Featured Articles

# Easy-to-use Atmospheric-pressure Scanning Electron Microscope for Food, Plants, and Tissue

## —AE1500 Tabletop Atmospheric SEM—

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*OVERVIEW: Hitachi High-Technologies developed the AE1500, a tabletop atmospheric SEM that is capable of scanning electron microscopy under atmospheric pressure, by using a membrane that allows an electron beam to pass while keeping the atmospheric-pressure chamber and evacuated column separate. Because the specimen does not come into contact with the membrane, a feature of the AE1500 is its ability to observe bulk specimens under atmospheric pressure. Hitachi High-Technologies also developed the ES-Corrector algorithm to correct the image for the electron beam scattering that occurs due to the presence of atmospheric gases between the membrane and the specimen. This means that bulk, high-moisture specimens such as food or biological samples can be observed with the electron microscope under atmospheric pressure without preprocessing.*

## INTRODUCTION

SCANNING electron microscopes (SEMs) (which are used to observe microscopic features) have become essential tools in numerous different areas of research and development. The specimen chamber of an SEM is typically maintained at a pressure between $10^{-5}$ Pa (hard vacuum) and $10^{2}$ Pa (soft vacuum). This is done to keep the path of the electron beam free of gases because of the scattering that occurs when electrons collide with gas molecules. On the other hand, there is strong demand for the use of SEMs for high-magnification observations of tissue and other soft materials that contain water. Unfortunately, because the saturated vapor pressure of water at room temperature is only about $2.3 \times 10^{3}$ Pa (2.3 kPa), it is difficult to observe specimens in a hydrated state using a conventional SEM because evaporation occurs even when used with a soft vacuum of $10^{2}$ Pa. While SEM observations under atmospheric pressure have previously been reported in the literature, their use has been subject to restrictions.

In response, Hitachi High-Technologies Corporation has developed a tabletop SEM that is easy to use for observation of water-containing specimens under atmospheric pressure. This article describes the AE1500 tabletop atmospheric scanning electron microscope.

## BACKGROUND

A number of methods for making SEM observations of a specimen under atmospheric pressure have previously been reported in the literature[(1) – (3)]. Most of these have exposed the specimen to the electron beam via a very thin membrane (tens of nanometers) that keeps evacuated column and atmospheric-pressure chamber separate, but in doing so, have required the specimen to be placed on the membrane [see Fig. 1 (a)].

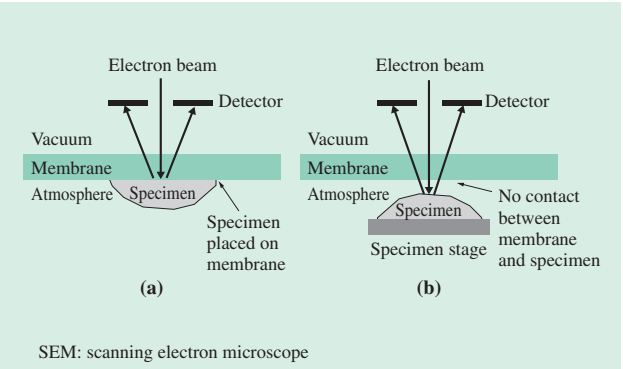



*Fig. 1—Techniques for Atmospheric SEM Imaging.*
*The observation of bulk material is difficult when the membrane and the specimen are in contact as in diagram (a). When there is no contact between the membrane and the specimen, as in diagram (b), the observation of bulk material is easy, however the electron beam is scattered by the intervening gas molecules.*

The problem with this approach is that it makes it difficult to observe bulk materials such as food, plants, or biological tissue.

In response, Hitachi High-Technologies has devised a technique for SEM observation under atmospheric pressure in which the specimen is placed on a stage, thereby avoiding any contact with the membrane [see Fig. 1 (b)][4]. Although this makes it easy to observe bulk materials, it has been assumed in the past that this will make SEM imaging impossible because the separation between the membrane and the specimen results in electron beam scattering due to the gas molecules in the atmosphere. However, by going back to the principles behind electron beam scattering, Hitachi High-Technologies determined that SEM images can be obtained even when there is a degree of separation between the membrane and the specimen. Hitachi High-Technologies has also developed the ES-Corrector image enhancement technique (which corrects for electron beam scattering) to produce crisp SEM images from which the effects of electron beam scattering have been removed.

