ATLAS OF SCANNING ELECTRON MICROSCOPY IN MEDICINE

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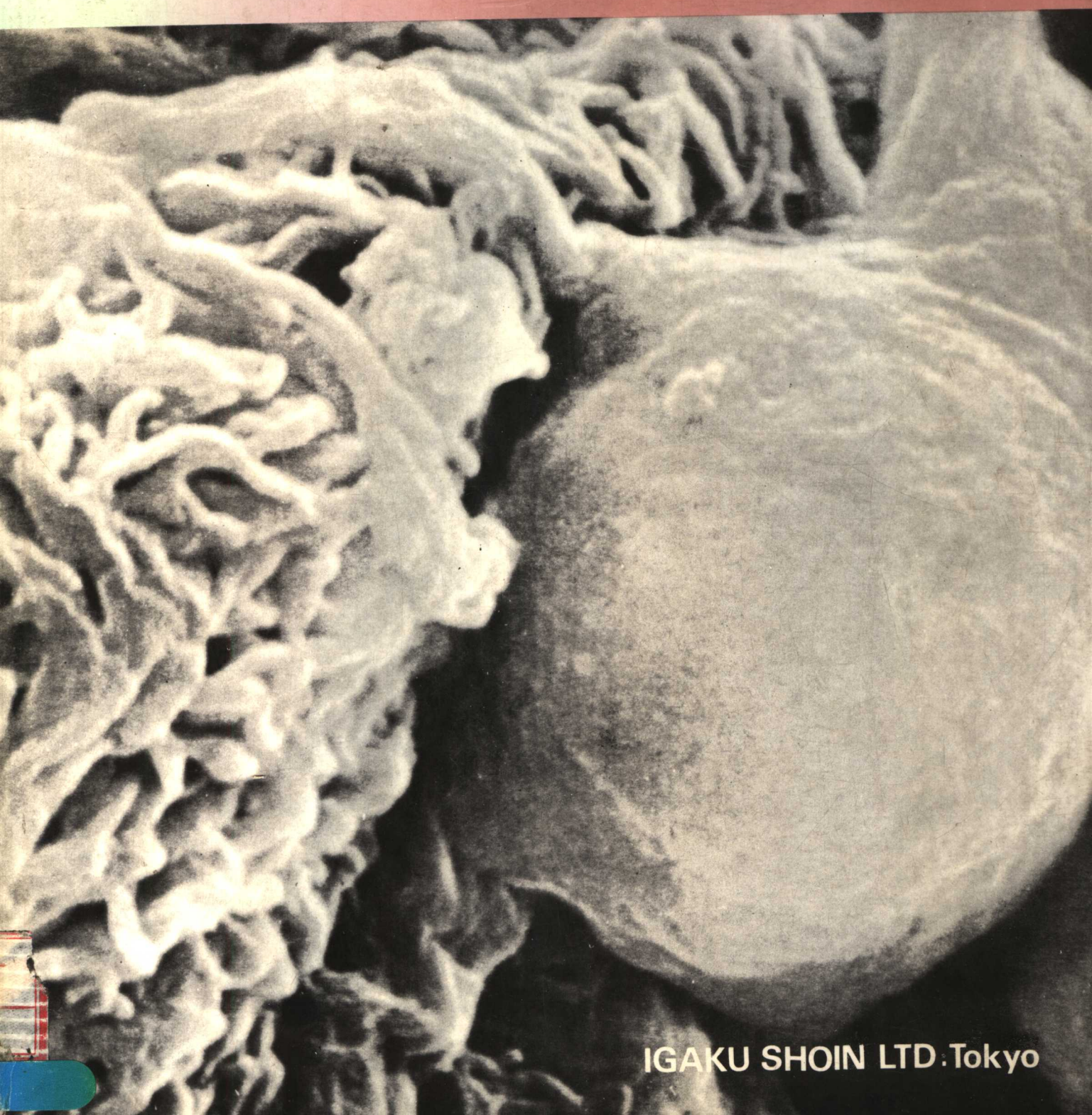

IGAKU SHOIN LTD. Tokyo

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PREFACE

Since the seventeenth century the magnifying glass has helped our observation of minute objects. In modern laboratories a binocular magnifying glass containing complex sets of lenses is used.

These optical instruments can extend the resolution of our naked eyes only to a certain extent. Though such structures as intestinal villi and renal glomeruli can be clearly observed, we come to the point where an identification of the individual cells forming these structures is desired—and much more. It was formerly but a scientist's dream to observe the delicate processes of those cells in their three-dimensional entity. The wall we meet is based on the limitations in the depth of focus as well as in the resolution, both limitations belonging to the intrinsic nature of light. The fine structures of cellular and subcellular levels have been studied almost exclusively by cutting the tissues into very thin sections and observing them under the transmission light or electron microscope. A large gap thus has occurred between macroscopic anatomy aided by magnifying glasses and microscopic anatomy based on the findings in the sections. This gap is also that between the three-dimensional and the two-dimensional fields.

The latest advance in electronics has shed light on this problem. The appearance of the scanning electron microscope, which is based on a mechanism fundamentally different from that in the previous transmission electron microscope, has realized that dream of ours to continuously raise the magnification of the magnifying glass to the level which the electron microscope achieves.

Observing things as they are with the naked eye is without doubt the most realistic and convincing method of study. The scanning electron microscope, though mediated by the action of electrons, may be said to extend simple and direct reality or, better to say, the feeling of attendance in the macroscopic observation to the microscopic fields. It may not be exaggerating to say that the wall we believed could never be overcome has been broken down by the advent of the scanning electron microscope.

In the last three years we have attempted to observe under the scanning electron microscope a variety of materials related to biomedical fields of study. Minute representatives of Nature, one after another, revealed their fascinating figures under this electron microscope. The octopus cells extended their tentacles to the renal glomerulus, the aspergillus fungi raised their gorgeous bouquets of conidiophores, the worms of isolated mitochondria appeared to be bending and stretching their bodies... Observation of one object drove us to the study of another.

Thus the photomicrographs accumulated to a considerable number and a selection of them is now to be bound in an atlas. They were not gathered so purposefully as to be arranged under a strict system, but may be considered a miscellaneous collection. This shortcoming of our atlas, we hope, will be rather an advantage in introducing the performance of our new weapon, the scanning electron microscope, and in showing its wide applicability to biomedical studies.

The main purpose of this volume, which includes pictures of those materials whose three-dimensional fine structures were first revealed to our research group, is to stimulate new investigations in related fields. This volume may, however, also serve

in the visual education of biomedical students and, further, perhaps amuse general readers as an "Album of Nature." These multiple characteristics of the scanning electron micrographs are believed not to decrease their purely scientific value but rather to indicate their many-sided merits.

