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Use of oolong tea extract staining of soft-tissue specimens in low-vacuum scanning electron microscope with a cooling stage

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Abstract For direct observation of the surface structures of soft-tissue specimens, we examined rat tracheal tissue in a low-vacuum scanning electron microscope (SEM) equipped with a cooling stage. In specimens fixed with glutaraldehyde and osmium tetroxide, back-scattered electron images of the surface structure could not be clearly observed in the low-vacuum SEM because of the disruption of fine structures and a low signal-to-noise (S/N) ratio. Processing of the specimens in 70% ethanol resulted in marked shrinkage, in contrast to results when processing in 30% ethanol. To overcome these problems, the trachea was initially fixed in 2.5% glutaraldehyde (0.1M phosphate buffer pH 7.4), treated with a mixture of 0.2% oolong tea extract (OTE) and 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide. The sample was immersed in 30% ethanol and examined in a chilled SEM at -10°C . The luminal contour of the tracheal epithelial cells was clearly observed because of the decrease in shrinkage. Cilia of ciliated cells and microvilli of nonciliated cells were also clearly observed. These specimens also showed a high S/N ratio, thus allowing the observation of samples without the need for complete dehydration, critical-point drying, or metal coating. This OTE-incorporated conductive staining method is simple and rapid, and should prove to be highly useful for rapid SEM analyses of biological specimens.

Key words Oolong tea extract staining · Trachea · Chilled scanning electron microscopy

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Introduction

For the observation of biological specimens with a conventional scanning electron microscope (SEM), the specimens are generally treated with a metal coating.¹ However, with a low-vacuum SEM, wet plants and insects can be directly observed without coating.^{2,3} A low-vacuum SEM, called a wet SEM or variable-pressure SEM, permits pressure in the specimen chamber to range from 1 to over 100Pa during operation, and creates images by detecting back-scattered electrons.^{2,4,5} The low-vacuum SEM has the advantage of being free of charge-related artifacts even in nonconductive materials. However, it has been difficult to demonstrate the surface structure of soft-tissue specimens, mainly because of the low electron density of the tissue components and because of tissue damage caused during sample preparation.

We present a new method for use with a low-vacuum SEM; this method, by incorporating staining with oolong tea extract (OTE), increases the signal-to-noise (S/N) ratio and prevents damage to the fine structure of soft-tissue specimens. In this study we used a low-vacuum SEM with a cooling stage (chilled SEM) in which specimens were cooled but were not completely frozen.

Materials and methods

The trachea obtained from a male Wistar rat (body weight, 200g) was cut into small pieces, about 2mm^3 in size, and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at room temperature (RT) for 10min. For the method reported here, the specimens were further fixed in the same glutaraldehyde solution containing 0.2% OTE (OTE powder; Suntory, Osaka, Japan) at RT for 2h, postfixed with 1% osmium tetroxide for 2h,⁶ rinsed in 0.1M phosphate buffer (pH 7.4), and dehydrated in 30% ethanol.

Control specimens were simply fixed in 2.5% glutaraldehyde at RT for 2h, postfixed in 1% osmium tetroxide for 2h, and dehydrated in either 30% or 70% ethanol.

The specimens were examined under variable pressures in an SEM (S-3000; Hitachi, Ibaraki, Japan) equipped with a cooling stage unit (Hitachi). The sample was first held on the cooling stage at -10°C , evaporated under a pressure of 10 Pa for several minutes in the specimen chamber, and observed in the SEM by the collection of back-scattered electrons. The operating condition was set at an accelerating voltage of 10 kV.

Results

In the direct observation of samples dehydrated in 70% ethanol without OTE staining, individual shapes of tracheal epithelial cells were detected because of shrinkage of the specimens. Surface structures, such as the microvilli of nonciliated cells, were disrupted and obscure. Cilia on ciliated cells were not clearly visible because of the fusion between cilia (Fig. 1a).

In the direct observation of samples dehydrated in 30% ethanol without OTE staining, the contour of each bronchial epithelial cell was well preserved (Fig. 1b), although microvilli were still obscure.

In samples dehydrated in 30% ethanol after OTE staining, the cell morphology and surface structures of each tracheal epithelial cell were clearly observed (Fig. 2a). Microvilli of nonciliated cells and their intracellular secretory granules were well preserved (Fig. 2b). Individual cilia in the tracheal epithelium were recognizable (Fig. 2a), although a cluster of cilia was compressed. The cilia had a diameter of about 180 nm (Fig. 2c). In addition, in specimens that were treated initially with OTE, the surfaces of the tracheal epithelial cells were covered with mucous materials.

Discussion

In the present study, we developed a new technique for direct observation of the surface structure of tissues, using a chilled SEM. Our findings indicate that the incorporation of OTE staining and dehydration in a lower concentration (30%) of ethanol was superior to conventional staining and dehydration in 70% ethanol for the preservation of the surface structure of the samples. These results suggested that hydrated specimens could be observed without the creation of considerable technical artifacts.

