Dressing living organisms in a thin polymer membrane, the NanoSuit, for high-vacuum FE-SEM observation

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Abstract

Scanning electron microscopy (SEM) has made remarkable progress and has become an essential tool for observing biological materials at microscopic level. However, various complex procedures have precluded observation of living organisms to date. Here, a new method is presented by which living organisms can be observed by field emission (FE)-SEM. Using this method, active movements of living animals were observed in vacuo ($10^{-5}$–$10^{-7}$ Pa) by protecting them with a coating of thin polymer membrane, a NanoSuit, and it was found that the surface fine structure of living organisms is very different from that of traditionally fixed samples. After observation of mosquito larvae in the high vacuum of the FE-SEM, it was possible to rear them subsequently in normal culture conditions. This method will be useful for numerous applications, particularly for electron microscopic observations in the life sciences.

Key words: field emission scanning electron microscope, high vacuo, living organism, surface-shield-effect, NanoSuit

Introduction

Scanning electron microscopy (SEM) has made remarkable progress since its initial days [1] and has become an essential tool for observing the fine structure of biological materials [2]. Because the electron microscope uses a beam of electrons to illuminate the specimen, it is necessary to evacuate the specimen chamber in order to prevent scattering by molecules in the air. To preserve and stabilize structures for SEM, cells, tissues and soft-bodied organisms usually require chemical fixation, followed by thorough drying.
Furthermore, nonconductive materials cannot be imaged by conventional SEM, so samples usually require coating with an ultrathin layer of an electrical conductor [2]. These various procedures have precluded observation of living organisms.

A new method is presented here to observe living organisms by a field emission scanning electron microscope (FE-SEM). We have previously reported that a simple surface modification by electron beams or plasmas can equip some multicellular organisms with a thin extra layer, coined a ‘NanoSuit’, and hence can keep them alive under high-vacuum (10^{-5}–10^{-7} Pa) conditions of an FE-SEM [3]. In the present report, to examine further the role of the NanoSuit, various living specimens were simply protected by the NanoSuit during FE-SEM observations. From the success of this technique, it is anticipated that this could be the starting point for more sophisticated observation of living organisms with the electron microscope and for the creation of new areas of biology, chemistry and physics in order to explain how the thin polymer membrane forms a gas and/or liquid barrier based on the surface-shield-effect to preserve life in vacuo.

Materials

Experimental organisms

The larvae of a chironomid midge, Chironomus yoshimatsui, were collected from the mud of a rice paddy (34°45’N, 137°43’E) and then cultured in the muddy water brought from the same paddy. The fourth larval instar (ca. 4 mm in body length) was used in the present study. In order to exclude any effects of the water, they were transferred to distilled water at 24 ± 1°C for 2 days prior to the experiment, with distilled water changes every 12 h. The larvae were rinsed thoroughly in distilled water 1 h before experiments began.

Specimens of the amphipod sandhopper, Talitrus saltator, were collected on a sandy beach in southern Tuscany (42°46’N, 11°6’E; Albegna, Grosseto, Italy), transported to the laboratory in plastic boxes containing wet sand and maintained in an aquarium containing sand moistened with artificial seawater at 20 ± 1°C. They were fed weekly with dry fish food placed on blotting paper. The experimental animals were rinsed thoroughly in distilled water 1 h before experiments began.

Preparation of Tween 20 solutions and sample preparation for FE-SEM to observe living specimens

The amphiphilic surfactant compound polyoxyethylene (20) sorbitan monolaurate (Tween 20; Wako Pure Chemical Industries) was used for all the experiments to mimic natural extracellular substances (ECS) [3]. To form the nanosuit, the organisms were dipped into 1% (v/v) Tween 20 solution dissolved in distilled water for 1 min, blotted briefly on a dry filter paper to remove excess solution. Then, the living specimen was introduced into the SEM to construct a Tween 20 film, without performing any traditional treatments such as chemical fixation or dehydration. Twenty animals were used for each experiment.

Preparation for standard SEM

For standard SEM observation, animals were prefixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO4 in the same buffer. The specimens were then dehydrated, freeze dried (JFD300, JEOL) and ultrathin coated with OsO4 (PMC-5000, Meiwa).

Microscopy

For FE-SEM, a Hitachi S-4800 was used at an acceleration voltage of 5.0 kV. The vacuum level of the observation chamber was 10^{-5}–10^{-7} Pa. To record the dynamic movements of animals, imaging data from the SEM were directly transferred to a Hi-band digital formatted video recorder (SONY, BDZ-EW500).