On the publication of this atlas, we express our cordial thanks to those who kindly contributed their precious photomicrographs. Dr. KÉIICHI TANAKA of the Tottori University Medical School provided the pictures of chromosomes and lens fibers; Dr. YOICHI ISHII of the Kyushu University School of Medicine, the figures of cercaria; Dr. NAOYA KOSAKA of the Okayama University Medical School, the figures of the inner ear; Dr. TAKUO OGATA of the same school, the surface views of the stomach; and Dr. TAKUO JOZAKI of the Yahata-Seitetsu Hospital, the picture of bacteria on the tongue.

Niigata, Kokura, Okayama,
July, 1970

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CONTENTS

What Is the Scanning Electron Microscope?

Although theoretical and experimental studies on the scanning electron microscope had already been initiated in Germany in the latter half of the nineteen-thirties, the industrial production of the scanning electron microscope was not successful until the early sixties in England and a little later in Japan.

In the scanning electron microscope an extremely narrowed beam of electrons is directed at the specimen and scanned to and fro across its surface. The secondary emissions emerging from the specimen are collected by a detector and the changes in their amount are transformed into an electric signal. This signal then is amplified and expressed in terms of a variation in brightness on the Braun tube. A picture is thus produced on a screen and can be recorded by indirect photography.

The pictures of the scanning electron microscope, as far as an even secondary emission is ensured, well represent the cubic appearance of the material surface and give an effect of illumination as if the light were put at the place of the detector and the eye of the observer at the site of the electron gun.

Materials such as metals which, when bombarded by electrons, emit ample secondary electrons can be observed under the scanning electron microscope without any treatment. However, as biological materials generally issue only scanty secondary emissions, they must be coated with a thin metallic layer beforehand. The practical procedure of coating will be described later.

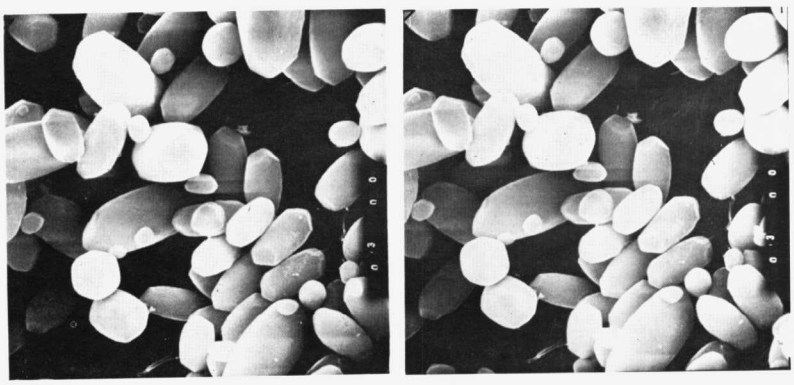


Fig. 1. An example of stereo-pair micrographs. Otoconia from the macula sacculi of the rabbit. ×1,500

The resolution of the scanning electron microscope is about 250 Å. It depends naturally on the technique of sharpening the electron beam. However sharpened it may be, the secondary emission emerges not from the very point hit by the electron beam but from a certain area and depth. This nature of electrons significantly affects the resolution of the scanning electron microscope. Furthermore, the coating itself, when it is made, substantially decreases the resolution. Because of these conditions, the highest effective magnification of the scanning electron microscope at present is about 30,000 times. This magnification can be and should be secured by careful maintenance and adjustment of the microscope, though many researchers today seem to be satisfied with a magnification of several thousand times.

One of the great merits of the scanning electron microscope is the conspicuously large depth of focus. Simply speaking, the depth of a field roughly corresponds to half of its width. When we have a field of 1 mm in diameter, a depth of 0.5 mm is sharply focused. The scanning electron micrographs are thus characterized by the sharp appearance of every elevation and depression on the surface insofar as the peak-to-valley height does not exceed the above-mentioned range. If we remind ourselves of the irritating experiences in close-up photography and light microphotography that are focused only to an extremely limited depth, the scanning electron microscope can be said to have a revolutionary performance.

By tilting the specimen holder through an adequate angle between two exposures, stereo-pair micrographs are obtained, which, when observed through a stereo-viewer, afford a real three-dimensional view of the specimen surface (*Fig. 1*). One may also measure the peak-to-valley height of given objects on the surface using a stereometer.

Scanning electron microscope observation of biological materials was retarded in comparison to that of hard materials such as metals and crystals, and it was only in 1967 that systematic studies began to be published. The hard tissues, such as teeth and bones, are relatively simple to treat, but in soft tissues, cells, parasites and microbes, it has been a large problem as to how to minimize the deformation caused by drying. The technique of metal coating should also be improved in order to clarify the fine structures of subcellular level.

Methodology in the sample preparation, including these problems, will now be briefly described.

Preparation of Specimens

The object of scanning electron microscopy is the *surface* of materials. The surfaces, however, are not necessarily natural and free ones, but may be artificial ones made by cutting, tearing and even by crushing. Free cells, subcellular fractions, as well as formed elements and crystals in body fluids and drugs are appropriate materials for scanning electron microscopy. Various kinds of impressions, such as simple celluloid replicas of surface reliefs and plastic casts of cavities and vessels, also provide useful specimens (FUJITA, TOKUNAGA and INOUE, 1969).

The potential materials for scanning electron microscopy thus may be said to be of infinite variety. This also implies a possibly large variety of methods in specimen preparation, and it should be understood that the following comment on the specimen preparation of animal tissues and free cells is just one possible choice.

1. Fixation

When the surface to be observed is covered by mucous and other substances, attempts should be made to eliminate them as completely as possible. Use of surface-activating materials or enzymes may be effective, but a simple and unexpectedly effective method is patiently rinsing the surface with a jet-stream of Ringer's solution or physiological saline.

As fixative, 2.5 per cent glutaraldehyde (in a 0.1 M phosphate buffer pH 7.2) is recommended for general use. A comparably good result for some materials may be obtained by simple fixation in 10 per cent formalin.

These non-coagulant fixatives have the merit of preventing proteinic fluids covering the surface from forming coagulations which would hide the surface appearance. On the other hand, however, the tissues and cells may be insufficiently hardened by these fixatives so that they may undergo severe shrinkage and deformation during the later procedures of dehydration and drying. This deficiency in aldehyde fixatives may be overcome by a post-fixation in 2 per cent osmium tetroxide, 0.2 per cent corrosive sublimate or in 2 per cent potassium permanganate (*Fig. 2*).

