Living Organisms under an Electron Microscope: 
the NanoSuit® Method aiming for 
Medical and Industrial Applications

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Scanning electron microscopy (SEM) has made a remarkable progress and is an essential 
tool for observing biological materials at microscopic level. However, various complex 
procedures have precluded observation of living organisms to date. Our recent discovery of a 
method coined the “NanoSuit®” is presented by which living organisms can be observed in 
a simple procedure by an SEM, which acts as a barrier to the passage of gases and liquids 
and thus protects the organism. Based on this discovery, we invented the coating method by 
the “biomimetic extracellular substances (ECS)” composed of biocompatible polymers for the 
organism which lack the natural ECS. The new “NanoSuit®” methods will be useful for medical and industrial applications.

Keywords: NanoSuit®, Nano-polymer, Electron microscope, Thin membrane, Morphology, Subcellular size, Biomimetics

1. Introduction

There is a proverb “Necessity is the mother of Invention”, but the “Scientific Desires” may also be 
a mother of invention. The light microscope was invented in the 17th century, and in the field of life 
sciences, the "cell theory" was raised, and many revolutionary scientific advances were progressed. However, the “Scientific Desires” are not limited with optical microscopes, but scientists have developed electron microscopes for higher resolution. The invention of the electron microscope by Max Knoll and Ernst Ruska in 1931 overcame the barrier to higher resolution that had been imposed by the limitations of optical microscopes. Comparing with the maximum resolving power of an optical microscope which is limited to about half the wavelength (ca. 300 nm), the accelerated electrons in an electron microscope are suitable to scrutinize the fine structures down to the 1 nm range. Ernst Ruska produced the first commercial electron microscope in the world in 1938 at Siemens, and he won a Nobel Prize in Physics in 1986. In Japan scientists gathered to decide to build an electron microscope, and the team of this group evolved into the Japan Electron Optics Laboratory (JEOL). Hitachi and Toshiba in Japan also played a major role in the early development process.

Since the development of the electron microscope, Helmut Ruska who was the brother of Ernst Ruska tried to visualize several submicroscopic biological structures, such as bacteria, parasites and different viruses [1]. Pease and Nixon started to combine several improvements of SEM in one instruments, and this was the prototype for the first commercial SEM called “Stereoscan” in 1965 [2]. Soon after the development, Pease et al. observed several stages of a living insect in an SEM (1966) without any pretreatment, and in most cases the specimens resumed their normal activity after being observed with SEM and underwent metamorphosis into the next stage [3].

Nowadays, electron microscopes are invaluable tools for the study of biological samples. Without an electron microscope, we would not have the current knowledge about the living things. Those samples
are placed, however, in a high vacuum condition, because electron microscopes use a beam of electrons instead of beams or rays of light. “Scientific Desires” which want to observe biological samples with high resolution equipment, has led to the development of techniques that can manage biological samples in high vacuum. Biological specimens are chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning for transmission electron microscope (TEM), and dehydrated specimens are coated with metal by sputter coaters to yield good contrast in a scanning electron microscope (SEM) [4]. These complex procedures preclude the observation of living organisms and often produce unwanted artifacts. Dehydration always results in shrinkage, and freeze drying (FD) and critical point drying (CPD) also give rise to shrinkage [5,6]. Therefore, researchers have tried to modify the SEM design to require lower levels of vacuum to circumvent such problems [7-10]. However, all these microscopes such as low-vacuum SEM or environmental SEM require reduced vacuums (<10⁻³ Pa) and result in markedly inferior resolution.

In spite of the former challenging experiments performed by Ruska and Pease et al., it has been thought that we cannot observe any living things using an electron microscope. We recently found an important method to cover organism with nano-thin film, coined “NanoSuit®” which could keep their structure with wet/alive condition in high vacuum. Here, we will summarize the recent experiments of the “NanoSuit®”.

2. Brief history of “NanoSuit®”

A biofilm can be said to be a familiar example of the slime that can be gathered in the kitchen sink and a plaque of teeth. Biofilms comprise any syntrophic consortium of bacteria in which cells stick to each other. In the medical field, the formation of a biofilm by microorganism, such as Staphylococcus aureus in the catheter is a major problem. This is because bacteria wrapped in a biofilm acquire resistance to antibiotics and immunity in the catheter. Among ecosystems, biofilms are also observed everywhere and play an important role in maintaining the ecosystem. Bacteria possesses a cell wall on the outside of the cell membrane, and a biofilm is formed on the outside.

On the other hand, many of eukaryotes release secretions, like the biofilm, to the outside of cells to protect individual cells. We, human beings also secrete outside the skin to enhance the moisturizing effect. Extremely abundant exocrine secretions are found in the larvae of Dipteran. The larvae produce secretions outside the body and the extracellular substances (ECS) of secretions maintains its external environment. Thus many organisms are able to withstand environmental changes by those secreted biofilms and/or ECS. Organisms might have acquired those drought tolerances in evolution.

