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Application of Ionic Liquid on Biological Samples in Correlative Optical Microscopy and Scanning Electron Microscopy.

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Correlative optical microscopy and scanning electron microscopy (SEM) combines two techniques enabling the use both optical and electronic analysis techniques on the same sample. For example, optical techniques, such as fluorescence staining, fluorescence in situ hybridization (FISH) could in principle be used with SEM and energy dispersive spectroscopy (EDS) [1]. Optical microscopy provides efficient species identification, biomolecule-labeling and live sample imaging. On the other hand, SEM enables nanometer-spatial resolution and elemental mapping [2]. Conventional biological sample preparation for SEM, which involves fixation, dehydration and coating with gold or other heavy-metal, make subsequent optical microscopy difficult. With SEM sample preparation processes it is difficult to relocate same field of view in optical microscopy. Gold coating renders samples opaque under an optical beam. Moreover, the heavy-metal coating prevents EDS analysis. Ionic liquids (IL) are highly conductive, stable under electron beam and have extremely low vapor pressure, which renders them suitable to use in SEM at vacuum conditions around 10^{-5} Pa [3]. Using IL may eliminate some of the problems with carrying out correlative microscopy of biological samples. The IL sample preparation processes may also be less expensive and simpler than conventional methods. This paper reports the preliminary results from a feasibility study using IL in correlative microscopy.

We used the Hitachi HiLEM IL1000 ionic liquid $C_7H_{19}NO_4S$ (Miyoshi Oil and Fat Co. Ltd), which is completely miscible with water or ethanol and very inert [4]. SEM was carried out using a Hitachi SU8230 cold FEG SEM at 0.5 and 1 kV. Concentrated biological samples (microbial cell pellet) were first fixed in 2% glutaraldehyde in cacodylate buffer and rinsed with Milli-Q water. After fixation, the pellet was re-suspended in 5% IL water solution. A droplet of the mixture was placed on an aluminum sample stub for 12 h to ensure sufficient liquid exchange. Excess IL was carefully rinsed off with water. After the evaporation of water at ambient temperature, the sample on Aluminum stub was analyzed by SEM and florescence microscopy.

We first applied this method to a *Sporomusa* culture. *Sporomusa* is an anaerobic bacterium, and we isolated a strain from a mixed culture fermenting methanol. Fig.1a shows the SEM micrograph of IL treated *Sporomusa* culture revealing detailed information on cell morphology. The image has desirable contrast and most of cells remain intact. Unlike the conventional SEM sample preparation, the IL treated cells are still “wet”, which may better represent the true morphology [3]. After the SEM observation, the sample was stained with DAPI on the sample stub and the stub was directly observed with an epifluorescence microscope. Fig.1 (b) shows that the IL treatment does not impact DAPI staining and

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fluorescence microscopy. Cells still can be clearly observed. Furthermore, IL treatment was also tested on a mixed culture containing a variety of anaerobic and methanogenic bacteria and archaea (*Methanoregula*, *Methanosaceta*, *Parcubacteria*, *Deltaproteobacteria*, *Treponema*, *Synergistetes* and some other novel unknown species [5]. The SEM micrograph of IL treated mixed culture clearly shows the morphologies of different species. In the future, the use of specific fluorescent stains (FISH) can help to identify the different species and correlate to SEM morphology. Nano-scale particles or proteins attached to cell membranes were also be observed, Fig.2b. A combination with EDS and other labeling techniques will also help to identify those particles.

This test case suggests that using IL may be a simple and effective sample preparation method for correlative microscopic analysis. Future research based will help to develop an efficient and effective protocols for correlative microscopy of biological samples.

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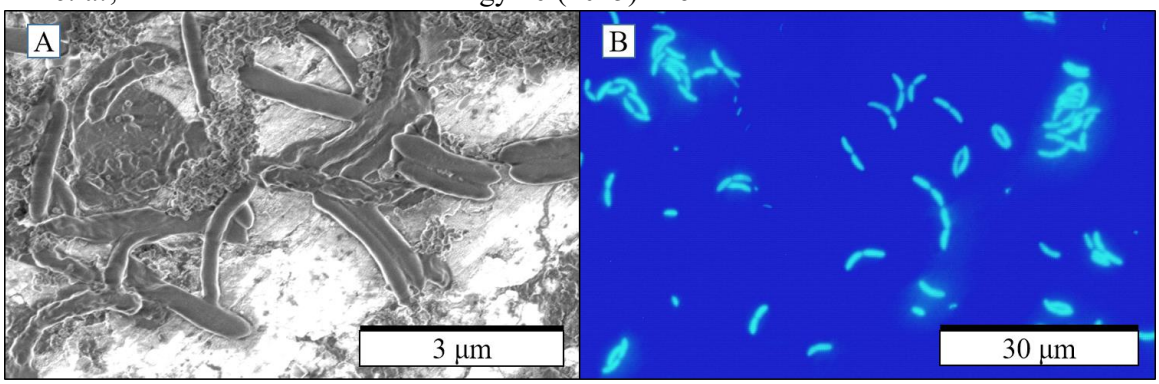

Fig.1. (a) SEM micrograph of IL treated *Sporomusa* culture; (b) Fluorescence micrograph of DAPI-stained *Sporomusa* culture after SEM observation.

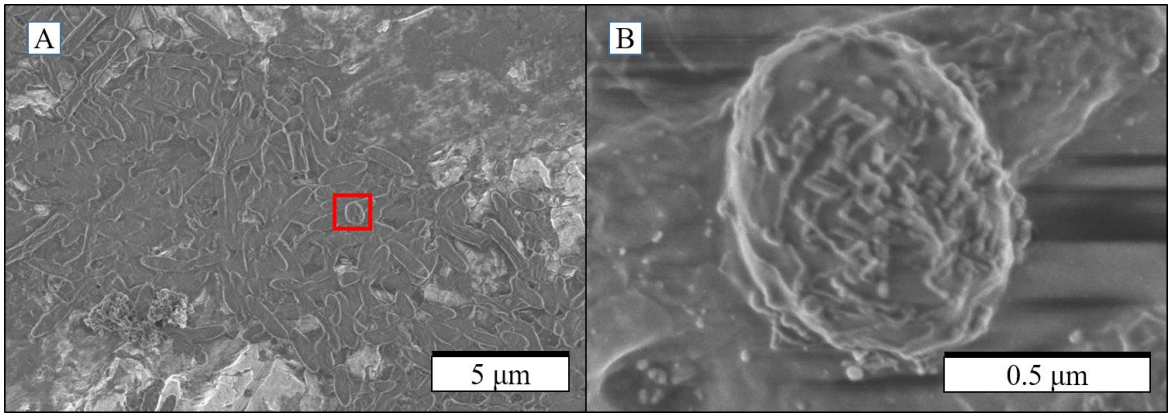

Fig.2. (a) SEM micrograph of IL treated mixed methanogenic culture; (b) High magnification image of red rectangle in left image.

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