Though both glutaraldehyde and formalin can be applied to free cells, our experience with red blood corpuscles, which are especially sensitive to osmotic pressure, indicates that a glutaraldehyde concentration of 1 per cent (in a 0.1 M phosphate buffer) preserves the most natural forms of cells (TOKUNAGA, FUJITA and HATTORI, 1969).

In the case of blood corpuscles and spermatozoa, it is of primary importance that the blood or sperm plasma be eliminated so that the cellular elements become virtually naked. Rinsing in physiological saline is effective, but a better and more simple method is to drop a small amount (2 or 3 drops) of blood or semen into a large volume (20 ml) of a non-coagulant fixative and to shake vigorously. The cellular elements are thus extensively dispersed in the fixative and, if one removes the supernatant after gentle centrifugation, the plasma which when coagulated would cover and bury the cells as "mud" under the scanning electron microscope, can be eliminated virtually completely (FUJITA, MIYOSHI and TOKUNAGA, 1970).

Specimens of subcellular fractions can be prepared by essentially the same method as described above (KURAHASI et al., 1969).

After the fixation the tissues and cells are rinsed and then dehydrated.

2. Dehydration and Drying

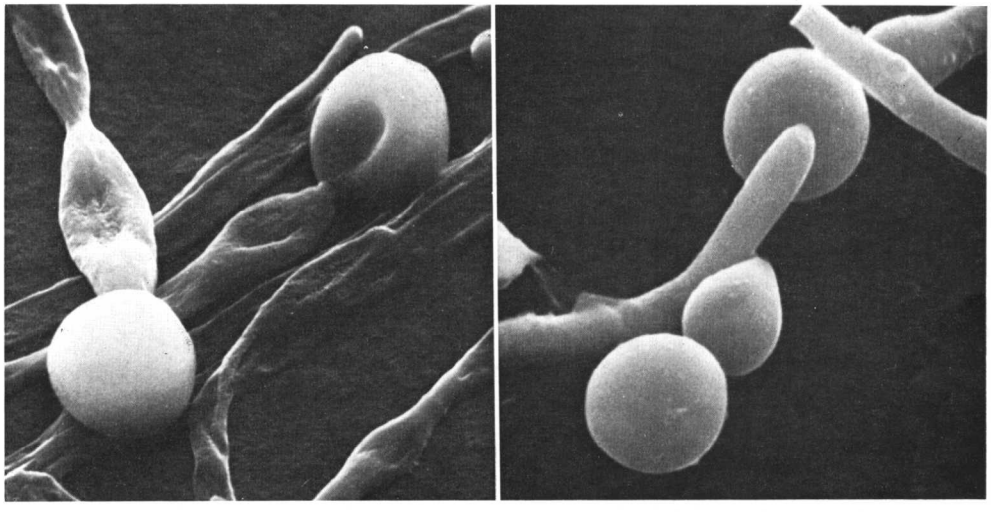
If fixed or unfixed tissues in a wet state are dried in air, a severe shrinkage and deformation is inevitable. In the case of free cells, drying water, with its high surface tension, gathers all the muddy substances from the vicinity to the surface of the cells and one may see under the scanning electron microscope only cells buried in the "mud." To minimize these changes, the following method of acetone dehydration and air drying is recommended.

The tissues and cells are dehydrated in acetone of ascending concentrations, i.e., in 30, 50, 70, 90, 95 and 100 per cent. The time for every concentration is probably 10 minutes, though naturally it depends on the size of the specimens. The final stage of dehydration should be done carefully through several changes of 100 per cent acetone and dried acetone. The specimens are then put on a filter paper and dried in air.

The method of acetone dehydration and air drying was reported by BARBER and BOYDE (1968). A little later, but independently, we came to know the usefulness of volatile media in drying tissues. Ethanol, acetone and propylen oxide were all found effective, but we found that moderately volatile acetone was most easy to handle, thus reaching the same conclusion as BARBER and BOYDE (FUJITA, INOUE and KODAMA, 1968).

Since 1969, however, BARBER and BOYDE have "changed more or less exclusively to freeze-drying of soft tissue specimens because of the great degree of shrinkage that can occur on air-drying." Drops of water containing either fixed or unfixed specimens were frozen in a bath of dichlorodifluoromethane cooled by liquid nitrogen. After being transferred to liquid nitrogen, they were put on a cold probe to be dried in vacuo.

We have experienced that freeze-drying may also cause relatively marked artifacts on one hand, and on the other hand, deformations from the acetone-drying method may be prevented to a considerable extent by various devices in the procedures of post-fixation and drying(*Fig. 2*). We attach importance to making the most of the merits of simple and rapid specimen preparation in scanning electron microscopy. From this standpoint we have dared to stay with the acetone drying and almost all the pictures in this volume are based on this method.



A B

Fig. 2. Scanning electron micrographs of *Candida albicans*.
A: Fixation in 2 per cent glutaraldehyde, acetone dehydration and air drying.
B: Postfixation in 2 per cent $KMnO_4$ was made after the glutaraldehyde fixation. The collapse and shrinkage of the fungal tissue which are obvious in Fig. A have been prevented by the postfixation as shown in Fig. B. ×3,250

Dehydration and drying of free cells (of blood, semen, ascites, etc.) and subcellular fractions are performed by the following procedures.

The fixed specimens are advanced, gentle centrifugation being repeated, through ascending grades of acetone. The final stage of dehydration in dried acetone should be done especially carefully. By vigorously shaking the centrifuge tube the specimen

is emulsified in a few ml of dried acetone. This emulsion is dropped with a pipet on small pieces (1 × 1 cm) of glass slide and dried under a warm breeze from a hair-dryer.

We recommend that the dried specimens be carefully checked before the next procedure of metal coating. In the case of tissues, the surfaces to be used should be surveyed under a binocular to see whether the structures of interest are free of coverage and what artificial deformations have occurred. In the case of free cells, specimens with fewer deformities and better scattering of cells covered by no muddy substance are selected under a transmission light microscope. Even such minute particles as blood platelets and isolated mitochondria can be clearly examined by this method. Dark field illumination is often useful for these materials.

3. Metal Coating

The next procedure is coating the specimens with carbon and metals. This procedure has two purposes. One is to ensure the electric conductivity from the specimen surface to the holder and another is to make a layer which will emit ample secondary electrons upon bombardment by electrons.

Though a metal coating was once made by simply dipping the specimens into a saturated ethanolic solution of auric chloride and drying them in air (JAQUES, COALSON and ZERVINS, 1965), the prevailing method now is vacuum evaporation.

