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Precision thin sectioning of silica phytoliths by Focused Ion Beam (FIB-SEM)

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Djanira Rodrigues Negrao[1,2], Julio Criginski Cezar[3], Fabiano E. Montoro[4], Jian Wang[5], Charles W. Rice[2], Carlos Driemeier[6]

[1]Brazilian Biorenewables National Laboratory (LNBR), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, SP, Brazil;
[2]Kansas State University, Department of Agronomy, Manhattan, KS, United States;
[3]Brazilian Synchrotron National Laboratory (LNLS), CNPEM, Campinas, SP, Brazil;
[4]Brazilian Nanotechnology National Laboratory (LNNano), CNPEM, Campinas, SP, Brazil;
[5]Canadian Light Source (CLS), Saskatoon, SK, Canada;
[6]Brazilian Biorenewables National Laboratory (LNBR), CNPEM, Campinas, SP, Brazil

Djanira Rodrigues Negrao
Kansas State University

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Disclaimer

This method was developed for the study and found **here**

Abstract

Phytoliths are microbodies of biogenic silica made by many plant species in all ecosystems over the globe. They serve critical functions in alleviating plant stresses and influencing the carbon and silicon biogeochemical cycles. The investigation of carbon occlusion within phytolith, as a potential source of long-term soil carbon storage, has long been hampered by a lack of direct experimental evidence. In this protocol, we employed a Focused Ion Beam coupled to a Scanning Electron Microscopy (FIB-SEM) to produce thin lamellas (approximately 15 × 10 μm^2 size, with thickness below 200 nm) enabling synchrotron scanning transmission X-ray microspectroscopy (STXM) analysis with ≈100–200 nm pixel size resolution at energies near the silicon and carbon K-absorption edges. Our results revealed the spatial distributions of carbon within phytoliths, highlighting its presence at lamella borders, within islands, and dispersed in extended regions. This protocol of phytolith slicing into thin lamella provides unprecedented insights into the spatial and chemical characteristics of carbon within phytoliths, offering a low-invasive alternative to wet-chemical digestion methods.

Guidelines

This technique represents a novel approach tailored for slicing silica phytoliths with FIB-SEM. Given that silica phytoliths are rigid structures produced by plants, this method holds potential applicability to various other phytolith morphotypes across phytolith research fields.

Materials

The recipe for the plant material fixation buffer we used in this protocol is as follows:
a) 10 mL of glutaraldehyde 25% - 1 ampoule of 10 mL
b) Paraformaldehyde 20% - 1 ampoule of 10 mL
c) 25 mL of 0.2 M sodium cacodylate at pH 7.2
d) 1 mL of 0.1% sodium chloride
e) Fill up to 100 mL mark in a volumetric balloon using ultrapure water

Troubleshooting

Safety warnings

Sodium cacodylate, formaldehyde, and glutaraldehyde are hazardous substances and require careful handling. Always ensure to wear safety goggles when working with these chemicals. Dispose them properly in designated containers that are accurately labeled to facilitate recognition at recycling facilities.

The dehydration and drying are critical steps. Take time to organize and label all microtubes and respective acetone gradients. Label the containers for critical drying point (CPD) with a solvent-resistant pencil. The dehydration and drying steps with CPD may take up to 4 hours to complete (depending on the number of samples you have). Reserve the CPD equipment in advance because you must proceed with the drying step as soon as you finish the dehydration. Do not let samples in 100% acetone for more than 20 min. Otherwise, samples will get damaged.

Before start

A few comments are delivered before manipulating plant material for imaging with electron microscopy:
1) Preparation of small pieces of sample (1 cm^2) will facilitate the fixation, dehydration, and drying steps. Furthermore, small samples are less prone to surface charging during imaging with an electronic microscope.
2) Plant tissues should be fixed (preserved) with specific types of buffers. In this work, we performed tissue fixation using a protocol based on Karnovsky (1965).
3) For hard plant tissues, like sugarcane epidermis stalk, acetone can safely be used in the dehydration steps (Negrão & Driemeier, 2022). Other alternatives consist of using ethanol.

