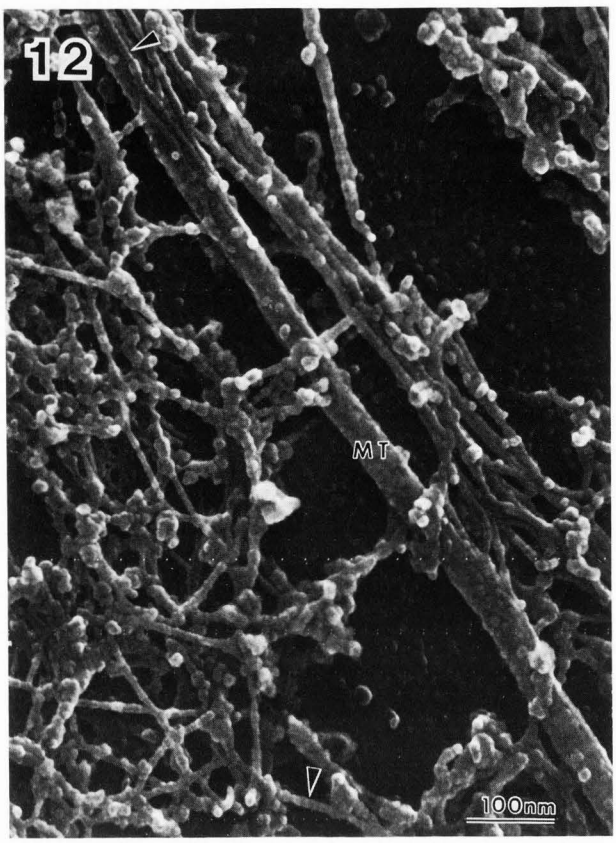


Fig. 11 Cytoskeletal structures of a cultured cell, prepared by the freeze polishing method. Cytoskeletal elements such as microfilament and microtubules (arrowhead) are observed together with mitochondria. MF: microfilamentous bundle M: mitochondria

at high resolution.

The rapid freezing, freeze substitution method we devised is also effective for observing in situ structures (Osatake et al., 1985). In this study, some differences due to the specimen preparation method were shown. This was evident in Golgi stacks and Golgi vesicles. In the chemically fixed lacrimal gland, the cisterna showed a somewhat wavy appearance, and the interval between the cisternae was not constant (Fig. 8). On the contrary, in the rapidly-frozen samples, the interval between the cisternae was constant in width, and Golgi stack consisted of closely packed cisternae (Fig.13). The Golgi vesicles in the chemically-fixed samples were less numerous than those of rapidly frozen samples (Figs. 8, 9 and 12). This indicates that Golgi vesicles may be incorporated into Golgi cisternae during chemical fixation, eventually producing less Golgi vesicles, and the wavy and dilated feature of the cisternae.



Concluding Remarks

As described above, the architecture of intracellular structures has been clarified three-dimensionally using high resolution SEM. Some structures thus far demonstrated represent new findings which cannot be appreciated by two-dimensional TEM images. High resolution SEM has opened new possibilities which are certainly worthy of the intracellular morphology. With further progress of specimen preparation as well as the SEM instrument itself, we are convinced that a new SEM cytology will be established in the near future.

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Fig. 12 A higher magnified view of cytoskeletal structures. A few actin filaments are associated to a microtubule (MT). Note the periodicity of actin filaments (arrowheads). The granular substances connected to fine filamentous network may be free ribosomes.

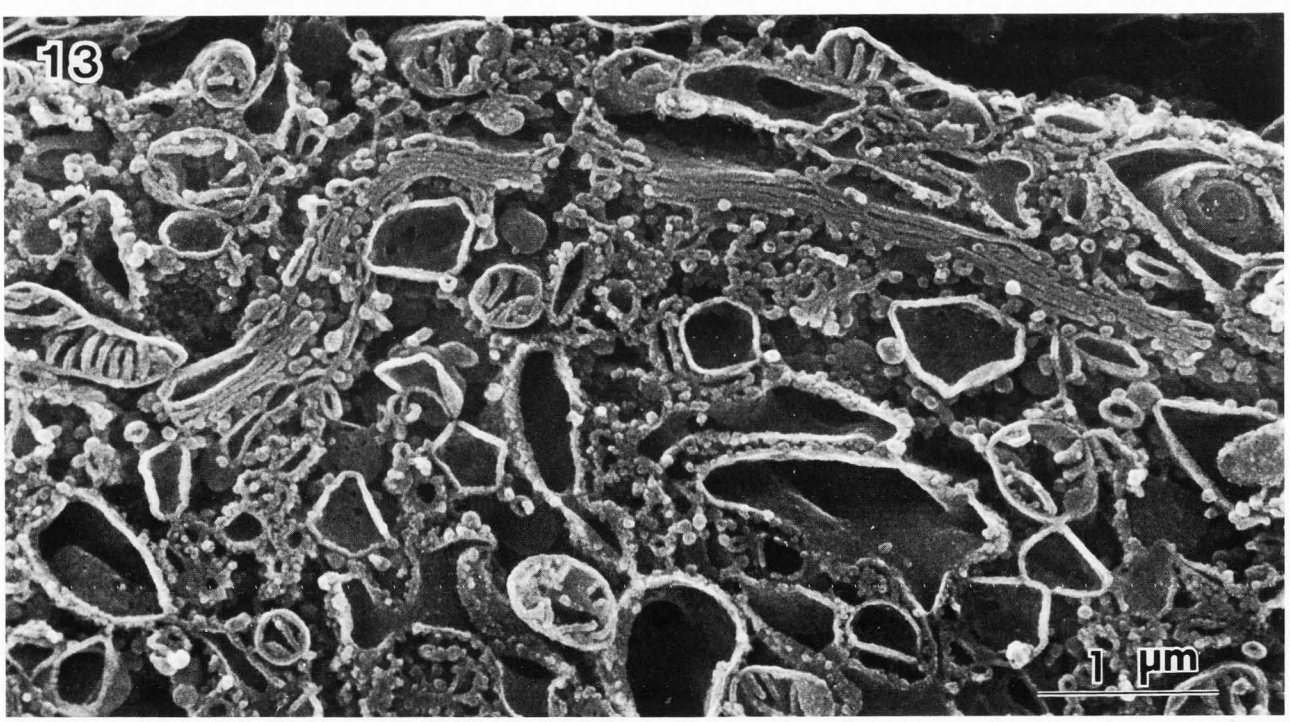

Fig. 13 A Golgi apparatus of a rat lacrimal gland cell prepared by the rapid freezing, freeze substitution method. The stack is consisted of closely packed cisternae, and many Golgi vesicles are discerned.

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