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Air-drying is sufficient pre-treatment for *in situ* visualization of microbes on minerals with scanning electron microscopy

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ABSTRACT

Scanning electron microscopy (SEM) is a powerful tool for observing microbe-mineral interactions *in situ*. Despite its wide usage in geomicrobiology there is no consensus on how the samples should be handled before visualizing in SEM. We compared response of artificial laboratory-grown bacterial community and natural *in situ* microbes on terrestrial basalt to different sample pre-treatment methods with the aim to preserve microbe-mineral interaction interface. Air-drying was the only method that maintained the location of loosely attached bacteria on a mineral surface, whereas chemical fixation and drying dislocated the cells. On the contrary, chemical fixation preserved the cellular morphology while air-drying caused the collapse of most of the laboratory-grown cells. Natural microbial communities on dry terrestrial basalt were composed of desiccation resistant microbes which remained attached to the surface and partially maintained their morphology regardless of the sample pre-treatment method. None of the tested methods allowed visualization of microbe-mineral interface in a biofilm. We suggest air-drying as a main sample pre-treatment method for visualizing microbes on mineral surfaces when loss of morphology is secondary to potentially dislocated cells and to potential chemical changes in the sample caused by the chemical fixation reagents.

1. Introduction

Recent rapid development in microbial ecology has uncovered rich and diverse microbial communities in the environments that were previously thought to be hostile and almost lifeless. This includes rocks in dry and cold environments, where nutrient supply is limited and living organisms rely on the elements extracted from the rocks themselves. In order to understand microbial interactions with lithosphere and their role in biogeochemical cycles we need to recognize and study microbe-mineral interactions. Vast majority of microbes cannot be grown in standard laboratory conditions, therefore, they need to be studied *in situ*. Scanning electron microscopy (SEM) is one of the most important direct visualization methods revealing microbes on mineral surfaces. It complements genetic methods that identify unculturable microbes and their potential metabolic activity, and also enables, using different analytical systems attached to SEM, *in situ* geochemical analyses of mineral-rock surfaces that reveal identity of minerals and microscale deviations in local chemical composition. SEM can be used to determine whether microbes prefer certain minerals or topographic features such as ridges, cavities and cracks, and whether microbial activity causes dissolution of substrates or secondary mineralisation.

SEM is a complex method of surface imaging, where the surface of a sample is scanned with an electron beam and secondary and/or backscattered electrons emitted from the sample surface are detected. Thorough introduction to the SEM technique is given in Goldstein et al. (1992). One of the most important prerequisites for imaging is a volatile-free sample. Any gas molecule in the SEM chamber would interfere with the primary electron beam and with secondary and backscattered electrons emitted from the sample compromising the image quality. Samples that are hydrated in their native state (*e.g.* biological tissues) must be dried prior to the observation. The most straightforward method for preparing geological samples is just air-drying or alternatively use of desiccator. However, it has been anticipated that air-drying is not suitable for biological specimens as the surface tension on the air-water interface disrupts the cells and causes cell lysis (Bennett et al., 2006). The most common method for preparing biological specimens for SEM involves successive (pre)fixation, dehydration, coating with an electron-conductive material and viewing (Bergmans et al., 2005). Usually aldehyde (glutar- or formaldehyde) in buffered solution is used to cross-link the proteins to preserve the structure of the cell and the remnants of fixative are removed in subsequent rinsing with buffer solution and dehydration with increasing concentrations of ethanol or

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acetone. Total removal of liquids is achieved with critical point drying (CPD) or chemical drying with hexamethyldisilazane (HMDS). CPD preserves cellular structures by avoiding liquid-gas interface and associated surface tension effects as the fluid is removed from the sample in supercritical state, whereas HMDS has a very low surface tension and its evaporation from the sample causes little distortion. HMDS drying and CPD have been found to perform equally for sample preservation in terms of cell morphology (Bray et al., 1993; Braet et al., 1997; Araujo et al., 2003). However, it has been estimated that up to 90% of individual cells not involved in a biofilm will be lost in CPD process (Bennett et al., 2006). Furthermore, HMDS drying is quicker and cheaper as it does not require specific equipment giving HMDS clear advantage over CPD. As a final step, samples must be coated with a thin layer of conductive material, usually carbon, gold or platinum, to prevent charge build-up on the surface of the sample and damage to the sample during imaging.

An alternative method to the conventional high-vacuum SEM is a variable-vacuum or environmental SEM where some gas (including water vapour) is allowed to leak into the specimen chamber creating a relatively high pressure (up to 2500 Pa). This gives an advantage of visualizing hydrated and non-conducting samples without any pre-treatment and eliminates the need for sample coating as surface charging is neutralised by positively ionised gas atoms (Danilatos, 1994). While environmental SEM has its advantages in visualizing biological samples it does not replace high-vacuum SEM but rather complements it (Muscariello et al., 2005). Environmental SEM provides a more realistic image of the sample, whereas high-vacuum SEM offers better resolution and resolves finer structures.

