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Atmospheric scanning electron microscope system with an open sample chamber: Configuration and applications

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ABSTRACT

An atmospheric scanning electron microscope (ASEM) with an open sample chamber and optical microscope (OM) is described and recent developments are reported. In this ClairScope system, the base of the open sample dish is sealed to the top of the inverted SEM column, allowing the liquid-immersed sample to be observed by OM from above and by SEM from below. The optical axes of the two microscopes are aligned, ensuring that the same sample areas are imaged to realize quasi-simultaneous correlative microscopy in solution. For example, the cathodoluminescence of ZnO particles was directly demonstrated. The improved system has (i) a fully motorized sample stage, (ii) a column protection system in the case of accidental window breakage, and (iii) an OM/SEM operation system controlled by a graphical user interface. The open sample chamber allows the external administration of reagents during sample observation. We monitored the influence of added NaCl on the random motion of silica particles in liquid. Further, using fluorescence as a transfection marker, the effect of small interfering RNA-mediated knockdown of endogenous Varp on Tyrp1 trafficking in melanocytes was examined. A temperature-regulated titanium ASEM dish allowed the dynamic observation of colloidal silver nanoparticles as they were heated to 240 °C and sintered.

Abbreviations: ASEM, atmospheric scanning electron microscope/microscopy; BEI, backscattered-electron imaging; EC, environmental cell; EM, electron microscope/microscopy; ER, endoplasmic reticulum; ESEM, environmental scanning electron microscope/microscopy; ETEM, environmental transmission electron microscope/microscopy; FOV, field of view; GUI, graphical user interface; MEMS, micro-electro-mechanical system; mStr, monomeric Strawberry; ND, neutral density; OM, optical microscope/microscopy; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; ROI, regions of interest; SEM, scanning electron microscope/microscopy; shRNA, small hairpin RNA; TEM, transmission electron microscope/microscopy; Tyrp1, tyrosinase-related protein 1; Varp, VPS9-ankyrin-repeat protein

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1. Introduction

The step from optical microscopy (OM) to electron microscopy (EM) required to image fine structures at higher resolution can have drastic consequences for the sample, especially if this is of biological origin. A series of more or less invasive methods is generally required to prepare the sample for the vacuum of an electron microscope (EM); imaging at ambient pressure is generally not possible. These time consuming and laborious pretreatments are necessary for cells, tissues, and all materials containing liquid. They include dehydration, drying and resin embedding, all of which might affect delicate structures. Moreover, the observation of dynamic phenomena in gas or liquid is not possible, and snapshots of these processes are difficult to obtain. Many attempts have been made to overcome these difficulties.

Already in 1944, Abrams and McBain [1] developed a capsule with two electron-beam transparent films between which a gaseous or aqueous sample could be placed for transmission EM (TEM). Such closed capsules, now known as environmental cells (ECs), have been developed further [2–4] and were recently combined with micro-electro-mechanical system (MEMS) technology [5–8], significantly improving the observation of samples in liquid [5,7,8]. A thin film has also been adapted for scanning EM (SEM) [4,9,10]. Although some ECs are semi-closed with inlet- and outlet-tubes, the capsule configuration generally tends to preclude the external administration of reagents during the experiment. The capsules are also very small, only holding up to 20 μl of liquid. This size and/or volume restricts the cell species that can be cultured within them. The limited volume also makes it hard to observe phenomena accompanying volume change, i.e., involving gas generation and liquid evaporation.

Pressure-limiting apertures have been developed to allow the observation of samples in a low pressure gas by TEM [2,11–13] and SEM [14]. These methods are called environmental TEM (ETEM) and environmental SEM (ESEM), respectively. They are applied to liquid samples or cells in aqueous solution [2,15–17]. However, great care has to be taken to ensure that the samples do not become much more concentrated (particularly important for the observation of chemical reactions) or dry out (particularly important for biological material) while being imaged in this low-pressure environment.