The following section describes the design and features of the atmospheric SEM, and the techniques that make it possible to perform SEM observations under atmospheric pressure.

## MICROSCOPE AND ASSOCIATED TECHNOLOGY

### Microscope Concept and Technical Challenges

The objective in developing the atmospheric SEM was to enable everyday items to be examined under atmospheric pressure in such fields as food, agriculture, pharmaceuticals, and medicine.

*Fig. 2—AE1500 SEM.*
*The AE1500 went on sale in September 2015.*

Hitachi High-Technologies has been selling its TM3030 tabletop SEM internationally since 2010. As the TM3030 can be placed on a desk, it is used in a wide range of fields, including things like science education for children as well as research and development, and industrial applications. The AE1500 tabletop atmospheric SEM was developed based on the TM3030, the latest tabletop model, and came about out of a desire to make an atmospheric SEM that would be easy to use for observing everyday items (see Fig. 2).

### Microscope Design

Fig. 3 shows the internal structure of the AE1500. Inside the part of the microscope that is in a state of vacuum is a chamber that can be kept under atmospheric pressure [see Fig. 3 (a)]. The specimen is placed in this atmospheric-pressure chamber. The membrane that separates the evacuated column from the atmospheric-pressure chamber is located at the top of this chamber. This allows the specimen to remain under atmospheric pressure (1 atmosphere = approximately 101 kPa) while the vacuum is maintained throughout the rest of the SEM.

The membrane (thickness: 20 nm) used for this purpose is made of silicon nitride ($SiN_x$), with electron beam scattering being one of the considerations behind this choice of thickness, as explained below.

The electron beam acceleration voltage is 15 kV. The electron beam is emitted by an electron gun located in the part labeled “SEM” in Fig. 3 and is focused on the specimen by an objective lens. After passing through the membrane and being scattered by atmospheric gas molecules, the electron beam reaches the specimen with an energy of approximately 15 kV. Because of the high energy of the electrons backscattered (reflected) from the specimen, they pass through the region of atmosphere and the membrane again to the backscattered electron detector. This design allows SEM observation under atmospheric pressure.

The specimen is aligned under the membrane by placing it on a holder and then inserting the specimen stage into the chamber [see Fig. 3 (b)]. Using its vacuum pump, the microscope is able to create a negative pressure around the specimen (ranging from a few kPa up to 101 kPa, corresponding to between 0.1 and 1 atmosphere) [see Fig 3 (c)]. Similarly, soft vacuum SEM observation (ranging between a few Pa and several tens of Pa) can also be performed by removing the membrane [see Fig 3 (d)].

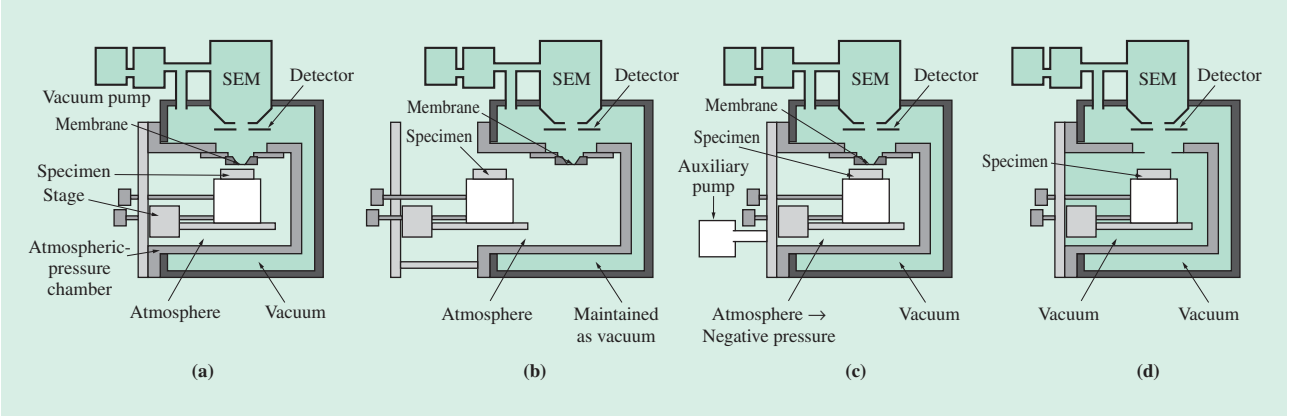



*Fig. 3—Internal Structure of AE1500.*
*Diagram (a) shows SEM observation under atmospheric pressure (101 kPa), (b) shows specimen insertion, (c) shows SEM observation with negative pressure (from several kPa to 101 kPa), and (d) shows vacuum SEM observation (several Pa to several tens of Pa). The AE1500 supports all of these different SEM observation modes: atmospheric pressure, negative pressure, and vacuum. Although not shown in the diagrams, the electron beam is generated by an electron gun and focused on the specimen by an objective lens in the SEM unit.*

