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Reprint of: Atmospheric scanning electron microscope observes cells and tissues in open medium through silicon nitride film ☆,☆☆

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ABSTRACT

Direct observation of subcellular structures and their characterization is essential for understanding their physiological functions. To observe them in open environment, we have developed an inverted scanning electron microscope with a detachable, open-culture dish, capable of 8 nm resolution, and combined with a fluorescence microscope quasi-simultaneously observing the same area from the top. For scanning electron microscopy from the bottom, a silicon nitride film window in the base of the dish maintains a vacuum between electron gun and open sample dish while allowing electrons to pass through. Electrons are backscattered from the sample and captured by a detector under the dish. Cells cultured on the open dish can be externally manipulated under optical microscopy, fixed, and observed using scanning electron microscopy. Once fine structures have been revealed by scanning electron microscopy, their component proteins may be identified by comparison with separately prepared fluorescence-labeled optical microscopic images of the candidate proteins, with their heavy-metal-labeled or stained ASEM images. Furthermore, cell nuclei in a tissue block stained with platinum-blue were successfully observed without thin-sectioning, which suggests the applicability of this inverted scanning electron microscope to cancer diagnosis. This microscope visualizes mesoscopic-scale structures, and is also applicable to non-bioscience fields including polymer chemistry.

1. Introduction

Direct observation of subcellular structures such as protein complexes and organelles is essential for understanding their physiological functions. Of the various microscopy techniques, Optical microscopy (OM) observes macro-organelles or very large molecular complexes. To increase the contrast of low-density structures, various staining techniques for the OM, including hematoxylin, eosin, and 4′,6-diamidino-2-phenylindole (DAPI) have been developed (Tanious et al., 1992). Using fluorescent labeling and other techniques, fluorescence microscopy can visualize and determine the localization of small proteins. However, light wavelength and other factors restrict the resolution of diffraction-limited OM to approximately 200 nm. Super-resolution OM such as STimulated Emission Depletion (STED) (Donnert et al., 2006; Hell and Wichmann, 1994), Saturated Structured Illumination Microscopy (SSIM) (Gustafsson, 2005), or REversible Saturable Optically Linear Fluorescence Transition (RESOLFT) microscopy (Hofmann et al., 2005) goes beyond this. The 'fitting with point-spread' function (including the PhotoActivated Localization Microscopy (PALM) (Betzig et al., 2006) and STochastic Optical Reconstruction Microscopy (STORM) (Rust et al., 2006) methods) also locates objects with higher accuracy. However, these techniques are limited to information from the fluorescent labeling, and resolution is still insufficient for the observation of fine intracellular structures or small pathogens. High-throughput observation with much higher resolution would greatly contribute to biology and medicine.

Electron microscopy (EM) has been widely used for the observation of subcellular structures. It can achieve sub-nanometer resolution, but the electron pathway should be under vacuum so as not to scatter electrons. This necessitates the pretreatment of biological samples for preservation in extremely low-pressure conditions. In one method, samples are frozen and coated with heavy metal, precluding direct observation of internal structures. In the Epon-thin-section method for the Transmission Electron Microscope

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(TEM), samples are fixed, dehydrated, resin-embedded, and thin-sectioned, followed by staining. Of these steps, hydrophobic treatments, including dehydration and resin-embedding, may be especially damaging to the delicate structures in cells. Various reagents have been developed for staining, such as uranyl acetate, lead citrate, phosphotungstic acid, and the recently-developed platinum-blue (Inaga et al., 2007). However, their variety is still limited compared with the enormous number of options for the OM.

Cryo-tomography is a superb method visualizing intact intracellular three-dimensional (3D) architecture at 4–5 nm resolution using the TEM (Leis et al., 2009; Medalia et al., 2002). However, the cryo-sectioning which is sometimes required for this method is generally inappropriate for mass research such as drug screening.

X-ray and electron crystallography have determined not only the structures of proteins, but also the structures of their conformers or complexes, at a resolution level of a few angstroms (Fujiyoshi, 1998; Henderson, 2004; McPherson, 1989). Single particle reconstruction using the TEM, which can be categorized into *in silico* crystallization (Frank, 2006; Rosenthal and Henderson, 2003; Sato et al., 2001) does not require crystallization, though the resolution is inferior to crystallography. However, these methods all require purified protein, at the least, which decreases the number of applicable proteins and also particularly slows throughput. Moreover, these methodologies are frequently inappropriate for molecular complexes of over 20 nm due to the difficulties in purification and/or crystallization, while optical microscopy is challenging at resolutions under 200 nm. Until now, the mesoscopic-scale range from 20 to 200 nm has not been easy to access, requiring a new, whole-cell microscopy. X-ray tomography addresses this resolution gap (Parkinson et al., 2008), having a resolution comparable with STED and SSIM. However, this method typically uses a beamline for the X-ray source.