The atmospheric SEM (ASEM) [18–24] was developed to overcome the disadvantages of ECs and the environmental EMs. The large sample dish of the ASEM (ASEM dish) is open to the atmosphere and separated from the vacuum of the inverted SEM by an electron-transparent, 100 nm-thick, SiN film window. The dish can hold up to 3-ml of liquid, facilitating cell culture. In the commercial instrument, the ClairScope™ (JEOL, Ltd.), an optical microscope (OM) is positioned in the open space above the ASEM dish to allow correlative microscopy; the axes of the two microscopes are mechanically aligned so that both fields of view (FOVs) correspond and are simultaneously shifted by a single two-dimensional movement of the specimen stage.

The ClairScope system and important additions and changes made to the ASEM hardware are reported in this paper, together with basic designs of the ASEM and ASEM dish. In particular the column protection system, recently developed to improve instrument reliability, is described. Further, operation of the ASEM specimen stage is now fully motorized, and not only the inverted SEM, but also stage positioning and OM operation are controlled via a graphical user interface (GUI). The following applications are also reported: the use of correlative OM/SEM microscopy to observe the cathodoluminescence of ZnO particles in water, to study the endoplasmic reticulum (ER) of COS7 cells, and to study the small hairpin RNA (shRNA)-induced suppression of the expression of a specific gene in melanocytes; the use of ASEM to follow the drying and sintering processes occurring in a silver paste and the effect of salt addition on the motion of colloidal silica particles. The possibility of following processes in real time in situ opens new fields of application for the ASEM.

2. Materials and methods

2.1. Basic configuration of ASEM and the ClairScope

A ClairScope™, JASM-6200 (JEOL, Ltd.; Fig. 1A and B) was employed for the experimental work. This instrument is equipped with an ASEM and an OM and has all the new features detailed in Section 2.2.

The principle and basic design of the ClairScope ASEM is briefly described in [18]. The instrument has an inverted SEM column; the electron gun is set at the bottom and a specialized sample dish (ASEM dish) at the top (Fig. 1C and D). The detachable, 35 mm wide and 13 mm high dish is made of polystyrene and has a hole at the center of its base that is closed by a SiN-windowed Si chip (Fig. 2A–E). Except for this window (Fig. 2D and E), the ASEM dish is essentially the same (material and size) as the polystyrene Petri dishes used for cell culture. MEMS technology [18] was used to make the SiN window; a thin layer of SiN was first deposited on a flat Si wafer using standard chemical vapor deposition. The Si substrate (Si chip) was then partly removed by wet etching (Fig. 2D) to make a 100 nm-thick window (0.25 mm × 0.25 mm). Afterwards, the windowed chip (4 mm × 4 mm) was positioned over the hole in the base of the ASEM dish and glued in place (Fig. 2A–C). The outersurface of the base of the plastic dish had previously been coated with aluminum to prevent charging during SEM imaging.

An alternative heated, temperature controlled ASEM dish has also been developed. It was fabricated by building a ring-shaped ceramic heater and a thermocouple into the body of a titanium dish with a central hole in its base (Fig. 2F). It was briefly reported by Suga et al. in 2011; only a vertical cut-away-view was schematically shown (see [20, Fig. 3C]). The SiN-windowed Si chip is positioned over the central hole and glued in place as above.

The combined ASEM and an OM system of the ClairScope™, JASM-6200 (Fig. 1A and B) allows a sample placed on the SiN window of the ASEM dish to be studied by OM/ASEM correlative microscopy. The inverted SEM projects an electron beam (at an acceleration voltage of 10, 20 or 30 kV) up thorough the film to the sample, and backscattered electrons emitted from the sample are captured by a disk-shaped, backscattered-electron imaging (BEI) detector located just below the film (Fig. 1D). The OM positioned above the ASEM dish and mechanically aligned with the axis of the inverted SEM (see below) allows corresponding lower magnification images to be recorded.

2.2. Improvement of the ASEM

The following new features were developed to make the ClairScope more robust and facilitate correlative OM/ASEM imaging.

2.2.1. Column protection system in the case of accidental SiN film breakage

The ASEM sample is at atmospheric pressure and separated from the vacuum of the inverted SEM by a 0.25 mm × 0.25 mm, 100 nm-thick SiN window. A 1 mm × 1 mm, 100-nm thick, SiN film window test-fabricated to determine the pressure-endurance, i.e., a window 16 times larger than the standard ASEM window, withstands a pressure differential of more than 1 atm and, as expected, this resistance decreases as the size of the window