FIXATION
Let samples be in contact with the fixation buffer for at least 2 days. This process will immediately deactivate the cell while promoting the conservation of the tissues.

POST FIXATION
Use a 0.05 M sodium cacodylate buffer at pH 7.2, adjusted with HCl, to perform a triple washing on the samples. Allow 10 min between each washing step.

DEHYDRATION & DRYING
Proceed with the dehydration by immersing the samples in a series of acetone solution concentrations (30, 50, 70, 90%). Allow 10 min between each immersion. Subsequently, perform three rounds of immersion in 100% acetone, with a 15-minute wait interval between each round.

Critical thickness for X-ray Transmission Scanning Microscopy (STXM)

1 We employed FIB-SEM sectioning to prepare thin slices of silica phytoliths for analysis with STXM. STXM is a sophisticated imaging technique used in a few synchrotron facilities over the globe. It is capable of pinpointing the precise location and chemistry of elements at a nanometric resolution. However, it is crucial to keep the thickness of the sample in check, especially for light elements like carbon, to accurately determine their chemical state. For this study, to effectively utilize STXM for carbon analysis, it was essential to ensure that the thickness of the sliced sample remained below 300 nanometers which is halfway the optimum thickness for Si and C K edges X-ray absorption measurements.

Plant material

2 The outer layer of the plant tissue (here we used sugarcane stalk) must contain numerous phytoliths. To extract sugarcane phytoliths, we manually peeled the stalk into strips of 2 mm thickness. These strips are subsequently cut into pieces of 1 cm^2 using a clean scissor. The plant material was then prepared following a the fixation, post fixation, dehydration and drying protocols (see guidelines & materials).

Mounting the Stubs for Electron Microscopy

3 Before mounting the aluminum stubs, it is important to label them with a pencil designated specifically for electron microscopy. Apply carbon tape to a small area of the stub, then carefully position the plant material onto the tape ensuring precision with the aid of a stereo microscope. Finally, apply a gold nanolayer of less than 10 nm thickness to the sample's surface to reduce charging during manipulation with FIB-SEM.

Preparing samples for FIB-SEM sectioning

4 Even though the plant material has already been coated with a gold nanolayer, it is necessary to provide additional protection to the specific surface intended for slicing. Therefore, a platinum strip measuring roughly 15 µm length × 2 µm width × 1 µm height was deposited onto the designated area of the phytolith (Fig. 1 b,c). This process was performed utilizing a beam current of 0.50 nA at 30 kV.

FIB-SEM milling

5 Subsequently, two trenches of roughly 25 µm length × 15 µm width × 15 µm height were carved out adjacent to the platinum strip via FIB milling (Fig. 1 d). This process involved employing a beam current of 30 nA at 30 kV, followed by a minor milling with 7 nA at 30

nA. Finally, lamellas of approximately 15 µm length × 15 µm height × 2 µm thickness were obtained (Fig. 1 f-l).

Detachment and transference of phytolith lamellas

6 The transfer of self-sustained lamellas onto the microprobe manipulator to a copper grid (Fig. 1 h-l) proceeded through the following steps using a 30 kV beam:

1. Cutting the right edge and the bottom of the lamella from the plant tissue with a beam current of 3 nA (Fig. 1 e).
2. Welding the microprobe manipulator with platinum at the upper left lamella corner with a beam current of 50 pA (Fig. 1 f).
3. Cutting the remaining left edge attached to the plant tissue with a beam current of 3 nA (Fig. 1 f).
4. Transferring and fixing the lamella to the FIB lift-out copper grid by welding platinum at its bottom's left and right corners with a beam current of 100 pA (Fig. 1 g-i).
5. Finally, all lamellas were thinned in the central region using currents ranging from 500 pA to 100 pA (Fig. 1 l).