TEM observation of wet samples is accomplished using Abrams' semi-closed unit, holding atmospheric or aqueous samples between two electron-transparent films made of collodion (Abrams and McBrain, 1944), or using the carbon film alternatives (Fukami et al., 1990; Fukushima et al., 1985). Since saturated vapor pressure (for example, 100 Pa) or atmospheric pressure is maintained inside this "environmental cell", the components of cell extracts can be observed at high resolution in their hydrated state (Butler and Hale, 1981; Fullam, 1972; Parsons, 1986; Swift and Brown, 1970). Dynamic liquid hydrogenation and polymerization reactions in the manufacture of polyamides are also imaged using the Environmental cell-TEM (Gai, 2002). Similarly, whole bacteria cells in water vapor were successfully observed (Daulton et al., 2001). The Scanning TEM (STEM) visualizes colloidal gold-labeled epidermal growth factors (EGFs) bound on the EGF receptors of fibroblast cells in an environmental capsule (Jonge et al., 2009).

The Scanning Electron Microscope (SEM), on the other hand, scans the surface of the sample with a narrow, high-energy electron beam. Backscattered or secondary electrons are detected for imaging, so the surface of the sample is observed regardless of its thickness (McMullan, 1953; Reimer, 1998; Zworykin et al., 1942). This unique characteristic has allowed development of the SEM in two different directions: the environmental SEM (Danilatos, 1981, 1991; Robinson, 1975) and the environmental cell (Thiberge et al., 2004a,b). The environmental SEM allows wet samples to be imaged in a low-pressure atmosphere of the 1000 Pa-order (1/100 atm) using differential pumping and gaseous electron detector technologies. This technique is only successful when the sample is surrounded by a very thin layer of water, but maintaining stable hydration of the sample is difficult due to evaporation. The second approach is an environmental capsule sealed with an electron-permeable polyimide film. Fully hydrated cells and tissues inside the capsule are observed using the SEM (Thiberge et al., 2004a,b). The volume of the capsule is, however, limited to only 15 μl, and the capsule is sealed. These characteristics prevent outside manipulation of cells or external administration of drugs. Further, the polyimide film used in this system, typically 145 nm in thickness, scatters electrons and reduces resolution.

Recently, very thin Silicon Nitride (SiN) films have been produced in the semiconductor fabrication process, and have been found to be almost electron-transparent (Green and Kino, 1991). Refining the process, we succeeded in manufacturing very thin, flat SiN film, 30 nm in thickness. This film allows good resolution while still sustaining a 1-atm pressure differential. Using it as an electron-permeable window in a specialized culture dish, we designed an inverted Atmospheric SEM (ASEM) with an open sample holder. This enables outside manipulation of cells, and allows optical microscopy to be tightly integrated with electron microscopy.

2. Materials and methods

2.1. Manufacture of electron-transparent film

The film must be thin enough to be electron-transparent and tough enough for stability against a 1-atm pressure differential. To achieve both of these goals, a thin layer of SiN (10, 30, 100 or 150 nm in thickness) was deposited on a flat Si substrate using standard Chemical Vapor Deposition (CVD) (Hwang et al., 1982). The Si substrate was then partly removed using wet etching (Hsu, 2008; Sze, 1998, 2001) to create a SiN window (Fig. 1A, right). 30 nm SiN film with *xy*-dimensions of 0.1 × 0.1 mm was used for the ASEM presented here. Even with *xy*-dimensions of 0.3 × 0.3 mm, the 30 nm SiN film has proved resistant to a pressure differential of as much as 3 atm. To check the pressure resistance of single-windowed ASEM dishes with various membrane thicknesses, their seals can be easily monitored by means of a sensor-equipped vacuum pump. Using this method, we tested and used more than 1000 ASEM dishes without any leakage.

2.2. Construction of the ASEM

The SiN-windowed ASEM dish was developed to hold samples in an open system (Fig. 1A–C). The window is built into the base of a standard plastic Petri dish 35 mm in diameter, with a maximum capacity of almost 10 ml. It is detachable from the microscope so that cells can be cultured as usual in a CO_2 incubator.

From below the ASEM dish holder, the newly designed inverted SEM (base machine, JSM-6490, JEOL Ltd.) projects an electron beam up through the SiN film to the sample. Backscattered electrons are captured for ASEM imaging by the disk-shaped Backscattered Electron Imaging (BEI) detector located just beneath the SiN film, encircling the electron beam. The full specifications of the ASEM (Fig. 1D) are given in another paper. As part of the imaging process, an optical microscope (BXFM, Olympus Co.) views the sample quasi-concurrently from above. The axes of both microscopes are mechanically aligned and fixed so that both fields of view are simultaneously shifted by a single two-dimensional movement of the specimen stage. The ASEM system, including the software, is named ClairScope (JASM-6200, JEOL Ltd.).

2.3. Cell culture, gold labeling and staining

Because SiN is in general a good substrate for cells, African green-monkey kidney fibroblast COS7 cells were cultured directly on the SiN film (30 or 100 nm) of the ASEM dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml kanamycin in 5% CO_2 atmosphere at 37 °C. The cells were fixed with 1% glutaraldehyde in