Imaging of organic signals in individual fossil diatom frustules with nanoSIMS and Raman spectroscopy

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ABSTRACT

The organic matter occluded in the silica of fossil diatom frustules is thought to be protected from diagenesis and used for paleoceanographic reconstructions. However, the location of the organic matter within the frustule has hitherto not been identified. Here, we combined high spatial resolution imaging by nanoSIMS and Raman microspectroscopy to identify where the organic material is retained in cleaned fossil diatom frustules. NanoSIMS imaging revealed that organic signals were present throughout the frustule but in higher concentrations at the pore walls. Raman measurements confirmed the heterogenous presence of organics but could not, because of lower spatial resolution, resolve the spatial patterns observed by nanoSIMS.

1. Introduction

Diatoms, a ubiquitous group of microalgae, construct frustules made of hydrated amorphous silica that can be preserved in the sediment record. The application of diatom frustule paleoproxies has increased greatly in the last decade. The nitrogen isotope composition of diatom frustules has been proposed as a proxy for nitrogen utilization and thus surface nutrient consumption (i.e., Horn et al., 2011a; Robinson et al., 2014). This proxy is based on the presence of different proteins (mainly pleuralsins, silaffins and long-chain polyamines) necessary for silica sequestration that are incorporated into the diatom frustule during growth (Kröger et al., 2002; Hecky et al., 1973; Kröger and Poulsen, 2008; Bridoux and Ingalls, 2010). During a period of growth, phytoplankton draw down nutrient concentrations, resulting in an increase of residual nitrate $\delta^{15}$N due to the fractionation of N isotopes during nutrient uptake (similar to Rayleigh distillation). The rise in $\delta^{15}$Nnitrate with nutrient depletion is expected to be recorded in the biogenic silica (opal) produced by diatoms (De La Rocha, 2006). As such, high $\delta^{15}$Nfrustule indicates a greater fraction of available nutrients (nitrate) has been consumed during the growing season (Shemesh et al., 1993).

It has been suggested that, in contrast to the bulk sediment, nitrogen encased within the diatom frustule is protected from diagenetic alteration and thus presents a more robust proxy (Shemesh et al., 1993; Sigman et al., 1999). These assumptions are based on observations such as similar relative amino acid abundances in the organic matter of fossil frustules and living diatoms (Shemesh et al., 1993), the apparent absence of diagenetic variability in frustule-bound N when compared to bulk sediment N content (Sigman et al., 1999), and high diatom-bound amino acid concentrations in silica-rich sediments relative to sediment traps (Ingalls et al., 2003). However, as of yet, it is unknown where exactly in the frustule this organic matter resides.

For $\delta^{15}$Nfrustule analysis, several precautions are generally taken to ensure that only the frustule-bound nitrogen is measured, including several harsh cleaning methods to remove any loose organic matter and diagenetic alteration (Hendry and Rickaby, 2008; Robinson et al., 2004). Despite this, questions remain regarding the nature of the occluded organic matter. The isotope composition of entombed organic matter may vary, for example, due to species-specific amino acid compositions (Des Combes et al., 2008) or species-specific fractionation (Horn et al., 2011b). Possible isotopic alteration of the occluded organic matter during diagenesis remains largely unexplored, but it has been shown that the diagenetic incorporation of metals does not affect the N isotope values of the frustule-bound organic matter (Ren et al., 2013).

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This study explores the use of high spatial resolution imaging techniques to identify where in the cleaned fossil diatom frustule the organic material is retained. Our approach involved the development and application of nanoSIMS imaging (nanoscale secondary ion mass spectrometry), thereafter additional validation was sought with Raman spectroscopy and Transmission Electron Microscopy (TEM). NanoSIMS allows imaging of elements with a lateral resolution down to 50 nm, but can be sensitive to 3D sample topography due to the low primary ion beam currents used to achieve high lateral resolution. In contrast, the Raman technique integrates signals over larger depths in the sample, but has a distinctly poorer (μm scale) lateral resolution (Liang et al., 2016). However, by probing of chemical bonds, Raman spectroscopy allows identification of the molecular structure of samples. The TEM technique used for this study had a high lateral resolution (<1 nm) but did not have the sensitivity to image the presence of the organic signal.

To further constrain the distribution of organics within the frustules we explored two ultrahigh resolution imaging techniques. First, we used an ultra-high resolution imaging technique with a 15-kV accelerating voltage to identify specimens suitable for further analysis (i.e., individual frustules with minimal 3D topography).