## Electron Beam Scattering

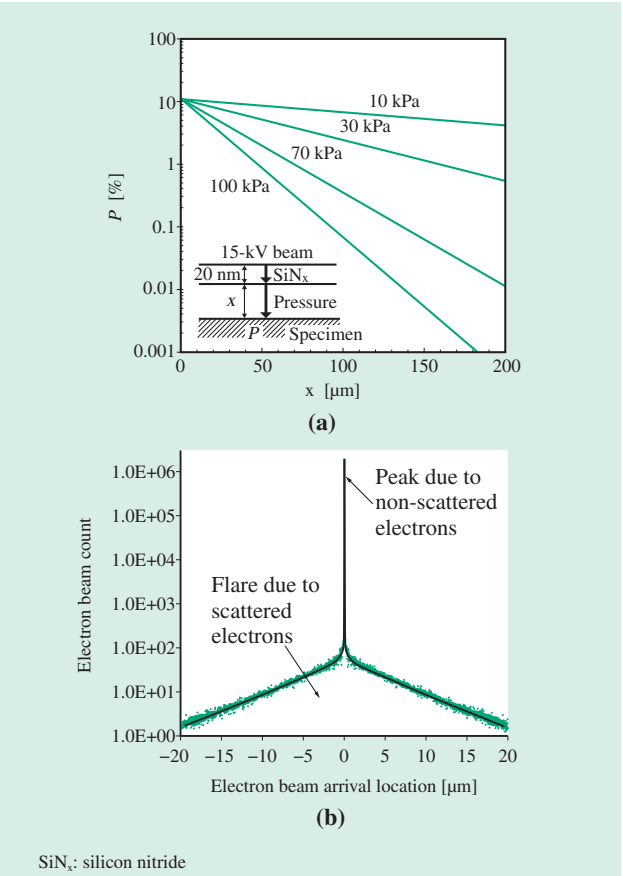
The distance between the membrane and the specimen in the new atmospheric SEM means that electron beam scattering due to atmospheric gases will invariably occur. As it was believed that this would make SEM images difficult to obtain, most atmospheric SEMs reported to date have used the technique in Fig. 1 (a). However, by going back to the principles behind electron beam scattering, Hitachi High-Technologies determined that SEM images can be obtained even when there is a degree of separation between the membrane and specimen. The following explains how.

An electron beam that passes through an atmosphere is scattered by the gas molecules. A certain proportion of the electrons, however, will not collide with any atmospheric gases stochastically and so will not be scattered. These are referred to as non-scattered electrons. The proportion $P$ of electrons that are not scattered is as follows[4].

$$P = exp\left(-\frac{N\rho\sigma}{A}x\right) \quad (1)$$

Here, $N$ is the number of gas molecules, $\rho$ is the density (g/cm$^3$), $\sigma$ is the scattering cross section area (cm$^2$), $A$ is the mass number, and $x$ is the distance traveled by the electrons (cm).

Fig. 4 (a) shows the proportion $P$ of electrons that are not scattered after passing through the membrane ($SiN_x$, 20-nm thickness) with an acceleration voltage of 15 kV and traveling the distance $x$ between the membrane and the specimen. Atmospheric pressure is 100 kPa, with the results for other pressures being




$SiN_x$: silicon nitride

*Fig. 4—Relationship between Distance from Membrane to Specimen and Proportion of Non-scattered Electrons and Results of Beam Profile Simulation.*
*Graph (a) shows the relationship between the distance from membrane to specimen and the proportion of non-scattered electrons, and graph (b) shows the results of a beam profile simulation. Because some electrons reach the specimen without being scattered, even under atmospheric pressure (100 kPa), the beam profile always includes a central peak.*

shown for reference. The important point to note here is that the value of *P* does not fall to zero even if the distance *x* between the membrane and the specimen is large. In other words, although the scattered electrons will end up somewhere other than the focal point targeted by the objective lens, those electrons that are not scattered will arrive at the focal point, with *P* being the proportion of such electrons.