Storage and transportation

7 The silica phytolith lamella is highly susceptible to damage during transportation due to its fragility. Storing the grids in dedicated containers and using membrane boxes for transport proved to be effective in safely delivering the phytolith lamella overseas.

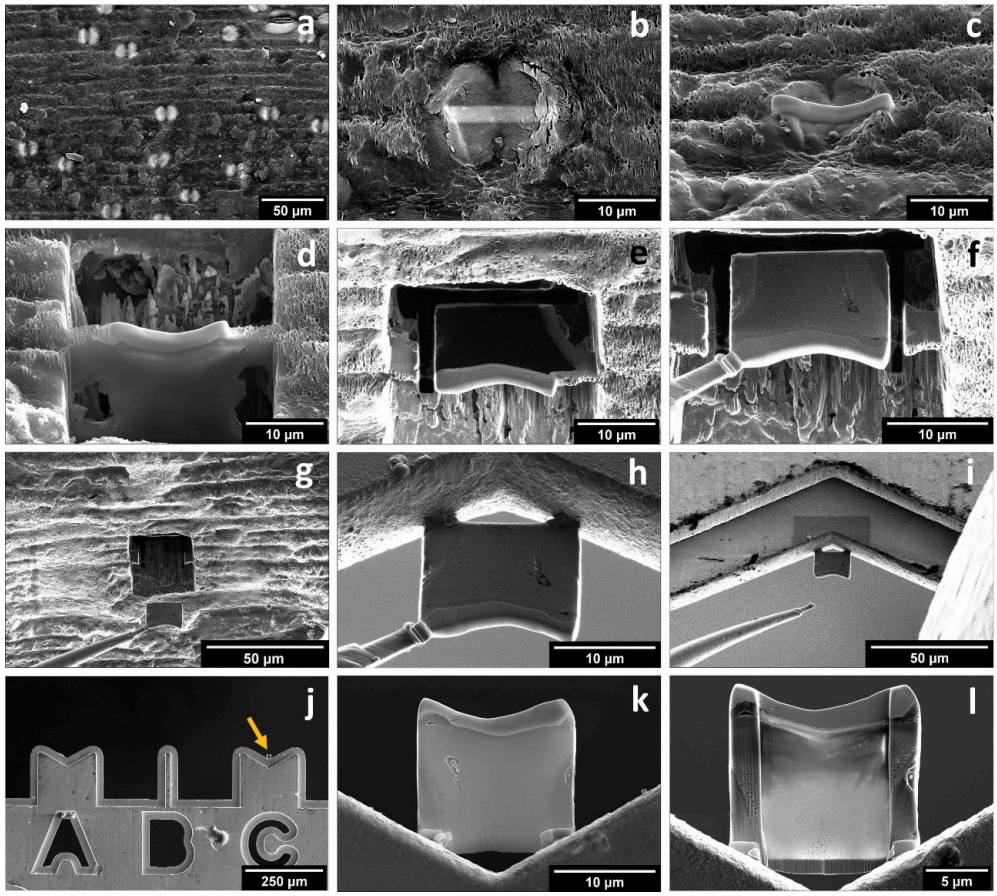

Fig.1 Scanning electron micrographs of the preparation steps of phytolith lamellas. (a) bilobate phytoliths at the surface of the sugarcane stalk (bright spots). (b-c) Platinum cover strip deposited along the longitudinal axis of a selected phytolith. (d-e) Trenches are carved by the focused ion beam to form the phytolith lamella. (f-g) Fixation of the micromanipulator to the platinum strip and removal of the lamella from the plant tissue. (h-i) Phytolith lamella fixed to a copper lift-out grid. (j) The orange arrow points to the lamella onto the "v" position of the grid. (k) Detail of the phytolith lamella fixed to the lift-out grid before (k) and after (l) thinning (<200 nm) the central region of the lamella under the focused ion beam.

Protocol references

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