2. Materials & methods

2.1. Materials

The samples for this study originate from sediment Core MD-022515. This core was retrieved from the Guaymas Basin (27°29.01′ N; 112°04.46′ W; 881 m water depth) during the MONA (Morges Ouest Nord Américaines) cruise of the R/V Marion Dufresne (International Marine Global Changes-IMAGES VIII) in June 2002 (Pichevin et al., 2012). The diatom frustules were collected from a core depth of 1161 cm, corresponding to an age of 12,450 years Before Present according to Morley et al., 2004). The chemical cleaning includes 3 steps and is performed on the isolated diatom fraction (Morley et al., 2004; Hendry and Rickaby, 2008; Polerecky et al., 2012), and comprised both mechanical and chemical cleaning steps.

2.2. Sample preparation and selection

Purification of the diatom samples for analysis was performed following published cleaning methods (Ellwood and Hunter, 1999; Morley et al., 2004; Hendry and Rickaby, 2008). The procedure includes both mechanical and chemical cleaning steps. Approximately 0.5 to 1 g of sediment was subsampled into a 50 mL centrifuge tube. Carbonates and organic matter were removed at temperature of 75 °C using 5% HCl and 40% H2O2, respectively, prior to mechanical separation of the diatoms from clays and other terrigenous components. The mechanical step consists of elimination of large debris by sieving (at 10 and 75 μm), differential settling using a heavy liquid (density set at 2.1 g/mL) as well as rinsing with diaphragm and slit settings of D1-3, ES-3, AS-2, and EnS-1. The analysis was carried out in the imaging mode by rastering a high-energy 133Cs+ beam (16 keV, 2 pA FCo, nominal beamsize 50–150 nm; electron gun enabled) over a target area on the frustule (between 4 μm × 4 μm and 55 μm × 55 μm in size) and measuring the sputtered secondary ion yields. After pre-sputtering, the instrument was tuned with diaphragm and slit settings of D1-3, ES-3, AS-2, and EnS-1. Data processing and analysis were done with an updated version of the Look@nanoSIMS software (Polerecky et al., 2012).

During the initial method development stage, measurements targeted frustules of different species of centric and pennate diatoms. However, pennate diatom frustules proved difficult to measure with the NanoSIMS due to their pronounced 3D topography. Therefore, only data obtained from the fossil frustules of various centric diatom species were presented. The combination of the primary ion current, dwell time, and the number of measured planes did not allow measurements through the entire thickness of the frustule. Therefore, the frustule interior and exterior were targeted by separate measurements.

2.4. Validation techniques

Raman measurements were performed with the Horiba LabRam spectrometer at the King Abdullah University for Science and Technology. The sample was excited with a 473 nm laser line in the backscattering configuration through a 100× objective allowing a lateral resolution of ~1 μm. Using a grating with 1800 grooves/mm, Raman spectra were collected in the range of 150–3300 cm⁻¹ with a spectral resolution of 1.2 cm⁻¹. Initially, the interior of an individual frustule was analyzed in three spots to identify the primary signals present. This was followed by imaging analysis of the interiors and exteriors of several frustules with the step size varying between 0.5 and 2 μm and the size of the imaged areas varying between 8 μm × 8 μm and 15 μm × 15 μm. During imaging with the lower step size (0.5 μm and 1 μm) only the lower frequency range (up to 1700 cm⁻¹) was recorded, whereas the larger step size imaging also covered higher frequencies (around 3000 cm⁻¹) to include a prominent organic peak between 2826 and 3021 cm⁻¹. Data analyses were performed using the manufacturer’s software LabSpec (version 6).

To further constrain the distribution of organics within the frustules we explored two ultrahigh resolution imaging techniques. First, we used an ultrahigh resolution (-1 nm) TEM to image the presence of organics at pore edges. For this, the FEI Titan 80–300 was used at the King...
Abdullah University for Science and Technology, which was equipped for electron energy loss spectroscopy (EELS) and energy-dispersive X-ray spectroscopy (EDS), in order to determine the elemental composition of the sample. However, it was not possible to acquire sufficient signal and therefore the results are not presented in this manuscript. Second, a FEI Helios Nanolab G3 SEM at Utrecht University was used for ultrahigh resolution (~1 nm) imaging after focused ion beaming cutting of a centric diatom frustule.

3. Results

3.1. Identification of organics by Raman spectroscopy

The presence of organics in the silica matrix of a cleaned fossil diatom frustule was confirmed by Raman measurements in multiple spots on the frustule (Fig. 1; Table 1). Besides Raman peaks associated with tetrahedral Si—O—Si, Si—O—Si network, Si—OH stretching and aluminium, there was clear evidence for an organic component characterized by C—H deformation and valence vibrations at wavenumbers around 1425 and 2910 cm⁻¹. An indication for N=O stretching at ~1600 cm⁻¹ was also present.