Fig. 4 (b) shows the beam profile calculated by a Monte Carlo simulation for an electron beam with an acceleration voltage of 15-kV after passing through the membrane ($SiN_x$, 20-nm thickness) and traveling the 100 µm distance between the membrane and the specimen. As shown in the figure, even after passing through 100 µm of atmosphere, the beam profile has both a wide tail (flare) due to scattered electrons and a sharp peak due to non-scattered electrons. That is, the beam profile of the atmospheric SEM at the specimen is the sum of scattered (beam flare) and non-scattered (central beam) components.

Fig. 5 shows a comparison of vacuum and atmospheric SEM images of a pattern of metal on silicon (Si). Fig. 5 (a) and 5 (b) are vacuum SEM images taken under a pressure of 10 Pa, and the image in Fig. 5 (c) is an atmospheric SEM image of the same location as 5 (a) taken with a distance of 100 µm between the membrane and the specimen. At first glance, image (c) appears blurred due to scattering of the electron beam by the atmosphere. However, when the magnification of image (c) is increased at the same position as image (b) and the brightness and contrast are adjusted, the detail of the metal pattern becomes clearly recognizable [see Fig. 5 (d)]. If the image really had been blurred by scattering of the electron beam, this detail should not be visible. From this it can be concluded that blurring due to scattering of the electron beam by the atmosphere is not the correct interpretation of the phenomenon.

The reason why the detail is still visible despite atmospheric scattering is believed to be as follows. Electrons that are not scattered by atmospheric gases are concentrated at the focal point. The diameter of the central beam of non-scattered electrons is the same regardless of whether the specimen is in an atmosphere or vacuum. Accordingly, the non-scattered electron beam serves to form the same detailed image as the vacuum SEM. The scattered electron beam, by contrast, travels in many different directions, and although it worsens the image contrast, it does not play any part in forming the detailed image, and therefore does not cause blurring. This means that, as long as the central beam is sufficiently powerful, it is possible to observe details in an atmospheric SEM image even if there is a gap between the membrane and specimen.

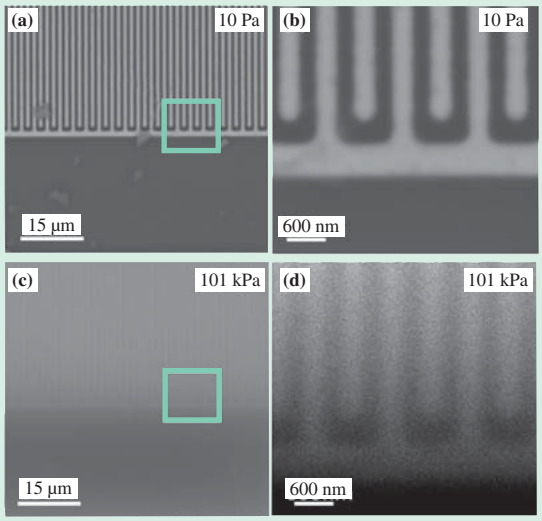



*Fig. 5—Comparisons of Vacuum SEM and Atmospheric SEM Images of Metal Pattern on Si.*
*Images (a) and (b) show vacuum SEM images of a metal pattern at × 5 k and × 30 k magnification, respectively. Images (c) and (d) show atmospheric SEM images of the metal pattern at × 5 k and × 30 k magnification, respectively. Details of the metal pattern can be clearly identified at high magnification in the atmospheric pressure image, just as in the vacuum image.*

## Image Enhancement Technique

This section describes an image enhancement technique for removing the influence of the scattered electron beam from the SEM image.

Fig. 6 (a) shows an image of the digit "9" formed in metal on Si, acquired under atmospheric pressure. Although the image appears blurred, the outline of the digit is clearly visible. This is due to the non-scattered electrons. Similarly, it can be assumed that the blurred appearance of the overall image (bright area around the digit) is due to the scattered electrons.