Another option for visualizing hydrated samples is cryo-SEM that comprises a conventional SEM with a cryo-chamber. Samples for cryo-SEM are prepared by rapid freezing in liquid nitrogen and optional freeze-fracturing or freeze-sectioning. Imaging is performed in the microscope chamber that operates at temperatures below 143 K (Walther and Muller, 1999). Cryo-SEM enables imaging of hydrated samples at higher resolution than environmental SEM, but is more time consuming and expensive to perform. Alternatively, special electron transparent specimen chambers/cells have been applied to study fully hydrated (wet) samples (de Jonge et al., 2009). Also, recently a rapid method using infiltration of samples with ionic liquid on conducting filter substrates was introduced (Golding et al., 2016) allowing SEM imaging of fully hydrated microbes with improved ultrastructural preservation, reduced dehydration and shrinkage. However, all the above-mentioned techniques require specialized instrumentation and are not suitable for imaging microbes *in situ* on environmental samples.

Despite plenty of literature about the usage of SEM for imaging microbes on rocks there is no consensus how samples should be treated for the best and most representative outcome. Often imaging conditions and even sample preparation methods have been reported incompletely, making it impossible to evaluate and compare the information on micrographs from different studies. Although the lack of consensus was pointed out already about a decade ago (Schädler et al., 2008), the situation has not changed much and comparisons and justifications are needed to conform to the common rules in the field of imaging natural microbes on solid surfaces. Studies comparing different sample pre-treatment methods have focused on aqueous single-species laboratory cultures (Schädler et al., 2008; Prakash and Nawani, 2014; Zeitvogel et al., 2017) that do not necessarily reflect the behaviour of complex microbial communities on environmental samples.

Here we compare different sample preparation methods for visualizing microbial cells on dry terrestrial basalts. Microbes naturally living on basalt and artificial bacterial community were used to compare the information between untreated samples in low-vacuum SEM (LV-SEM), and air-dried and chemically fixed samples in conventional high-vacuum SEM (HV-SEM) mode. Chemically treated samples were fixed in glutaraldehyde, dehydrated with series of ethanol solutions and eventually either air-dried or HMDS-dried. As a result, we present suggestions what should be considered before planning using SEM for visualizing microbes on mineral surfaces and encourage researches to report their sample treatment protocols as the treatments might have crucial influence on the final outcome.

2. Materials and methods

Basaltic rock sample was collected from Rauðhálsahraun lava flow in Ljósufjöll volcanic system, Iceland in August 2011. The fist-sized rock was broken into 0.1–0.5 cm^3 pieces. The pieces were washed twice by soaking into sterile water to remove the dust and open debris.

A mock community of 34 bacterial strains (Supplementary Information, Table S1) was grown with rock chips in 10% R2A medium (Difco) on a rotary shaker for 16 h at room temperature at 40 rpm. The mock community was composed of environmental bacteria that were identified by 16S rRNA gene sequencing (J. Cairns, personal communication).

Both the original rock chips and the samples incubated with the mock community were visualized either in low (LV-SEM) or conventional high vacuum (HV-SEM) mode with Zeiss Evo MA15 variable pressure electron microscope equipped with Oxford AZTEC energy dispersive X-ray spectroscopy (EDS) detector. For low vacuum mode, no pre-treatment was applied to the samples. For high vacuum mode, different pre-treatments were applied: i) air-drying for at least 2 h; ii) chemical fixation followed by drying in HMDS; iii) chemical fixation followed by air-drying. For chemical fixation, the samples were incubated in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h, then samples were rinsed twice with 0.1 M cacodylate buffer for 15 min followed by 15 min incubations in 25%, 50%, 75%, 95%, 100% and 100% ethanol solutions. Half of the chemically fixed samples were treated twice with 100% HMDS for 10 min, another half were air-dried. All steps were carried out at room temperature. Samples that had been inoculated with the mock community were washed twice with 0.1 M cacodylate buffer before glutaraldehyde fixation to remove growth media.

Samples were visualized at following specifications: chamber pressure 45 Pa in low vacuum mode and $< 10^{-5}$ Pa in high vacuum mode, beam current 30.0 μA and accelerating voltage 20 kV. Working distance varied from 8 to 10 mm due to the natural roughness of the sample surface. For low vacuum mode, the samples were attached to the sample holder with double-sided tape and aluminium tape. For high vacuum mode, the samples were attached to the stub with silver adhesive and coated with 5–10 nm thick platinum layer with Leica EM SCD500 high vacuum sputter coater.

At least two independent experiments were performed with natural microbial communities and three independent experiments with laboratory mock community. Number of observed areas exceeded 50 per rock chip. Microbes were ubiquitous throughout the samples allowing numerous observations per sample, the only exception being natural biofilms that were difficult to find and only one to three observations per treatment could be made.

3. Results and discussion

3.1. Mock laboratory community

LV-SEM imaging of wet (drained but not dried) basalt chip from bacterial culture revealed evenly spread bacteria on the sample surface (Fig. 1A). Most of the cells appeared rod-shaped, about the same size and in contact with neighbouring cells. Air-drying before HV-SEM imaging caused the cells to collapse and lead to some clumping of the cells (Fig. 1B). Most of the cells appeared flattened in the middle with thicker edges and were spread in groups across the surface (Fig. 1B). However, the level of flattening was not uniform as not all the cells had collapsed (Fig. 1C). Chemically fixed cells retained the cell shape better than air-dried cells (Fig. 1D–F). Best preserved cylindrical or round