Results

We have previously reported that a thin-film NanoSuit is formed when living organisms are covered with either a layer of natural ECS or an artificial substance that mimicks ECS (such as a 1% aqueous solution of Tween 20), subsequently polymerized by plasma or electron beam irradiation. With the assistance of the NanoSuit, organisms have the potential to tolerate high-vacuum environments without any electric charges [3]. The experiments reported here test a variety of living organisms in an FE-SEM to investigate the role of the NanoSuit.

Figure 1a shows the overall shape of a midge larva. When observed in the SEM sample chamber, slight shrinkage was already detectable (Fig. 1b) but the specimen showed slight movements and no apparent electrostatic charging. Subsequently, the larva quickly shrank along its entire length and all movements had ceased within 5 min (Fig. 1c). Charging commenced about 10 min after movements had ceased (Supplementary Movie 1). By comparison, midge larvae washed in distilled water and subsequently dipped in Tween 20 showed no observable change in their appearance when introduced into the FE-SEM, but remained alive and showed rapid movement (Fig. 1d and e; Supplementary Movie 2). Even after active movement ceased, the specimens showed no electrostatic charge for at least 1 h.
To investigate what happens to fine structure when an organism is covered with a Tween 20 NanoSuit, careful observations were made at high magnification (Fig. 2). With the conventional method, nonconductive specimens tend to be charged when scanned by the electron beam, especially at steep edges. In contrast, with the newly developed method, the tip of an antenna showed no electrostatic charge (Fig. 2a). Even when observed at high magnification, structural detail was clearly observed without any charging (Fig. 2b). No collapse was observed in any part of the body (Fig. 2c) and the large bore of the stigma located at the tail seemed to have been preserved completely (Fig. 2d). These results indicate that the NanoSuit maintains neatly ordered structures on the surface and greatly reduces electrostatic charging.

After SEM observation, control specimens treated in distilled water alone did not survive. However, when larvae protected by the NanoSuit were returned to the muddy water under atmospheric pressure, all the animals were found to be still alive and reinitiated movement within a few
minutes. After rearing these survivors for 3–4 days, around 70% of them pupated and developed successfully into adult mosquitoes (N = 25) (Fig. 3). These results indicate that the NanoSuit plays an important role in keeping the animals alive in the SEM, presumably because it acts as a barrier to gas and liquid release from the organism: a property that is called here the ‘surface-shield-effect’.

To investigate the ‘surface-shield-effect’ morphologically, a comparison was made between specimens treated by traditional methods (Fig. 4a–d) and those coated with a NanoSuit (Fig. 4e–h). The sandhopper *T. saltator* is abundant on sandy beaches around the Mediterranean and has been used in many scientific investigations, in particular concerning the mechanisms of orientation on land, in which evaporative water loss is an important issue for such a semi-terrestrial amphipod [4,5]. In the lateral view of the animal at low magnification, the outline of each scutum appears more clearly defined following preparation by traditional methods (Fig. 4a) compared with those coated by a NanoSuit (Fig. 4e), where the appearance is much smoother. Note also the presence of indentations indicating shrinkage in the head and anterior segments in Fig. 4a, whereas no such shrinkage is apparent in Fig. 4e. Considering the tip of the antenna at higher magnification, clear morphological differences are apparent. Each antennal segment has protrusions consisting of setae which are seen to be separated when prepared by traditional methods (Fig. 4b and c) but neatly straight and aligned following preparation with a NanoSuit (Fig. 4f and g). The tip of the antenna similarly has a much neater appearance when protected by a NanoSuit (Fig. 4h; cf. Fig. 4d).

![Fig. 3. Newly hatched adult mosquito developed from a larva 3 days after it had undergone SEM observation. Scale bar: 0.5 mm.](image)

![Fig. 4. SEMs comparing animals treated by traditional methods (a–d) and when protected by a NanoSuit (e–h). The tip of the antenna, indicated by a large square in (a and e), is enlarged in (b and f), respectively. Each segment of the antenna tip possesses long protrusions composed of three setae (arrows). These setae appear separated (b and c) using traditional methods, but show consistent neat alignment in specimens coated with a NanoSuit (f and g). The antenna tip (arrow heads in b and f) has a bundled structure which is splayed when viewed following traditional treatment (d), but is aligned straight when the specimen is protected by a NanoSuit (h). Scale bars: 0.2 mm (a and e), 20 µm (b and f), 10 µm (c and g) and 5 µm (d and h).](image)
The imaging system of the present SEM was designed for inactive materials, so only the ‘video mode (scan speed 10–15 frames/s)’ could be used to observe active movements of the animals (cf. movements of some animals shown in Supplementary Movies 3 and 4). However, observations at high magnification could be performed only when the living animals happened to stop moving (compare the blurred image in Fig. 5 with the stable image in Fig. 6). The thorax is very smooth with no holes in the cuticle in *T. saltator*, but the basiopod of the caudal pereiopods has many small depressions on the surface of the plate. The fine structure of these depressions in the plate of the sixth pereiopod was compared in traditional and NanoSuit preparations. In the former, many clear black holes were observed (Fig. 6a), associated with patches of white debris (Fig. 6b). A similar number of depressions were observed on the surface of the NanoSuit coated plates (Fig. 6c). However, the edges of these depressions are smooth (Fig. 6d) and there is no debris apparent, suggesting that the NanoSuit maintains the surface intact whereas traditional methods result in disruption of the surface by removing material associated with the depressions, separating them into well-defined ‘holes’ and ‘debris’.