Double coating with carbon and gold by vacuum evaporation (BARBER and BOYDE, 1968) seems to be most widely performed. Carbon assures a tough layer of a high electric conductivity and is said to be evaporated going not directly but "around corners." Gold is easy to treat in evaporation, emits ample secondary electrons and is free from degeneration. The specimens shown in this volume are, for the major part, doubly coated with carbon and gold, but a part of them are singly coated with gold. It is difficult to come to a definite conclusion but we are inclined to find no significant difference between the simple and double coatings. Platinum-palladium, silver, copper or aluminum may be used for gold.

It is of critical importance for successful scanning electron microscopy to have a coating layer cover the specimen as evenly as possible in spite of the roughness in its surface. This purpose is attained to some extent either by setting two or three evaporation sources above the specimens or by repeating evaporation from different directions.

Much more effective is the use of a specimen disk which can be rotated around the vertical axis (BARBER and BOYDE, 1968). The evaporation source is set obliquely above the disk (at an angle of 40–60°) and the disk, during the deposition of carbon and metals, is rotated quickly by hand or, still better, by motor drive. Recently a more advanced evaporator has been available in which the specimen disk is motor driven into a combined movement of a horizontal rotation around the vertical axis and a rocking (to a desired extent of angle) around the horizontal axis. This device guarantees much more even coating than in the case of simple rotation. Carbon is evaporated in a vacuum of about 5×10^{-5} Torr, while gold in a vacuum a little lower than this.

According to the combined scanning and transmission electron microscope study by BARBER and BOYDE (1968), an appropriate coating layer consists of 200 Å thick carbon and 300 Å thick gold in the case of double coating of soft tissue surface. Some authors, however, believe that the thickness of the coating should be as thin as 100 Å.

Recently an evaporator equipped with a "thickness monitor" has been provided which, by the use of a quartz oscillator, enables the detection of ultra-fine weight changes caused by metal deposition. With this instrument one can attain an adjustable and repeatable deposition of coating layers.

When the coating is too insufficient to ensure a good electric conductivity on the specimen surface, the image produced is severely disturbed by an undesirable charging effect. When the coating is superfluous, the surface structures to be observed are covered "under the snow."

4. Electron Microscopy

The specimens are, either before or after metal coating, mounted on a copper stub with conductive silver-paste and inserted into the specimen chamber of the scanning electron microscope. A beam accelerating voltage is selected between 5 and 25 kV. In a low magnification, a high contrast in images free of charging effect can be obtained at 5 or 9 kV. The highest accelerating voltage, 25 kV, is needed only for

magnifications higher than 20,000 times.

When one compares the images of the same specimen under the same magnification at different accelerating voltages, a significant difference may be noticed. A lower voltage generally gives a sharper picture of the surface structure. The difference is ascribed to the fact that at lower voltage only the secondary emissions from the very superficial layer of the specimen are collected, whereas at higher voltage, information from deeper layers becomes mingled.

Though the scanning electron micrographs have the merit of being very simply understood, the following points may be worthy of attention:

First, the scanning electron micrographs are, so to speak, "snow-covered landscapes." The surface structures appear thicker because of the depth of the snow or coating layer. There is no significant problem in structures larger than cellular level but minute matters of subcellular level should be carefully analyzed. Projections appear thicker and depressions smaller than they really are. Very narrow spaces may be buried.

Second, the more or less marked shrinkage of the specimens during the drying procedure should be taken in account. A comparison of the dimensions of structures in scanning and transmission electron micrographs thus requires careful consideration.

Reference

Barber, V.C. and A. Boyde: Scanning electron microscopic studies of cilia. Z. Zellforsch. 84: 269-284 (1968).

Boyde, A. and V.C. Barber: Freeze-drying methods for the scanning electron-microscopical study of the protozoon *Spirostomum ambiguum* and the statocyst of the cephalopod mollusc *Loligo vulgaris*. J. Cell Science 4: 223-239 (1969).

Boyde, A. and C. Wood: Preparation of animal tissues for surface-scanning electron microscopy. J. Microsc. 90: 221-249 (1969).

Cosslett, V.E.: Scanning microscopy with electrons and X-rays. J. Electron Microsc. 16: 51-64 (1967).

Fujita, T., H. Inoue and T. Kodama: Scanning electron microscopy of the normal and rheumatoid synovial membranes. Arch. histol. jap. 29: 511-522 (1968).

Fujita, T., J. Tokunaga and H. Inoue: Scanning electron microscopy of the skin using celluloid impressions. Arch. histol. jap. 30: 321-326 (1969).

Fujita, T., M. Miyoshi and J. Tokunaga: Scanning and transmission electron microscopy of human ejaculate spermatozoa with special reference to their abnormal forms. Z. Zellforsch. 105: 483-497 (1970).

Jaques, W.E., J. Coalson and A. Zervins: Application of the scanning electron microscope to human tissues. A preliminary study. Exp. molecul. Pathol. 4: 576-580 (1965).

Kurahasi, K., J. Tokunaga, T. Fujita and M. Miyahara: Scanning electron microscopy of isolated mitochondria. I. Arch. histol. jap. 30: 217-232 (1969).

Tokunaga, J., T. Fujita and A. Hattori: Scanning electron microscopy of normal and pathological human erythrocytes. Arch. histol. jap. 31: 21-35 (1969).

Tokunaga, J., T. Fujita and H. Inoue: Medical application of scanning electron microscopy (in Japanese). Igaku no Ayumi 68: 485-491 (1969).

Scanning Electron Microscopy 1968. Proceeding of the Symposium on "The Scanning Electron Microscope — the Instrument and Its Applications" (Symposium Director: O. Johari). IIT Research Institute, Chicago, 1968 (185 pages).

Scanning Electron Microscopy 1969. Proceeding of the Second Annual Scanning Electron Microscope Symposium (Symposium Director: O. Johari). IIT Research Institute, Chicago, 1969 (525 pages).

Scanning Electron Microscopy 1970. Proceeding of the Third Annual Scanning Electron Microscope Symposium (Symposium Director: O. Johari). IIT Research Institute, Chicago, 1970 (534 pages).