In order to find the nature of tolerance of living organisms in high vacuum condition, we introduced several living organism of various taxa directly into an SEM to see whether they could survive under high vacuum conditions. We found that the larvae of the fruit fly Drosophila melanogaster (Oregon-R) were tolerated in high vacuum condition, when they were irradiated by electron beam immediately in an SEM. Although the larvae possess a soft cuticle, they continued to move actively around 60 min in high vacuum condition. The result led to the hypothesis that electron-beam enhanced cross-linking within the ECS to form a durable polymer on the surface and that this polymer increased resistance to vacuum conditions. This finding could shed a light on the new observation technique for
various living/wet organic samples with a high resolution SEM [11].

From a biomimetic point of view, solutions containing nontoxic amphiphilic molecules, polysorbate 20 (Tween 20, TW20) as main chemical substance (NanoSuit® solution) were tested in an attempt to mimic the ECS layer. To test the barrier properties of the NanoSuit® membrane made by this solution, the surfaces of several different animals previously unable to survive SEM exposure were provided exogenous materials by immersing them briefly in the solution before electron or plasma irradiation. When live larvae of mosquito were observed under the SEM without any additional treatment, they quickly shrank and ceased to move. Larvae treated with a nontoxic amphiphilic solution but not irradiated in the SEM showed the same collapsed structure when observed 30 min later. However, larvae covered with nontoxic amphiphilic solution and observed by SEM ab initio retained their morphology and exhibited active movements for 30 min. The plasma irradiation for the before introduced them into an SEM also showed the same results (Fig. 1) [11,12].

3. A petal of cherry blossom

As described above, polymerization of a natural ECS on the outer surface of Dipteran by electron beam or plasma irradiation, can give rise to the NanoSuit®, which can keep small animals alive under the high vacuum of an SEM. We examined the ability of plants whether they have any natural ECS, which can make the NanoSuit® tolerant against high vacuum condition. Using petals of cherry blossoms as experimental specimens and examined their behavior under high vacuum conditions (Fig. 2). Experiments on healthy living petals have demonstrated that without any pretreatment, the overall morphology of specimens is well preserved and intact after imaging in an SEM, suggesting that natural substances on the petal surface behave like animal ECS and form a NanoSuit® following irradiation with an electron beam. Furthermore, we have shown that the surface material can be extracted with chloroform and polymerized into a free-standing membrane by plasma irradiation. From our results, we conclude that surface materials, which have the ability to prevent water loss under natural conditions, increase the barrier ability and can protect plants under high vacuum conditions [13]. Because plants have evolved from the sea to the land, they must develop surface materials to protect themselves from environmental stresses.

![Fig. 2. Observations of petals of a cherry blossom by light microscopy (LM) (A, E), an SEM (B, C, F, G) and a TEM (D, H). The rectangles in B and F indicate the position of images magnified in C and G, respectively. A-D: No treatment (freshly collected) petal, E-H: Gentry immersed in chloroform solution to remove the surface wax of petal. Comparing with the no treatment specimens (B, C), the specimens treated with chloroform (F, G) showed that the cells in petals shrank and collapsed, and showed electrostatic charging during SEM observation (G). D, H: TEM images of cross sections through petals, no treatment petal with electron beam irradiation (D), and treated with chloroform and electron beam irradiation (H). Those specimens were then fixed and sectioned for TEM observation. The layer between the arrowheads in (D) indicates the newly formed NanoSuit® membrane.]

4. The tissue observation for pathological diagnosis

As described above, we used the natural ECS substances or TW 20 based NanoSuit® solution to form the NanoSuit® membrane on the living organisms, which of the outer surface was covered with epithelium or cuticle [11-14]. By contrast, excised tissues or single cells do not have such a protective cover, so that an alternative barrier was needed. We used a surface shield enhancer (SSE) solution with glycerin as a main component. Since glycerin is strongly hygroscopic, it has been used as a humectant in cosmetics [15]. To increase the barrier effect, we have combined glycerin with electrolytes and polymerized a thin liquid film over the sample in order to prepare a different type of NanoSuit®. In some cases, this treatment yielded an effective NanoSuit® membrane and permitted imaging in the SEM. The clear surface structure of pathological tissue (Fig. 3) and cell (Fig. 4) are observable. The SSE constitutes a very effective diffusion barrier and it is very thin (less than 10 nm) [16]. The SSE based surface shield is much thinner...
than the TW 20-based NanoSuit membrane (50–200 nm) that we previously developed [13]. As a consequence, it is possible to image surface structures at much higher resolution. It seems likely that it interacts with protein and/or proteoglycan on the surface of cells and tissues to form a stabilizing polymer coat after plasma irradiation or exposure to the electron beam in an SEM [16]. There have been several previous attempts to adapt SEM for the observation of wet samples. Thiberge et al. used polyimide or silicon nitride membranes to protect the sample from the vacuum [17]. However, this method required the use of high acceleration voltages (15–30 kV) to penetrate the relatively thick membranes. The intense radiation of the electron beam during high magnification imaging was sufficient to cause damage to the specimens. The SSE-based NanoSuit® method can be applied to both living specimens and to fixed tissue and requires only use of low voltage electrons. Imaging occurs in a hydrous/wet state closely approximating the natural condition.

