# COMBINATION OF DIFFERENT MICROSCOPY TECHNIQUES FOR THE INTEGRATED STUDY OF EXTREMOPHILE ENDOLITHIC MICROORGANISMS AND THEIR HABITATS

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## ABSTRACT

Some micro-organisms are able to withstand extreme environments and form communities within rocks. To characterise these endolithic micro-ecosystems, several microscopy and microanalytical approaches need to be combined, including scanning electron microscopy with back scattered electron imaging (SEM-BSE), low temperature scanning electron microscopy (LTSEM), confocal scanning laser microscopy (CSLM), and the X-ray energy dispersive spectroscopy (EDS) microanalytical system. These techniques have allowed the simultaneous observation of these micro-organisms and their habitats. SEM-BSE and LTSEM serves to evaluate the biodiversity of the rock from a morphological and ultrastructural perspective. LTSEM also permits water localisation in the cells and their microhabitats. Information on the spatial distribution of the micro-organisms inside the rock is provided by CSLM. Lithobiontic communities have been shown to interact with their substrate. The EDS technique coupled to SEM-BSE permits the chemical characterisation of mineral features, the detection of biomobilisation and biomineralisation processes, and yields information on the chemical environment. These techniques are also applicable in the search for fossilised microorganisms.

## INTRODUCTION

Some micro-organisms from extreme habitats adapt to harsh conditions of temperature, ultraviolet radiation, water availability, etc., forming communities within rocks. Indeed, one of the main outstanding features of terrestrial life in the Antarctic continent, is the predominance of rock as a substrate for living organisms. Although the study of micro-organisms colonising the inside of lithic materials confronts with considerable difficulties, the detailed mineralogical and biological characterisation of endolithic microhabitats is essential in the understanding of the adaptation of rock-dwelling micro-organisms to their macro- and microhabitats. The adaptation and survival of the endolithic Antarctic micro-organism depends upon a precarious equilibrium of biological, geological and climatic factors. Any, discrete, unfavourable change in external conditions can result in the death and disappearance of microscopic organisms. When microbial life decays, mineralised microorganisms and biomarkers can point to the previous existence of life. The ability to recognise these fossils and/or other traces left behind may have important astrobiological implications.

To study live, mummified, fossilised micro-organisms and biomarkers, different microscopy and microanalytical approaches need to be combined, such as light microscopy (LM), scanning electron microscopy with back-scattered electron imaging (SEM-BSE), low temperature scanning electron microscopy (LTSEM), confocal scanning laser microscopy (CSLM), transmission electron microscopy (TEM), environmental scanning electron microscopy (ESEM) and the X-ray energy dispersive spectroscopy (EDS) microanalytical system. These techniques generate different kinds of information (Fig. 1) and have allowed the simultaneous exploration of these micro-organisms and their habitats. Observations must be carried out *in situ*, that it to say, without separating the biological components from their lithic substrate. In this way, live micro-organisms are examined in their own microhabitat and fossils observed in the place where mineralisation took place.

## SEM-BSE AND EDS

SEM-BSE images have allowed us to discover that rocks from these extreme habitats present several

fissures and cavities which harbour a great diversity of micro-organisms (Fig. 2, 3). The "SEM-BSE technique" (Wierzchos & Ascaso, 1994) is the basis for the integrated study of lithobiontic communities and their microhabitats. Different features of the micro-organism and substrate can be investigated simultaneously. The ultrastructure of live microorganisms can be visualised and this allows their morphological and ultrastructural identification (Fig. 3). Different patterns of microbial distribution may indicate the different adaptability of each micro-organism to certain conditions. In several zones, the lithobiontic community constitutes a complex biofilm, which interacts with the lithic substrate both geophysically and geochemically. Not all the substrate is affected equally by the proximity and growth of lithobiontic micro-organisms. Interaction with the lithic substrate gives rise to mineral transformation and biomobilisation and biomineralisation processes. These biogeochemical alteration processes can be explored by combining the "SEM-BSE technique" with the EDS microanalytical system. Biomobilisation (changes in certain chemical elements of the mineral substrate) and biomineralisation (formation of new minerals) processes are frequently detected in the vicinity of lithobionts (Ascaso et al., 1995; Ascaso et al., 1998; Wierzchos & Ascaso, 1996, 1998). The EDS technique coupled to SEM-BSE has permitted the detection of these processes in a lithic Antarctic biofilm (Wierzchos & Ascaso, 2001). Figure 2 is an SEM-BSE image of calcium oxalate and silica gel deposits confirmed by EDS scan-line analysis (Fig. 4). Both arise from biomineralisation processes and, therefore, act as biomarkers.