VISCERAL ORGANS

I. Filiform Papillae of the Tongue

The back of the tongue feels rough as it is covered with tiny spine-like protrusions armed with cornified epithelial cells. These protrusions are named filiform papillae to be differentiated from the larger and less numerous fungiform, folliate and vallate papillae. In contrast to the latter forms, the filiform papillae lack taste buds and serve simply as a mechanical file in licking off things. As slight movement and pressure on

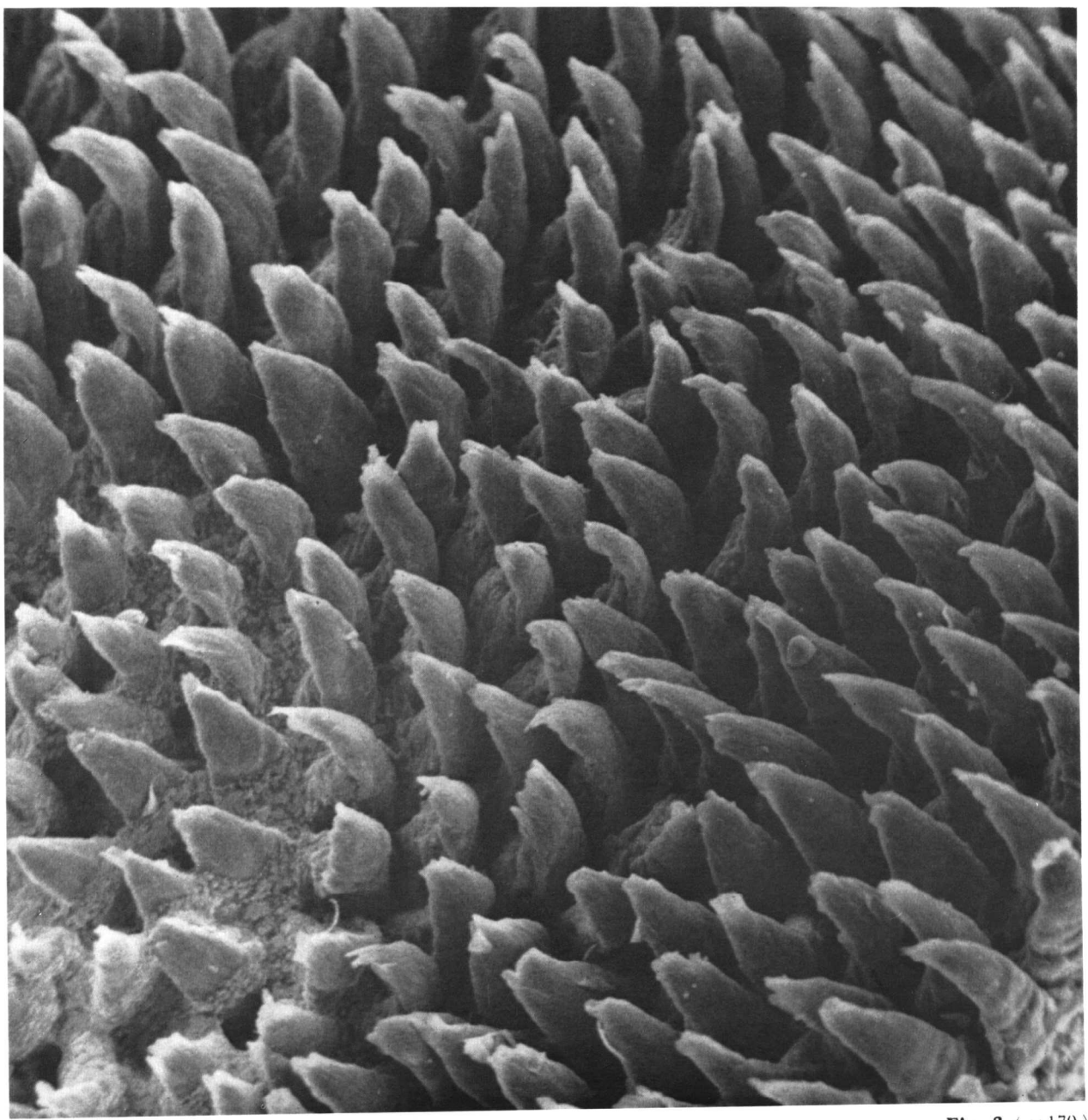


Fig. 3 (×170)

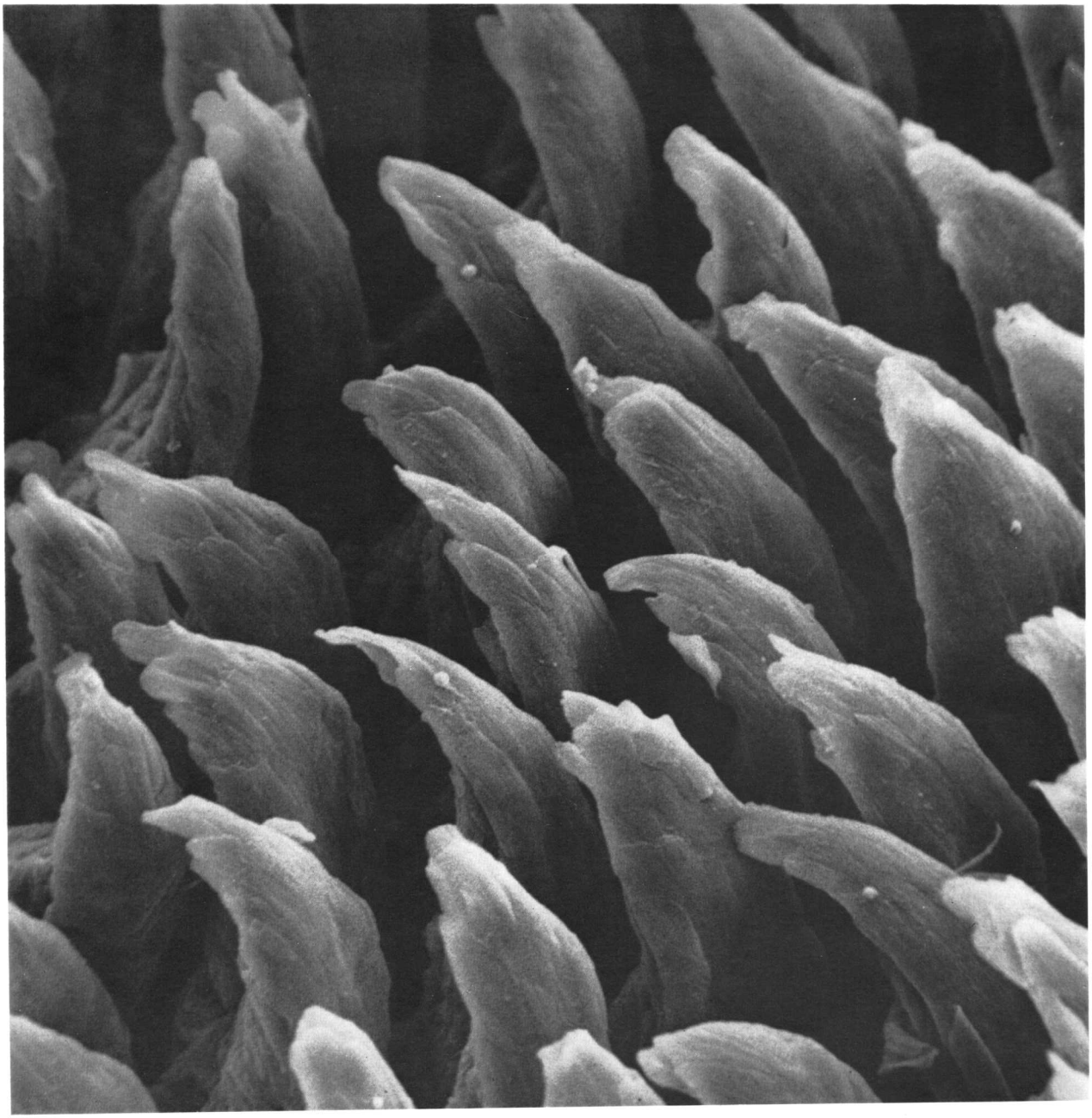


Fig. 4 (×430)