Given this interpretation, the problem was assumed to be caused by electron beam scattering between the membrane and the specimen, and it was concluded that, in practice, this could be treated as an electron scattering (ES) field whereby the electron beam is reliably scattered in a repeatable manner (mathematically, this field can be represented by a single transfer function). Recognizing that significant image enhancement would be possible if this ES field could be determined and removed from the image,

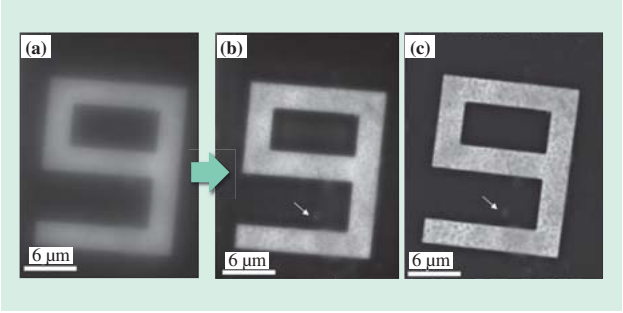



*Fig. 6—Digit Formed in Copper on Si Substrate.*
*Image (a) shows the atmospheric SEM image of the digit "9" formed in copper on an Si substrate, image (b) shows the image after correction by the ES-Corrector image enhancement algorithm, and image (c) shows the vacuum SEM image. Image enhancement makes it possible to see the contaminant indicated by the white arrow.*

Hitachi High-Technologies developed the ES-Corrector algorithm that corrects for electron beam scattering[6]. Fig. 6 (b) shows the image produced by this image enhancement algorithm and Fig. 6 (c) shows an image of the same specimen taken in vacuum. Images (b) and (c) show sub-micron details of the metal film surface, including a contaminant at the location indicated by the white arrow in the figure that is nearly invisible in image (a). This demonstrates the high level of image enhancement achieved in image (b).

Fig. 7 shows a variety of enhanced specimen images acquired under atmospheric pressure. Images (a), (b), and (c) show the original atmospheric SEM images (before correction by ES-Corrector), and images (d), (e), and (f) show the corrected images. This demonstrates the improvement in image quality that results from eliminating the effect of electron beam scattering and enhancing the contrast.

## EXAMPLE OBSERVATIONS

As noted above, SEM imaging of specimens under atmospheric pressure can be performed even when there is a gap (no contact) between the membrane and the specimen. The advantage of this technique is that it can be used to observe bulk materials such as food, plants, or biological tissue simply by placing the specimen on the stage. The following describe examples of such observations.

(1) Observation of food

By removing the membrane from the AE1500 and changing from the configuration shown in Fig. 3 (a) to that in 3 (d), a specimen viewed under atmospheric pressure can also be viewed in vacuum. Fig. 8 shows two images of the same location on the surface of dried pasta taken under atmospheric pressure and in vacuum respectively. Whereas the starch grains of the pasta are visible in image (a), the vacuum image in (b) includes a large number of cracks that are not visible in image (a). This indicates how exposure to vacuum can result in cracking even in an already dry specimen. From this, it can be concluded that specimens that

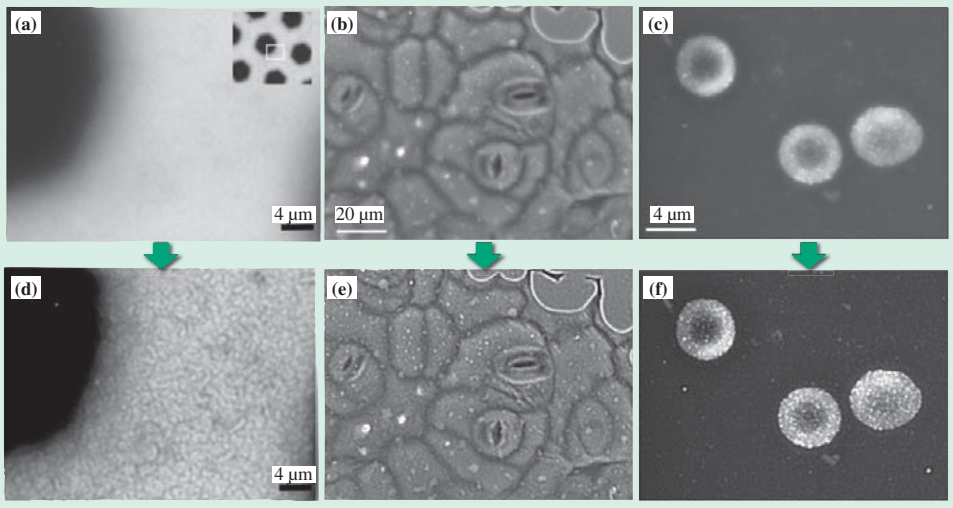



*Fig. 7—Image Enhancement of Specimen Observed under Atmospheric Pressure.*
*Image (a) shows a copper mesh, (b) a daikon radish leaf, and (c) the red blood cell of a rat immunostained with colloidal gold. The images were obtained with an acceleration voltage of 15 kV, at room temperature, and at one atmosphere.*

