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CLEM of Neurons, Tissues and Biofilms immersed in Liquid using The Atmospheric Scanning Electron Microscope (ASEM): Dual Gold-Labeling

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Environmental SEM (ESEM) and environmental-capsule EM (EC-EM) were developed to realize the direct observation of wet cells and tissues. However, the small size and volume (< 20 μl) of the sample holder limits the manipulation during observation and correlative light-electron microscopy (CLEM). We have developed atmospheric SEM (ASEM) for observation of fixed samples immersed in liquid in open environment [1,2]. In ASEM, an optical microscope (OM) positioned above the inverted SEM. In between a specimen dish with 8 silicon nitride (SiN)-film windows is set on the X-Y stage (Fig.1A).

Using ASEM in combination with immuno-labeling, we have visualized intracellular molecular-complex formations including axonal segmentation in primary culture neurons [5,6] and accumulation of calcium sensor STIM-1 proteins in response to Ca^{2+} store depletion in T cells [3]. The distribution of leg protein complex in Mycoplasma [4], and Fas proteins in ES cells [5,6] were observed using immune-fluorescence gold labeling. By placing sample on the SiN film, we observed various kinds of tissues including brains, cancer metastasized ling [7], secretory tissues [8] immersed in aqueous liquid. In material science field, metal sintering [2] and electrochemical depositions [9] were imaged using ASEM.

ASEM observation using dual gold-labeling was performed. Bacterial biofilms formed by methicillin-resistant *Staphylococcus aureus* (MRSA) were successfully immuno-labeled [10]. Biofilms of clinically isolated *S. aureus* MR10 cultured for four hours were labelled with colloidal gold-anti-dsDNA antibody, counter-stained with positively charged Nanogold (anti-dsDNA/PCG), and gold enhanced (Fig.1b, c). Labeled strings in the extracellular matrix suggest dsDNA as a component of biofilm. Axonal segmentation is proposed to be critical in early brain development. In cultured *drosophila* neurons, BP102 [5,6] fluorescence border representing the axonal segmentation is correlated with a crossing of immuno-gold labeled microtubule bundles imaged by ASEM (Fig.1d, e).

Guided by an inspection using OM, various tissues immersed in aqueous solution were targeted in the center of the ASEM dish, and imaged using ASEM. After PTA-staining, various symbiotic bacteria species are visualized on the mucosal side of mouse stomach (Fig. 2a), suggesting applicability to bacteria flora study in our digestive tract. Chronic complications of diabetes mellitus can be triggered by thickening the basement membrane of microvessels. We observed intraperitoneal adipose tissue of 10 week-old female ob/ob fat model mice (Fig. 2b) and the islet of Langerhans in pancreas (Fig. 2c). Blood cells, especially Erythrocytes, were prominent in the capillaries. The work is supported by grants from CREST JST, Kakenhi ‘Sparse modeling’ and 15K14499, CANON and AIST to C.S.

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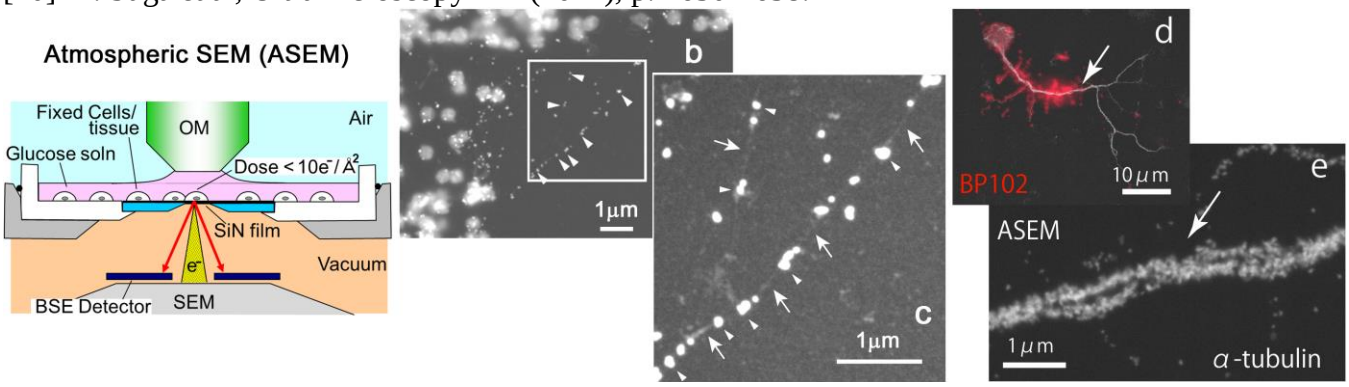