3.2. NanoSIMS imaging of frustules

After establishing the presence of organics with single point Raman analyses, the elemental distributions were imaged with nanoSIMS. Secondary ions CN⁻ (detected as ¹²C⁴N⁻) and O⁻ were used as a proxy for organics and silica, respectively. Generally, CN⁻ ions were detected throughout the frustule but showed significantly higher counts in ring-like structures around the pores and in hotspots scattered across the frustule or near the edges of the frustule (Fig. 2b and f). The patterns were similar for both the frustule interior and exterior, although the surface morphology differed markedly between the two sides (compare Fig. 2a–b and e–f). Similar patterns were also observed for other elements associated with organics including C and S (Fig. 2c–d and g–h).

High-resolution SEM images revealed that, on the frustule interior, the ring-like structures were associated with a clear thickening of the silica matrix around the pore (see inset in Fig. 2a). The difference in contrast in the SEM images suggested that the silica matrix in these ring-like structures was different from the rest of the matrix, consistent with the ring-like patterns in the organic signals observed by NanoSIMS.

![Fig. 1. Single point Raman spectra obtained from a cleaned fossil frustule of a centric diatom. Raman spectra were collected at three different locations on the silica of the interior frustule wall of a single specimen. Peak assignments are summarized in Table 1.](image-url)

3.3. Mapping of organics by Raman spectroscopy

Although the signal around 2910 cm⁻¹ had a stronger presence in the Raman spectra (Fig. 1), subsequent mapping focused on the peak at 1425 cm⁻¹, which allowed reduction in the frequency range and thus improved lateral resolution. Images obtained by Raman measurements showed pronounced heterogeneity in the signals corresponding to both the silica matrix and organics (Fig. 3). The intensity of the peaks corresponding to Si—OH and Si—O—Si appeared less variable than the intensity of the 5C-Hx peak. Images of the peak ratio 5C-Hx/Si—O—Si were heterogeneous (Fig. 3e), but even the lowest step size (0.5 μm step size) did not resolve the spatial patterns detected by NanoSIMS (compare Figs. 2 and 3). Raman images of the frustule interior and exterior appeared similar (Fig. 3).

4. Discussion

After performing the established mechanical and chemical cleaning steps, microscopic fossil diatom frustules were analyzed with different high-resolution imaging techniques to study the presence of remaining organics tightly bound to the silica matrix. For the first time, the presence of these organics was confirmed through imaging techniques. Furthermore, the chemical mapping capabilities of the techniques made it possible to resolve, to a variable degree, the patterns in the distribution of organics in the frustule. This revealed heterogeneity and it is suggested that this is due to enrichments around the pores.

4.1. NanoSIMS measurements

In this study, NanoSIMS proved to be the best option to map the spatial distribution of elements within the fragile frustules of diatoms. NanoSIMS images revealed intensified organic signals at the pore edges of the (centric) diatom frustules (Fig. 2). The CN signal in particular, but also the signals of C and S, were clearly higher around the pores, and this spatial pattern remained as more material was sputtered away during the measurement (Supplementary Figs. S3 & S4). On the frustule interior, this signal enrichment is likely linked to the thickening of the silica matrix, which was often observed at the pore boundaries (Fig. 2a). As similar patterns were also observed on the frustule exterior, it is likely that the entire pore wall is marked by an increased organic presence (Fig. 4).

However, nanoSIMS measurements are known to be potentially sensitive to 3D topography of the sample surface, leading for instance to higher secondary count rates at or around the edges of the studied sample (e.g., Roepert, 2019; Nuñez et al., 2018). Thus, quantification should be avoided and a degree of caution is required when interpreting nanoSIMS data. Although we made a thorough and critical assessment of our data (Supplementary Figs. S3–S5), including the analysis of lateral and depth variation in ion counts and ion count ratios, we cannot exclude that these “edge effects” played a role during the NanoSIMS measurements Other techniques without this caveat but with a comparable sensitivity and spatial resolution are needed to verify our results.
4.2. Validation

The nanoSIMS results presented in this study provide the first glimpse of the spatial distribution of organic matter bound to the silica matrix in fossil diatom frustules. In an attempt to validate these results, we looked towards additional high spatial resolution imaging.

Fig. 2. NanoSIMS imaging of organic signals in cleaned fossil frustules of centric diatoms. Shown are SEM images of the frustule interior and exterior post NanoSIMS measurement, and the corresponding images of ion count ratios CN/O, C/O and S/O measured by NanoSIMS. White square in the SEM images indicates the area measured by NanoSIMS. Inset in panel a) shows a detailed SEM image of the pores using the FEI Helios Nanolab G3, measured on a different frustule than the one shown by the larger image (contrast slightly enhanced through image processing). Ion count images from which the ratio images were calculated are shown in Supplementary Fig. S1. Note that the intensity of the hue in the ratio images was modulated by the O ion count image to suppress noise from the background.