**Discussion**

In order to observe living organisms by conventional SEM in high vacuum, there are two major problems to solve: how to produce a sufficiently thin barrier of gas and/or liquid through which to observe surface fine structure and how to increase the electric conductivity of the surface of the animal, without any toxicity or damage to the living tissues. Recent research to improve techniques such as low-vacuum SEM [6] and use of an environmental SEM (ESEM) have been developed [7]. However, although both are elegant techniques, low-vacuum SEMs allow semi-wet samples to be imaged without coating but are unable to attain the resolution of conventional SEMs. ESEMs, being wet or contained in low vacuum or gas, can also be used to observe specimens without coating and produce better images than a low-vacuum SEM. However, it is not reliable enough to investigate the movements of living organisms at this level of resolution. We previously found that, with the assistance of a polymerized thin-film NanoSuit, organisms possess the potential to tolerate high-vacuum environments without any electrical charges [3]. The advance that we have made is to preserve life in high vacuum long enough to observe active movements of living specimens and at good resolution. The improved clarity in fine structure seen with living specimens is due to a surface-shield-effect caused by the thin NanoSuit film on the surface of the animal *in vacuo*.

In the present experiments, in addition to the apparent barrier effect, no electrostatic charging was observed on any of the animals during their active movements. Animals treated in distilled water alone showed electrostatic charge build-up quickly after they ceased moving (Fig. 1b and c; Supplementary Movie 1). In contrast, charging commenced about an hour after movements ceased under NanoSuit protection. Traditionally, biological samples are coated with ultrathin electrical conductors such as gold, palladium, platinum or osmium, to prevent the accumulation of electrostatic...
charge at the surface [2]. The living specimens in the present study were observed at good resolution and with no electrostatic charging, which suggests that living organisms have their own electrical conductors and/or possess certain properties on the surface which inhibit charging. The NanoSuit seems somehow able to preserve life and to prolong the charge-free condition.

Until our discovery, most scientists believed that traditional viewing with the SEM represented the true appearance of the surface of living animals. However, the structures used as examples in Figures 4 and 6 showed many differences between the images of living and fixed specimens. These morphological differences apparently result from the presence of the NanoSuit on the surface of the living animal providing the surface-shield-effect. One intriguing apparent contradiction here is the appearance of well-separated setae in the traditional method (Fig. 4c and d), which seems to show more detail than in the NanoSuit condition where the setae adhere to each other (Fig. 4g and h), implying a more poorly resolved structure. However, the differences suggest that, in the method presented here, the natural surface structure of the living organism might be conserved.

At present, however, the NanoSuit component Tween 20 is not omnipotent. It is obvious that, in addition to the Tween 20 solution, much more suitable components must be found for each species to live longer in high vacuum. In a different study, this research group has proposed other possible candidates for forming a NanoSuit, and various monomer solutions were fabricated into thin films by plasma polymerization [8]. It is also necessary to investigate whether or not the surface-shield-effect is accomplished by a combination of different substances including unknown compounds which might be produced by the living animal itself. Nevertheless, it is apparent that the NanoSuit surface-shield-effect preserves the morphology of the living animal, decreasing the evaporation rate of gas and/or liquid and facilitating electrical conductivity in the electron beam. When the many intriguing aspects of the surface-shield-effect have been resolved, it is easy to predict that this may be the start of a new era of improvement in the depth of our understanding not only in biology but also in many other fields of science.

Concluding remarks

The NanoSuit plays an important role in keeping animals alive in the FE-SEM, thus permitting the use of high vacuum to reveal the fine structure of living specimens at high magnification (×500 000). Since the NanoSuit maintains the organism’s surface intact during high-resolution imaging, it will be a useful tool for all future work involving SEM observations on living organisms. Furthermore, the NanoSuit represents a simpler, less time-consuming procedure, and should be suitable for numerous applications, especially in the biological sciences.

Supplementary data

Supplementary data are available at http://jmicro.oxfordjournals.org/.

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References