Scheme 1. Workflow for CLEM analysis of paraffin sections with the NanoSuit® method. ① Standard slide glass for light microscope with cover glass. ② Remove the cover glass. ③ Apply electronic coloring dye such as 0.5% gold chloride to understand the localization of specific organelle. ④ Apply NanoSuit® solution to stabilize the specimen for SEM observation. ⑤ Observe by an SEM at the same position observed by a light microscope. ⑥ Re-stain and encapsulate with mounting medium and a cover glass.

5. A novel histological approach for examining paraffin sections in a nondestructive manner by correlative light and electron microscopy (CLEM)

Histological examination using the light microscopy is currently the gold standard for life science research and diagnostics. However, magnified observations are limited because of the limitations intrinsic to light microscopy. Thus, a dual approach, known as correlative light and electron microscopy (CLEM), has emerged. Because there is the enormous accumulation of research using light microscopes, it is hoped that new research will be built on or compared with those data obtained by light microscope. We applied the NanoSuit® method to CLEM analysis of paraffin sections. Workflow is very simple; first we use the light microscope to determine the position where we need to observe by high magnification. After light microscopic photos are taken, the cover glass is removed immersing Xylene solution. And then, just
add NanoSuit solution and make thinner layer using spin coating and observe by an SEM. Removal of the SSE solution after observation is a further advantage, as this allows slides to be re-stained and stored (Scheme 1). Thus, the NanoSuit method represents a novel approach that will advance the field of histology [18].

Figures 5 and 6 show examples of pathological specimen observed by NanoSuit®-CLEM method. This method helps easily to observe the same position by light and electron microscopes. Therefore, the researchers can use excellent points both of light and electron microscopes, as it were, this method can take advantage of excellent things and make up for bad ones. The light microscope can observe the stained target organelle, but only flat image and the magnification is only by 1000 times, however, the SEM can observe more than 10,000 times in 3D image, but the history of staining technique is short. Now we can combine those microscopes easily using NanoSuit®-CLEM method.

Fig. 5. Cervical intraepithelial neoplasia (CIN1) case. A: Immunostaining with anti-HPV L1 antibody was performed on paraffin sections of CIN1 cases, and observed by diaminobenzidine (DAB) coloring using light microscope. B: The same position indicated by red circle in A was observed by an SEM using SSE-NanoSuit® CLEM method. The distribution of nuclear gold particles (indicated by white arrows) was observed by an SEM using a secondary antibody (biotin-labeled anti-mouse antibody) with avidin attached to 40 nm gold particles.

Fig. 6. Laryngeal papillomatosis tested positive for HPV 6. The specimen was applied 0.5% gold chloride, and observed by NanoSuit®-CLEM method. The brawn spot in the red circle using light microscope (A) was observed as brighter area in an SEM (B), because of the treatment of gold chloride (white arrow).

6. Energy-dispersive X-ray spectrometry (EDX) analysis of paraffin sections

Scanning electron microscopy combined with energy-dispersive X-ray spectrometry (EDX) is a spectral technique that provides visual identification of multiple elements simultaneously. To analyze the elemental components of paraffin sections by SEM/EDX, the procedure described in scheme 1 is applicable to observe identical H&E specimens. SEM/EDX analysis can reveal the existence of multiple elements such as Al, Si, Mg, O, and even C [18,19].

The NanoSuit®-CLEM observation method of paraffin sections has various applications. In the nearest future, it is expected that many new discoveries will be added by adding three-dimensional high-resolution information and elemental analysis by SEM/EDX as diagnostic information combining with the data of optical microscopes with a history of more than 100 years. The spread of CLEM observations by the NanoSuit® method will generate new knowledge and may be developed as new pathological
7. Industrial application of NanoSuit® for biomimetics

The NanoSuit® membrane for an electron microscope described above can form a free-standing film. A self-standing film can be formed along the substrate and adheres it, because it can be formed from a solution. The film is a pinhole-free and can prevent drying and prevent invasion of bacteria. Using this simple fabrication method of self-standing membrane, the applications such as food protection will be invented in various industries.

8. Conclusion

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. The “NanoSuit®” is presented by which living organisms can be observed by an SEM. A simple surface modification to extracellular substances (ECS) of certain multicellular organisms by electron beams or plasmas can coat of thin polymer membrane made of ECS, and acts as a barrier to the passage of gases and liquids and thus protects the organism. The new “NanoSuit®” methods will be useful for numerous applications, particularly in medical and industrial applications. We would like to develop new applications continuously with our glowing “Scientific Desires”.

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