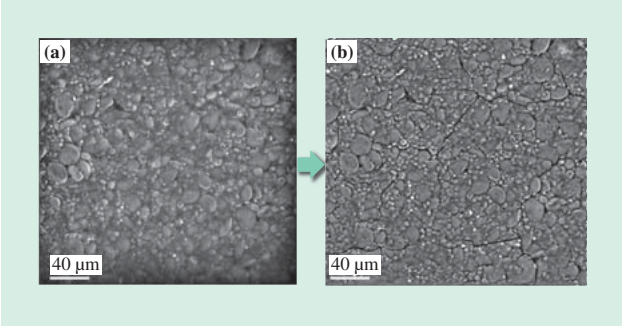



*Fig. 8—Observation of Dried Pasta.*
*Image (a) shows a surface image of dried pasta under atmospheric pressure, and image (b) shows a surface image taken in vacuum. As cracking occurs even in a dry specimen exposed to vacuum, it is preferable that specimens containing even a small amount of moisture be observed under atmospheric pressure.*

potentially contain water are better observed under atmospheric pressure.

(2) Observation of cosmetics drying process

Fig. 9 shows images of sunscreen used to protect the skin against ultraviolet rays. Sunscreen contains silica, titanium, or other fine particles suspended in a fluid. Atmospheric pressure observations were made of the drying of sunscreen applied to a substrate. The images show how the large quantity of water or other liquid present at 30 seconds after application has largely evaporated after 5 minutes, and is almost entirely dry after 15 minutes, leaving large numbers of residual fine particles adhering to the substrate. This behavior of fluid on a substrate was something that could not be observed using conventional vacuum SEM. It is also common in the case of cosmetic, pharmaceutical, and other similar observations that drying of the substrate (such as skin) must be avoided as well as that of the liquid specimen. Achieving this requires that SEM observations be made under atmospheric pressure.

(3) Observation of tissue

This example involves the observation of animal tissue[5]. A scalpel was used to cut the large intestine from the gastrointestinal tract of a rat fixed in formalin [see Fig. 10 (a) and (b)] and the intestinal cross section was viewed using a stereoscopic optical microscope [see Fig. 10 (c)]. The same cross section was then viewed using the AE1500 without preprocessing such as staining or vapor deposition [see Fig. 10 (d)]. Fine details that are not visible through the stereoscopic microscope can be clearly observed using the AE1500. Next, the SEM specimen was dehydrated, embedded in paraffin, sliced, the paraffin was removed, and then specimen was stained with hematoxylin and eosin (H&E), a standard practice for preparing thin-slice tissue specimens. The image of the resulting specimen viewed through a biological microscope is shown in Fig. 10 (e). Features such as intestinal villi are clearly visible, demonstrating that the process of atmospheric SEM observation preserved the state of the tissue without drying out the specimen.

The reason why fine details could not be seen using the stereoscopic microscope is because of the strong tendency for light to pass directly through the specimen, so that rather than only showing the surface structure, images of the interior are also superimposed on the result. The usual way to make these details visible is to first slice and stain the specimen as in Fig. 10 (e). The problem with this is that procedures like embedding and slicing require a lot of work. Atmospheric SEM involves the detection of electrons that are backscattered from the surface, with the