## CSLM

This technique is essential for visualising endolithic micro-organisms in natural, undisturbed conditions. Certain micro-organisms may be identified by the autofluorescence of some of their components or different affinity to certain stains. Figure 5a is a confocal image of algae within a rock fissure visualised by the autofluorescence of chlorophyll. The SEM-BSE image in the left corner (Fig. 5b) corresponds to the same area. The use of CSLM and the SEM-BSE technique on the same zone allows, what Ascaso et al. (1998) termed the "correlative microscopy strategy", to be applied. Moreover, sets of sectional images may be used to create three-dimensional pictures, such that the spatial relationship of the different micro-organisms can be established (De los Rios et al., 1999; Ascaso et al., 1998; Wierzchos & Ascaso, 2001). CSLM allows determination of the spatial distribution of the organic components of the biofilm formed by live microorganisms (algae, fungi, bacteria, etc.) and extracellular organic polymers, along with estimation of the number of micro-organisms per unit volume.

## LTSEM

Using LTSEM, ultrastructural details of cryosectioned cells within the lithic substrate may be obtained (Fig. 6). With LTSEM, samples are examined frozen, and in this way, micro-organisms may be observed in their natural state of hydration (De los Ríos et al., 1999). The technique also permits the localisation of water in the microhabitat. The precise location of minute quantities of water that Antarctic micro-organisms obtain from the microenvironment of their rock habitat has also been achieved with this technique (Ascaso et al., in preparation).

The simultaneous application of two or more of these microscopy techniques to the same area ("correlative microscopy") is an excellent way of performing an integrated study of these lithobiontic communities (Ascaso et al., 1998). Further, integration of all the information generated by such methods provides deep insight into the ecological functioning of this form of life under extreme conditions.

## FOSSILISED MICRO-ORGANISMS

The most appropriate techniques for the analysis of mineralised micro-organisms are SEM coupled to EDS, and TEM. Figure 7 shows a fossilised algal cell in which thylakoids and lipid bodies can be distinguished. To date there is no other method of examining a fossilised (totally mineralised) micro-organism on the micrometer scale (Ascaso, 2000; Wierzchos & Ascaso, 2001).

## CONCLUSIONS

In conclusion, the combination of diverse microscopy techniques is a reliable method of studying the complete life cycle of extremophilic endolithic micro-organisms, since it permits the *in situ* analysis of the different components of the organo-mineral phase within which the lithobiontic community is integrated, as well as the processes occurring in the lithic substrate and their effects.

Every technique developed for the study of endolithic microorganisms (alive or fossilised), represents an advance in the development of adequate methods of detecting the presence of micro-organisms, fossils or biomarkers in hard extraterrestrial substrates. Initial samples from Mars will probably be rocks. If

presumptive micro-organisms from Mars are alive or mummified, molecular biology, bioengineering, computer simulation of membrane function, fluorescence techniques, etc., could be of great interest, in addition to all the microscopy approaches discussed here. However, if the rocks from the Mars Return mission planned for 2011 only contain fossilised (mineralised) micro-organisms or their mineral biomarkers, physiological and molecular techniques will be of no use at all. Microscopy studies carried out in situ on Mars will be essential for the collection of appropriate samples. Nevertheless, if we are to confirm the biological origin of some structures, the subsequent use of high resolution microscopy by expert observers will be crucial.

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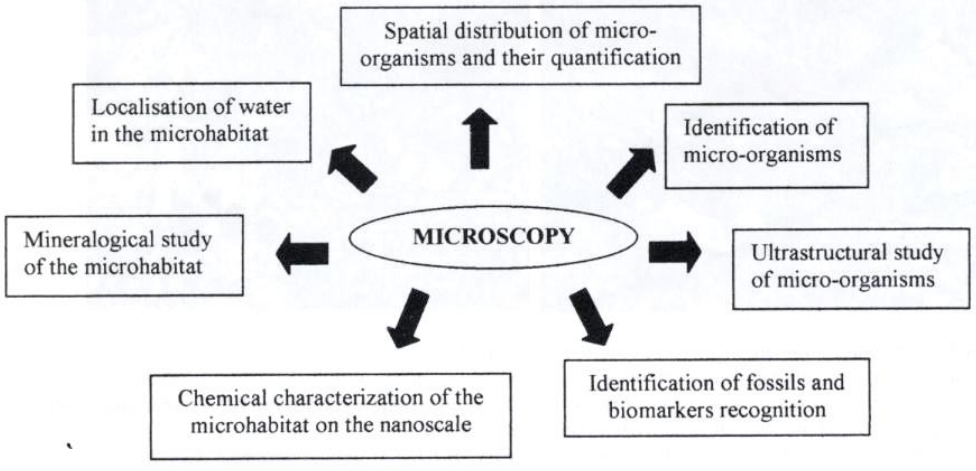


*Figure 1. Diagram summarising the different type of information provided by microscopy*