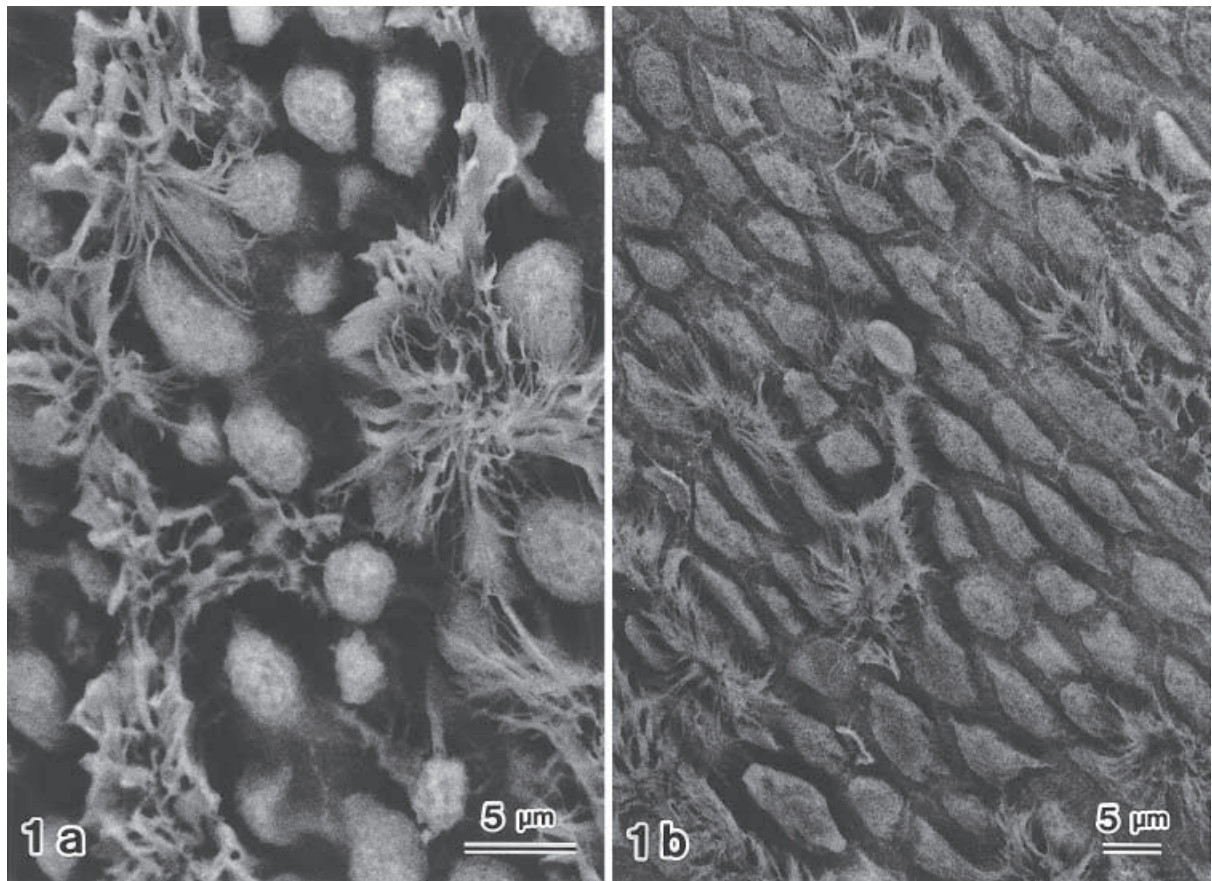


Fig. 1a,b. Rat trachea fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. Surface structures, such as microvilli, in nonciliated cells are not seen clearly. **a** Dehydration in 70% alcohol, and **b** dehydration in

30% alcohol. The contour of each individual tracheal epithelial cell is clearly recognized in **b**