their ends may be transmitted to the nerve endings richly distributed at their base, it has been presumed that they are also a kind of tentacle for the perception of touch sensation.

Figs. 3 and **4** show the filiform papillae densely covering the back of the tongue of a rabbit. Every papilla is curled with its end pointed to the throat, a form suited for the licking-off mechanism of the tongue.

Fig. 5 (× 1,700)

Fig. 5 which is a closer view of the middle portion of the preceding micrograph reveals scale-like epithelial cells covering the papilla. In **Figs. 6** and **7** an exquisite relief on the surface of these cornified cells is shown highly magnified. Fig. 7 includes three of these cells piled like slates.

The micrographs shown in this section clearly indicate the two merits of the scanning electron microscope: that it can demonstrate the surface structures of matters from the level of a magnifying glass to that of an electron microscope, and that it affords such a large depth of focus as could never be expected in light microscopy.

Note

The back of the rabbit tongue was carefully washed with water and fixed in 10 per cent formalin. Small blocks of the tissue were dehydrated in acetone, dried in air and coated with gold. EM: JSM-2

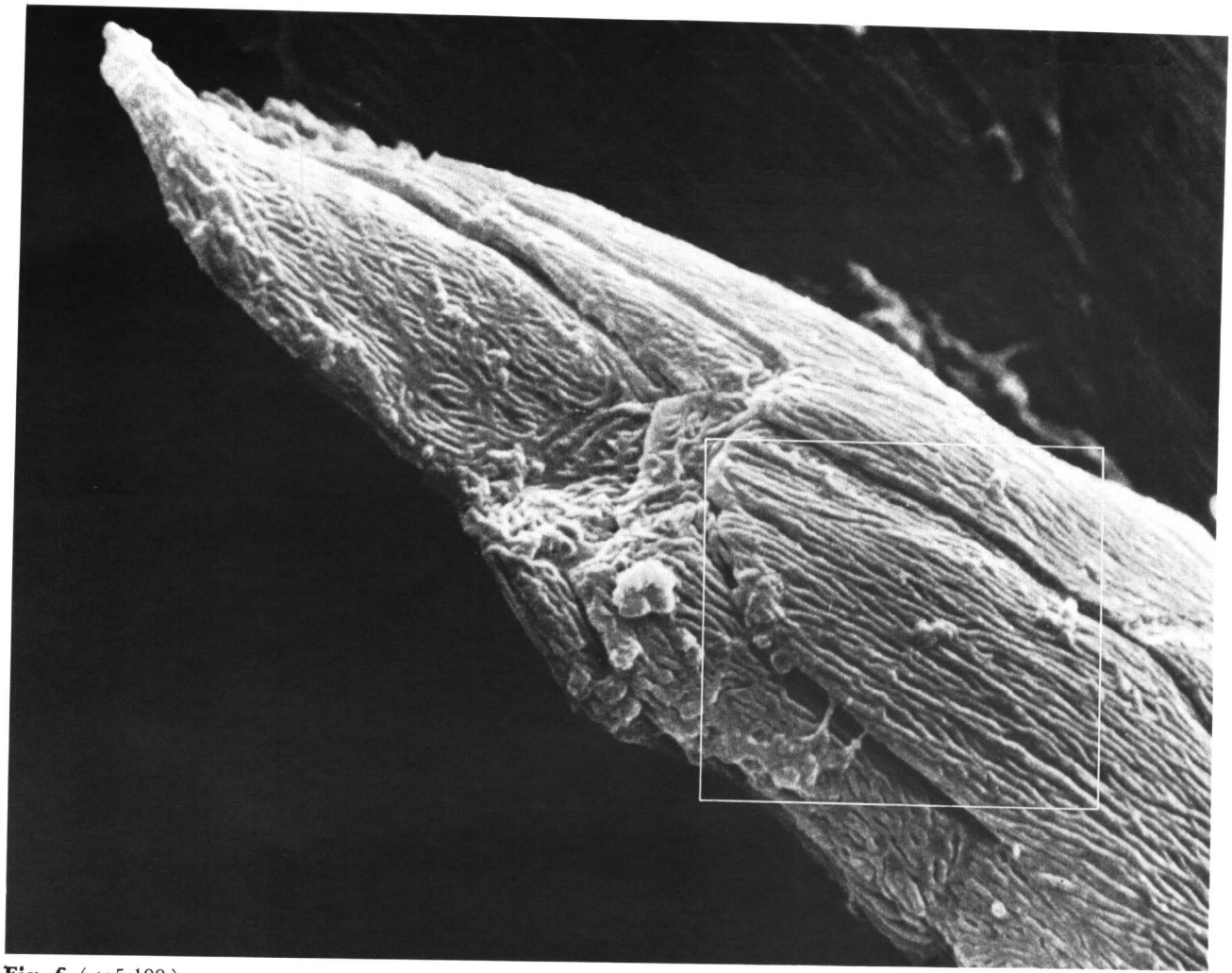


Fig. 6 (×5,100)