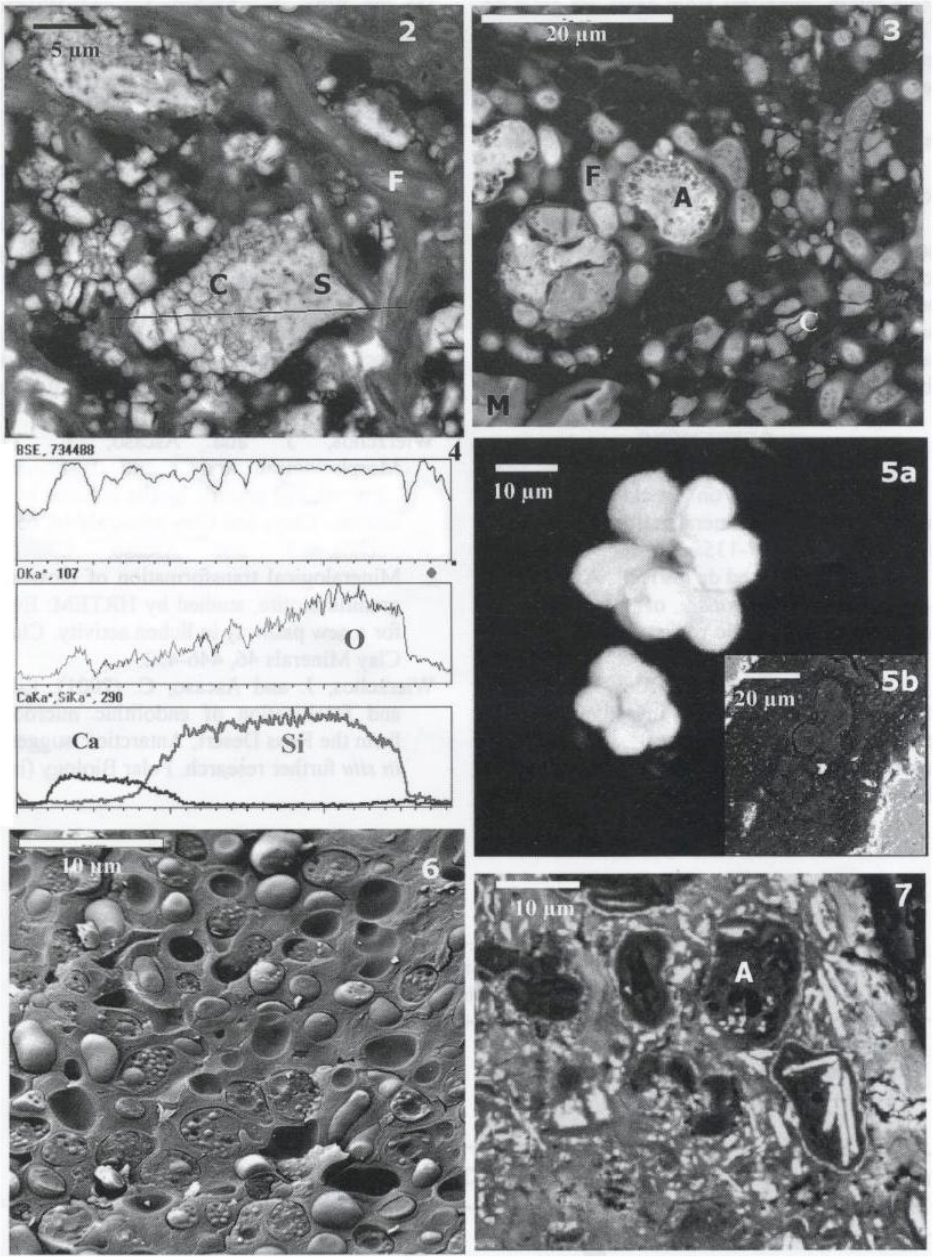


***Fig. 2:*** *SEM-BSE image showing deposits of calcium oxalate and silica gel among fungal hyphae. F, fungus; C, calcium oxalate; S, silica gel.* ***Fig. 3:*** *SEM-BSE image of algal and fungal cells within the lithic substrate. A, alga; F, fungus; M, mineral fragments; C, calcium oxalate crystals.* ***Fig. 4:*** *EDS microprobe line profiles of O, Ca and Si along the transect indicated by the black line in Fig. 2.*

***Fig. 5:*** *Correlative microscopy. Fig. 5a: Confocal image of algae within the lithic substrate. Fig. 5b: SEM-BSE image corresponding to the same zone as Fig. 5a.*

***Fig. 6:*** *LTSEM image of fungal endolithic cells.*

***Fig. 7:*** *SEM-BSE image of an endolithic fossilised community. A, alga*