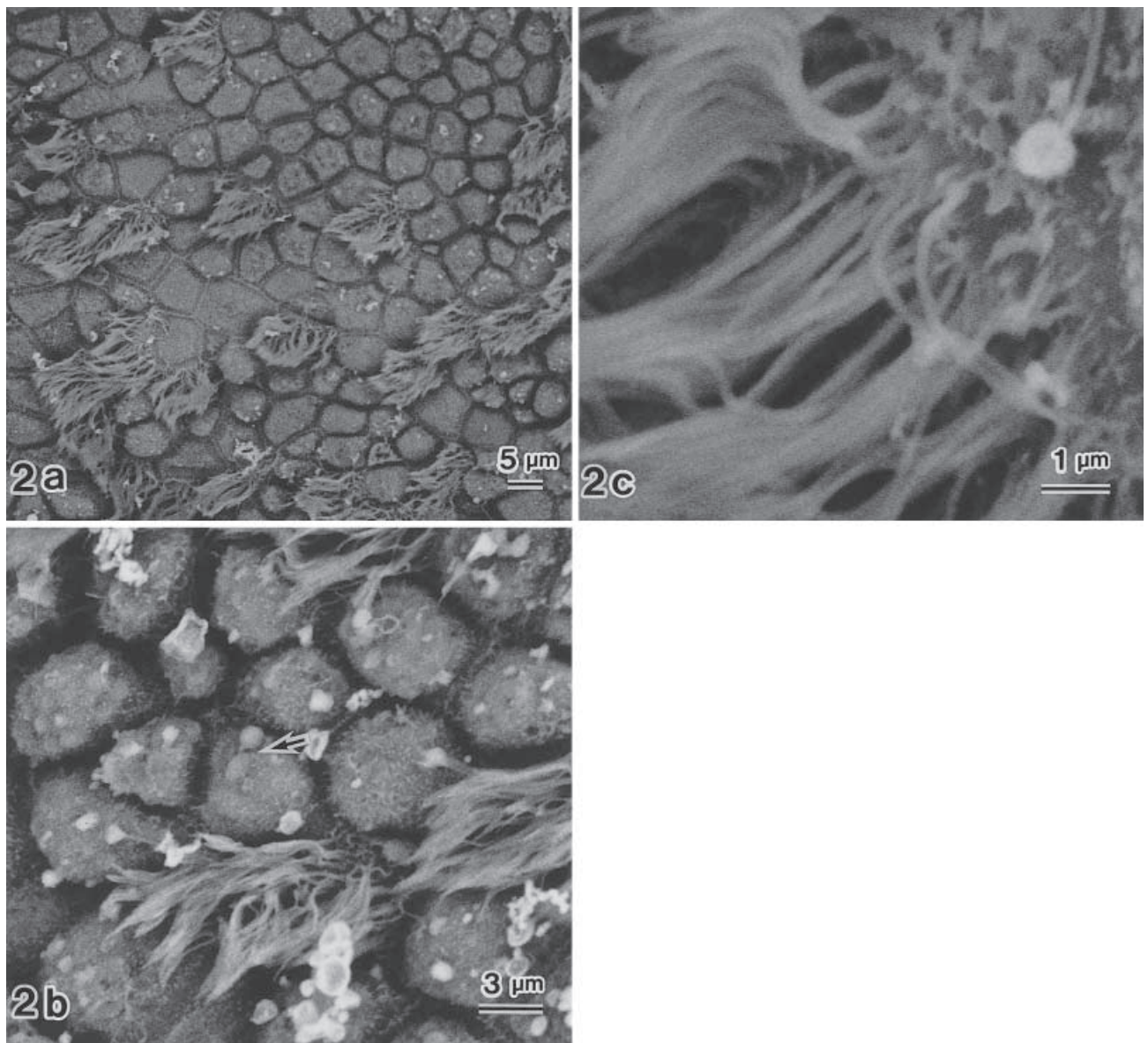


Fig. 2a-c. Rat trachea processed by staining with oolong tea extract and dehydration in 30% ethanol, as observed in a chilled scanning electron microscope at -10°C . **a** The morphology of each tracheal

epithelial cell is well preserved. **b** Microvilli of nonciliated cells are clearly observed. The outlines of granules inside nonciliated cells are also seen (*arrow*). **c** Cilia of ciliated cells are clearly visible (\O : 180 nm)

To observe the surface structure of soft-tissue specimens at high magnification, Murakami⁷ introduced a revised tannic acid-osmium method for staining and SEM observation. But, he did not employ this method for a low-vacuum SEM. Hashizume et al.⁸ examined frozen soft-tissue specimens fixed simply with glutaraldehyde and osmium on the cooling stage in a low-vacuum SEM, and succeeded in visualizing the surface structures. T-Butyl alcohol has been used as an organic solvent for freezing, as described previously.⁹⁻¹¹ However, the specimens had to be observed at accelerating voltages of 5–6 kV in a chamber pressure of 20 Pa, because they had a low S/N ratio. We found that specimens stained with OTE showed a high S/N ratio, thus allowing for observation at an accelerating voltage of 10 kV in a chamber pressure of 10 Pa, on a cooling stage at -10°C .

As shown in the present study, the S/N ratio was also low in the specimens fixed simply with glutaraldehyde and osmium tetroxide. These specimens also incurred severe damage during processing. However, we showed that these technical artifacts could be prevented using the new OTE staining method. OTE contains various polyphenols, similar to those in tannic acid. Oolong tea polyphenols in OTE have a lower molecular weight than those in tannic acid.¹² In addition, tannic acid can enter the cells, whereas OTE polyphenols do not enter the cells. It has also been suggested that tannic acid injures the cell membranes. A recent transmission electron microscope study revealed that, in block staining with OTE, lung epithelial surface films,¹³ cell membranes, collagen fibrils, and elastic fibers were stained, but the intercellular components were not stained.⁶

In conclusion, the incorporation of OTE staining allows soft-tissue specimens to be accurately observed in a chilled SEM without the creation of severe artifacts. The present method should prove to be useful for rapid SEM analyses of biological materials.

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