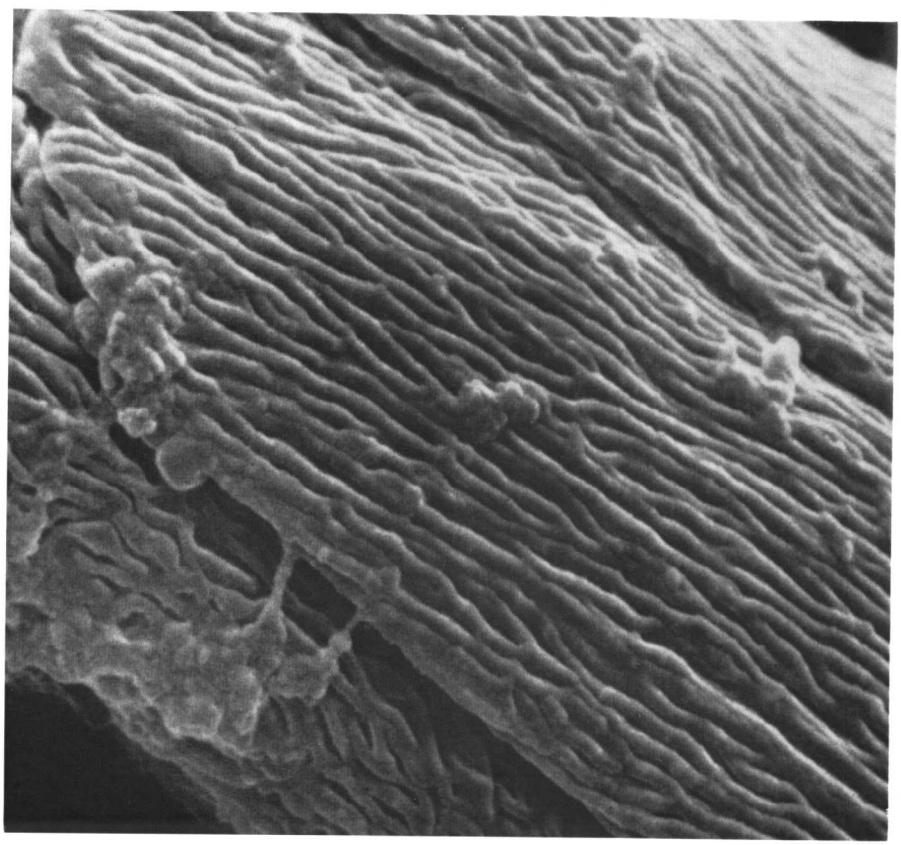


Fig. 7 (×12,000)

2. Sensory Papillae of the Frog Tongue

In the frog, which has no taste buds on the tongue, the fungiform papillae play the role of taste receptors. It is also known that these sensory papillae, dispersed on the back of the tongue, are contractile in response to various physical and chemical stimuli.

Under the scanning electron microscope a fungiform papilla looks like a round table (**Fig. 9**). The main central part of the table is called a sensory epithelial disc and is covered by a number of hexagonal sensory cells. Surrounding this epithelial disc there are a few rows of ciliated cells and the side of the table is covered by large rounded epithelial cells.

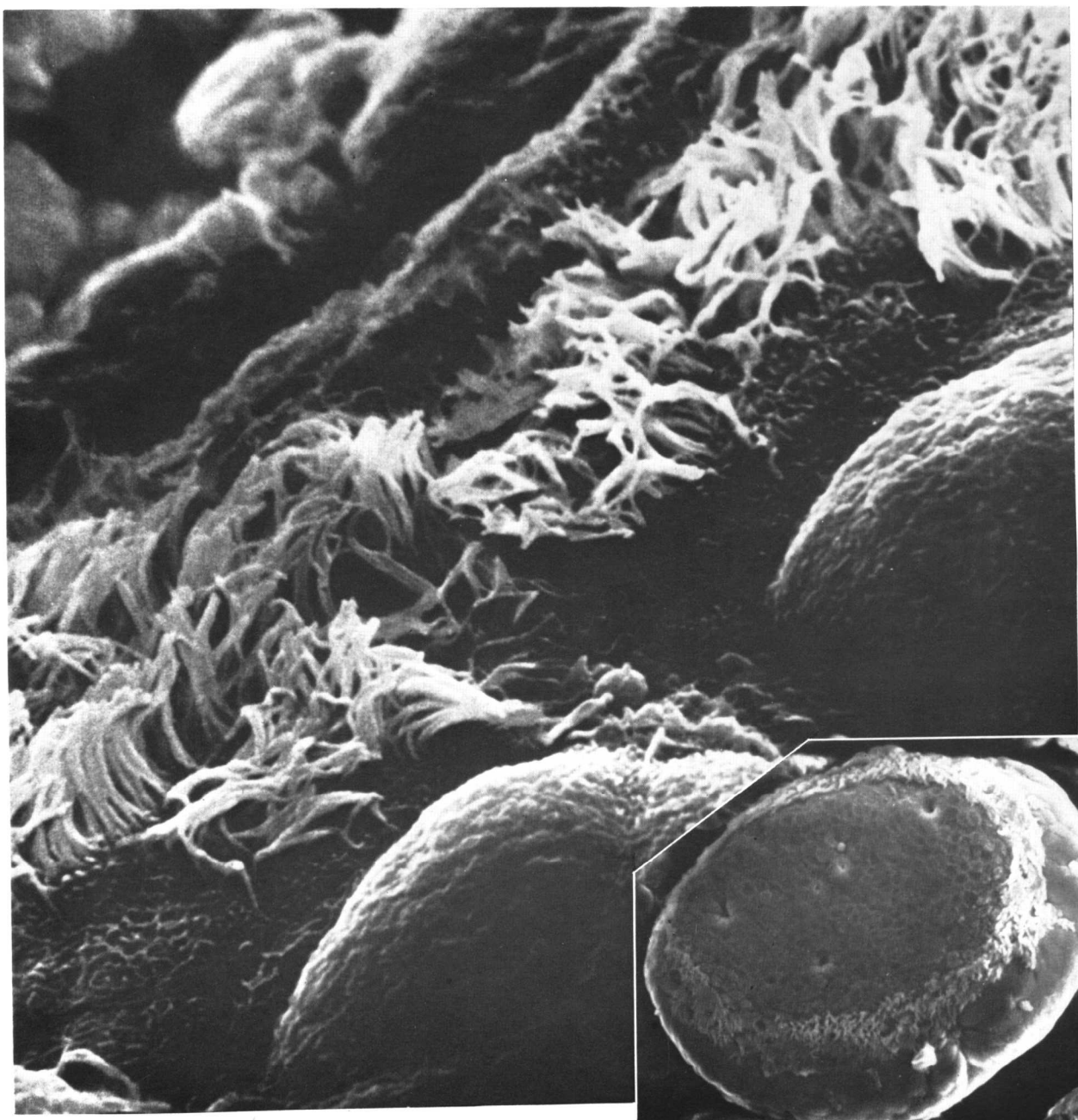


Fig. 8 (×5,000)

Fig. 9 (×400)

Fig. 10 (× 15,000)