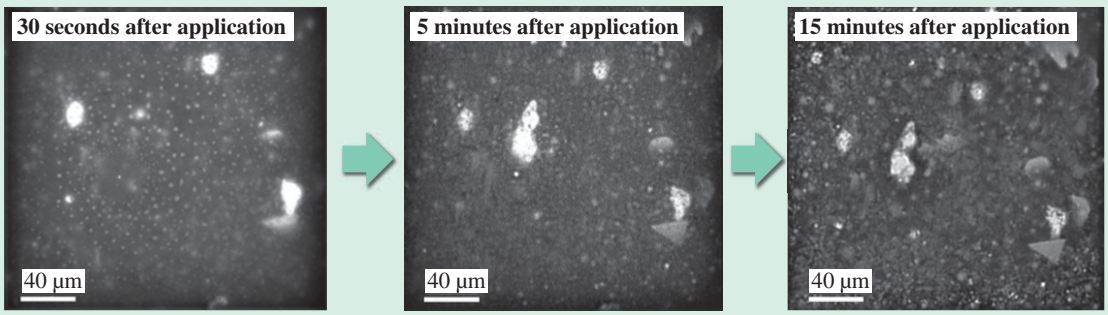



*Fig. 9—Observation of Cosmetic in the Process of Drying.*
*The drying of liquid sunscreen is evident 30 seconds after application, together with the deposition of the fine particles contained in the sunscreen. The image after 15 minutes shows that drying is complete and fine particles have adhered to the substrate.*

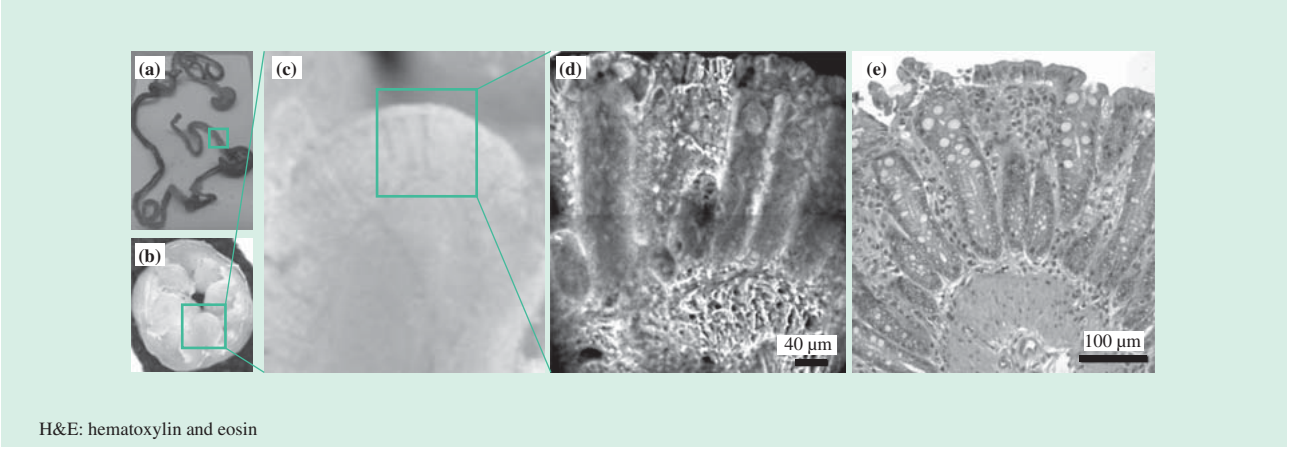



*Fig. 10—Images of Rat Intestine.*
*Image (a) is a gastrointestinal tract that has been removed from a rat, (b) is a cross section of its large intestine, (c) is a stereoscopic microscope image, (d) is an atmospheric SEM image, and (e) is a biological microscope image taken after the specimen was preserved in paraffin, sliced, and stained with H&E. The atmospheric SEM can obtain information about the fine structures on the surface of tissue without preprocessing.*

electron beam by its nature not passing through the specimen. In other words, it is a more sensitive way of observing surfaces than optical microscopy. This is why atmospheric SEM can view fine details that are not visible by a stereoscopic microscope that uses light [see Fig. 10 (d)]. The use of atmospheric SEMs to obtain information about fine details and identify suitable samples prior to preparations such as embedding the tissue in resin or slicing it has the potential to be a very useful technique for the examination of tissue samples.

## CONCLUSIONS

Hitachi High-Technologies found that an SEM image could be obtained from the non-scattered electrons even when the membrane and specimen are not in contact and atmospheric gas molecules are present in the electron beam path. Hitachi High-Technologies also developed the ES-Corrector algorithm for electron beam scattering correction that can remove the effect of electron beam scattering from atmospheric SEM images and demonstrated that this significantly enhanced atmospheric SEM images. By making it possible to view specimens under atmospheric pressure, which in the past could only be viewed in vacuum, SEM observations can now be made of solid water-containing specimens without preprocessing.

It is anticipated that this will contribute in the future to fields where little use has been made of SEMs in the past, such as food, cosmetics, pharmaceuticals, and medicine.

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