Fig. 3. Raman spectral mapping of cleaned fossil frustules of centric diatoms. Shown are maps of peak intensities corresponding to silica (Si–OH, Si–O–Si) and organics (δC-Hx), and the corresponding maps of the peak ratio δC-Hx/Si–O–Si. Maps from the frustule interior and exterior were acquired with the step-size of 0.5 μm and 1 μm, respectively. The measured areas (diameter of 8 μm and 15 μm) are marked in the corresponding optical image. Note that the images used an interpolation of values between the individual measured pixels. Lower resolution images are presented in Supplementary Fig. S2.
techniques. As mentioned in Section 2.4 (Material and Methods), we have explored the use of the ultrahigh resolution FEI Titan 80–300 TEM to image the presence of organics at pore edges with an even greater spatial resolution (<1 nm). However, it was not possible to acquire sufficient signal for organics and thus the results are not presented in this manuscript. Focused-ion beam SEM imaging at high resolution confirmed differences in density between pore-edges and the remaining of the diatom frustule (Fig. 2A inset), consistent with nanoSIMS results. Being aware of the limited spatial resolution, Raman spectroscopy was employed for resolving the concerns surrounding potential 3D-topography in nanoSIMS measurements. The Raman technique is also surface-specific, but it integrates the signal over a larger depth interval (~μm) and is thus less likely affected by potential edge effects. Raman measurements confirmed the presence of organic signals in cleaned fossil frustules, and indicated that they are heterogeneously distributed within the silica matrix. While the heterogeneity observed by nanoSIMS was confirmed, the lateral resolution of the Raman imaging (down to 0.5 μm) was insufficient to confirm or reject the spatial patterns observed around the pores.

While we applied several state-of-the-art techniques, there are additional techniques, or combinations of techniques, that could be explored in future research now that the presence of organics has been confirmed. There is clear value in combining, for example, scanning probe microscopy and Time-of-flight SIMS. Liang et al. (2016) gives an overview of the different techniques and how they can be used to simultaneously acquire morphological and chemical information. This may be a promising avenue to learn more about the nature of the observed organics around the pores.

4.3. Implications

An increased presence of organics at the pore walls (Fig. 4) may be due to a combination of the biomineralization process and the exchange of nutrients, through the pores, between the diatom cell and the surrounding water. During silicification, a protein template forms the basis onto which the silica frame is secreted (Hildebrand et al., 2018; Thamdrakol and Hildebrand, 2008). An imaging study of the insoluble organic matrix (Tesson and Hildebrand, 2013) found that the pores of a diatom valve can be occluded by this material. Based on these observations, it is hypothesised that, during growth, the pores are not open channels but are instead lined or completely occluded by the organic material.

The samples investigated were thoroughly cleaned involving well-established multiple mechanical and chemical cleaning steps (Pichevin et al., 2014) that should have removed any superficial organic material remaining in fossil frustules. Furthermore, additional thorough pre-sputtering of the sample would aid in the removal of potential contaminants. Indeed, high-resolution imaging, in particular with nanoSIMS, clearly showed organics (in particular 12C-14N) that remained throughout the measurement (Supplementary Figs. S3 & S4) and are likely embedded in the silica matrix and concentrated around pore walls. This material is most likely the carrier for proxies such as δ15N.

5. Conclusions

This study explored the combination of high-resolution imaging techniques to document, for the first time, the presence and distribution of organic signals inside the silica matrix of individual fossil diatom frustules. NanoSIMS imaging suggested that organic signals were present throughout the frustule but in higher concentrations at the pore walls. Raman measurements confirmed the heterogeneous presence of organics but could not resolve the spatial patterns observed by nanoSIMS. This study shows that the organic material embedded in the silica matrix of fossil frustules can be imaged. The combination of nanoSIMS, Raman spectroscopy and other high-resolution imaging techniques may be used for future studies to improve our understanding of the δ15N diatom paleoproxy method. However, nanoSIMS analysis should be combined with additional high spatial resolution chemical mapping techniques to confirm and better understand the distribution of organics within the frustule of fossil diatoms.

Author contributions

JJM, SA and SPA designed the experiments. L. Pichevin cleaned the diatoms. SPA prepared the samples and conducted NanoSIMS analyses. GD performed the Raman analyses. SPA and L. Polerecky performed NanoSIMS data analysis. GD and SPA performed the Raman data analyses. SPA interpreted the data, prepared the figures and wrote the manuscript text with contributions from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marchem.2020.103906.

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