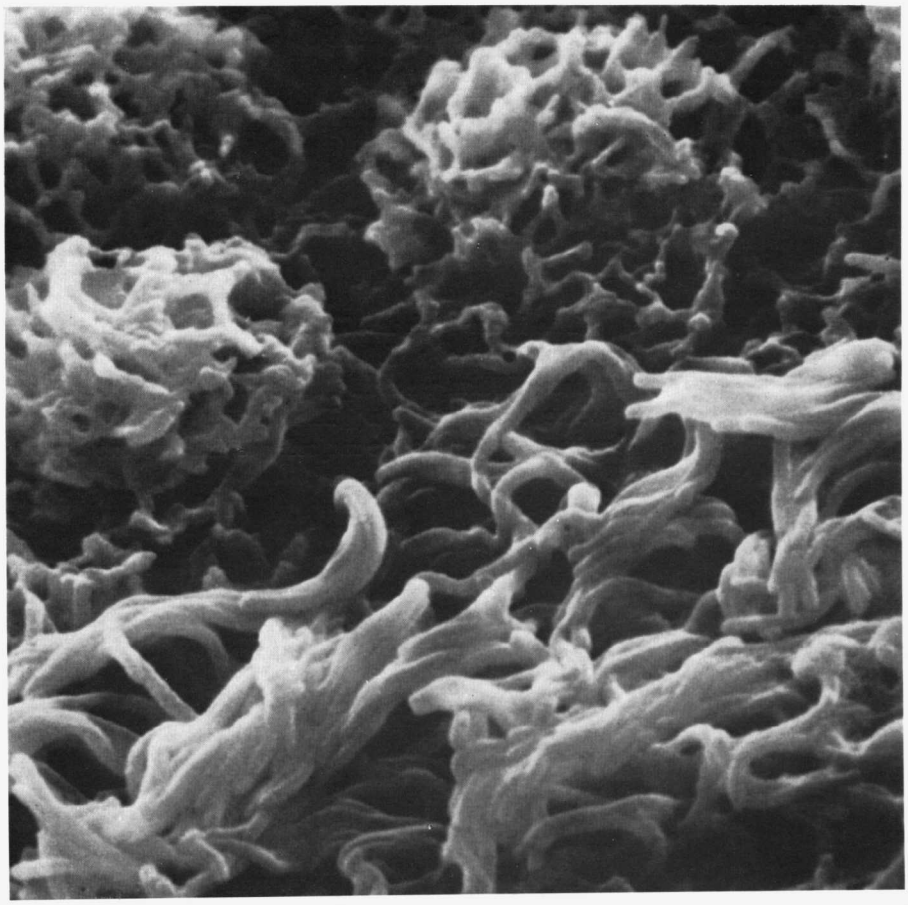


Fig. 8 is a view of the peripheral part of the table showing the latter cells in the foreground and the cilia in the back.

Fig. 10 is a close-up of the border of the sensory epithelial disc. Noodle-like cilia are seen in the lower half of the picture. In the upper half three sensory cells are recognized by their round heads covered with shaggy microvilli.

Fig. 11 shows an aberrant ciliated cell occasionally found among the epithelial cells of the table side.

These findings were obtained in collaboration with Dr. AKITATSU SHIMAMURA, Department of Oral Histology, and Dr. SATOSHI NAKAHARA, Department of Physiology of the Kyushu Dental College.

Note

The tongue of the bullfrog was removed and kept in physiological saline for several hours. The surface of the tongue meanwhile was washed repeatedly with a jet-stream of the saline in order to remove the mucus on the tongue as completely as possible. The specimen then was fixed in 2.5 per cent glutaraldehyde (0.1 M phosphate), post-fixed in 2 per cent osmium tetroxide, dehydrated in acetone, dried in air and coated with carbon and gold. EM: JSM-2

Reference

SHIMAMURA, A. and J. TOKUNAGA: Scanning electron microscopy of sensory (fungiform) papillae in the frog tongue. Proc. 3 rd Annu. Sympos. SEM. 1970. Chicago, IIT Res. Inst. (p. 227-232).

Figs. 9 and 11 by courtesy of the Editor of the Symposium Proceeding and the IIT Research Institute.

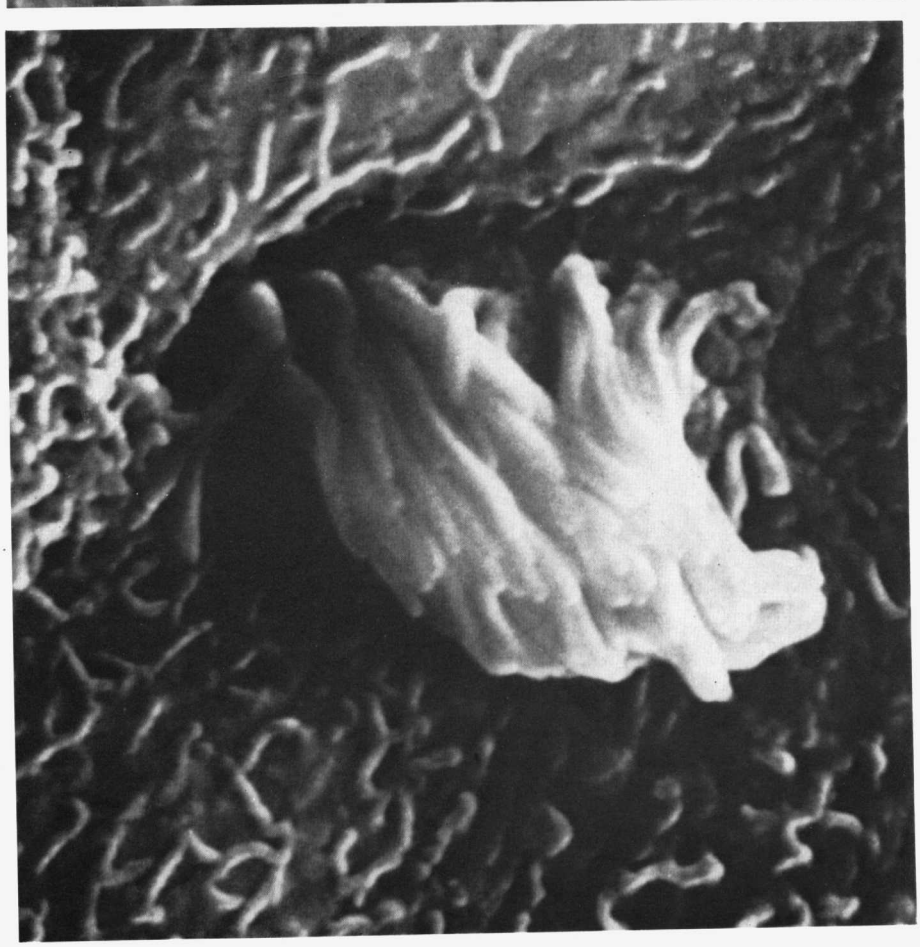


Fig. 11 (× 12,000)