Figure 1. ASEM and optical microscopy of axonal segmentation and MRSA biofilm formation. (a) ASEM system used to image aldehyde-fixed cells and tissues in radical scavenger, dextrose solution. An optical microscope (OM) is above the inverted SEM, with the 2-3 ml-capacity specimen dish in between. The detachable, 35-mm ASEM dish has eight 100-nm thick (250×250)-μm SiN film windows in its bottom plate. The electron beam projects from underneath onto the cells through the SiN film. (b, c) MRSA biofilm labelled both with colloidal gold-anti-dsDNA antibody and positively charged Nanogold, and gold enhanced. A higher magnification image of the rectangle is shown on the right. Arrowheads and arrows indicate linearly aligned colloidal gold particles and PCG-positive fibrils, respectively. (d, e) Fluorescence images representing the axonal segmentation in cultured neurons, imaged by fluorescence microscope and ASEM. Arrows indicate axonal segmentation, filtering protein species. (e) Higher-magnification ASEM image of the indicated area in (d).

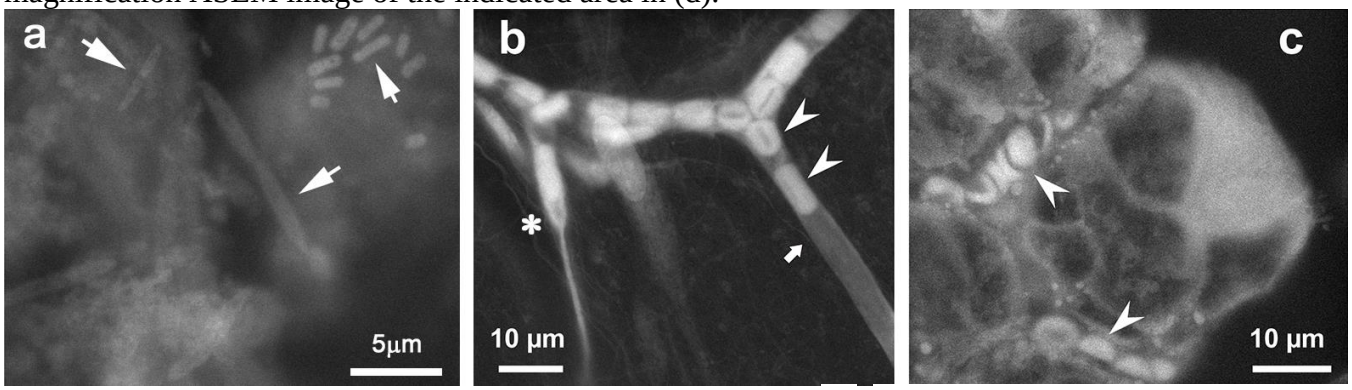

Figure 2. ASEM images of PTA-stained mouse tissues. (a) Mucosal side of stomach. Various symbiotic bacteria species are observed (arrows). (b) Capillaries of intraperitoneal adipose tissue of ob/ob mice (arrow). A pericyte is indicated by star. (c) Islet of Langerhans in pancreas. Erythrocytes can be seen in the capillaries (arrowheads in b, c).

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