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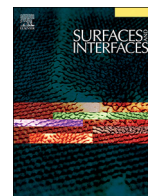
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Analysis of diatoms by holotomography

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A B S T R A C T

3D X-ray tomography was used to unravel the inner and outer morphology of a centric diatom namely the *Coscinodiscus* sp. We show how holotomography carried out with a 40 nm voxel size can provide detailed information about the sizes of the different parts of the diatom frustule. We have in particular analyzed quantitatively the pore size in the cribrum and cribellum parts of the diatom together with an estimation of the wall size. We evidence that in center of the diatom, walls are forming a hexagonal arrangement that is containing some pentagons to promote paving the space in 3D.

1. Introduction

Diatoms are among the most fascinating organisms found in nature [11]. These unicellular photosynthetic protists that belong to the stramenopiles are found in a large number of freshwater or marine environments and live inside an intricately silica shell. A rough estimate suggests from 50,000 to 200,000 species, making them among the most species-rich lineages of eukaryotes. They have several important ecological roles, contributing to various biogeochemical cycles with estimates between a fifth and a quarter of all photosynthesis on our planet [13]. By fixing carbon or converting it from carbon dioxide into sugar, diatoms also reduce the amount of carbon dioxide in the atmosphere just as terrestrial plants [13,4]. Diatoms are also a key source of food and energy for other organisms in many ecosystems so that many animals depend on diatoms either directly or indirectly for their growth. Diatoms are the principal marine organisms involved in the cycle of silicon, which constitutes about one-quarter of the Earth's crust.

As mention above, diatoms are capable of synthesizing a transparent cell walls made of silicon dioxide hydrated with a small amount of water ($\text{SiO}_2 + \text{H}_2\text{O}$). As silica is the main component of glass, they are often called "algae in glass houses". The cell wall or thecae which is named frustule consists of two overlapping parts known as valves. Since silica is impervious, diatoms have evolved elaborate patterns of perforations in their valves to allow nutrient exchange with the environment, and motility in some species. The proposed evolutionary

functions for these intricate shell designs include nutrient acquisition, control of diatom sinking rate, control of turbulent flow around the cell, and protection from grazing and viral attack [2]. These patterns have also an unprecedentedly high specific strength, exceeding that of all other reported natural biomaterials, which we attribute to the combination of the honeycomb sandwich plate architecture and extremely low flaw density in the constituent biosilica [1]. The ornamentation pattern of the valve, which are very aesthetic, are use for classifying diatoms. Three different classes were proposed by Round et al. [11]: the *Coscinodiscophyceae*, are centric diatoms with frustule that present a radial symmetry, *Fragilariophyceae* are pennate diatoms with bilateral symmetry but lacking a raphe, and *Bacillariophyceae*, pennate diatoms with a raphe [10,8].

Here we have focused our attention to a specific centric gender, the *Coscinodiscus*. Our major interest was to image in 3D this diatom in order to get a full description of its 3D architecture. This was achieved by using 3D- Holotomography at the ID16-B beamline of ESRF. With this technique we have investigated the frustule topography of *Coscinodiscus* sp. With the 3D array of this object we have determined many morphologic properties of the diatom such as the pores size, the domain walls and the shape of the outer and inner frustule membranes. With a better understanding of the diatom frustule structure from the nanometer scale up to whole cell we can foresee that we will have a powerful tool to unravel some key physical properties of porous biosilica membranes and thus anticipate potential technological

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applications. Comparison with other imaging technique is provided.

2. Experimental

Coscinodiscus sp. (referenced CCMP 584) come from the National Center of Marine Algae and Microbiota located in Maine-USA. *Coscinodiscus* sp. is maintained in laboratory seawater environment as described in Vartanian et al. [14]. The medium is sterilized by autoclave (120 °C, 25 min) and then completed at cold by the addition of orthosilicic acid ($C_{\text{final}} = 175 \mu\text{mol L}^{-1}$), hydrogen phosphate dipotassium ($C_{\text{final}} = 0.3 \text{ mmol L}^{-1}$) as well as a mixture of vitamins (biotin at $50 \mu\text{g L}^{-1}$, vitamin B12 at $50 \mu\text{g L}^{-1}$ and thiamine at $0.1 \mu\text{g L}^{-1}$). *Coscinodiscus* sp. culture is maintained for two weeks at 18 °C in polycarbonate flasks in an incubator with a constant day-night cycle (12–12 h) and a luminous intensity equivalent to $80 \mu\text{Em}^{-2} \text{ s}^{-1}$ during the day. The frustules of *Coscinodiscus* sp. are purified in order to remove organic matter to finally obtain the isolated inorganic frustules. The chemical purification protocol is based on 10 mL of *Coscinodiscus* sp. solution. Cells are washed 3 times with distilled water, filtered and are then dispersed in a mixture of 5 mL of sulfuric acid (96%) and 1 mL of nitric acid (80%). After 30 min of treatment, the pellet with silica is washed 5 times with filtered distilled water. The acid treatment separates the different parts of the frustule, the two valves and their girdle bands. *Coscinodiscus* sp. frustules are then suspended in a mix solution of ethanol:water with a ratio [1:4]. By a micropipette, 200 μL of solution are then spread on a microscope slide. *Coscinodiscus* sp. valves are mounted on a cylindrical micrometer silica tip for X-ray tomography analysis. This step was performed using a micromanipulator consisting of an optical microscope, a camera and a micromanipulator plate (xyz).

The 3D volumes have been acquired using X-ray nano-tomography [3] at the nano-analysis endstation ID16B at the ESRF having a focused beam of $50 \times 50 \text{ nm}^2$ [7]. Phase contrast imaging was performed using this focused beam as secondary source producing a conic and monochromatic beam with an energy of 17.5 keV and a high flux of $6.10^{11} \text{ ph s}^{-1}$. Thanks to the geometry, the magnification i.e. pixel size and field of view depends of the sample position between the secondary source and the detector, in this experiment, pixel size varied from 10 nm to 120 nm. For each tomography, about 2560 projections, were recorded on a PCO edge camera (2560×2160 pixels) along a 360° rotation with an exposure time of 100 or 200 ms per step, depending of the sample. 3D reconstructions were achieved in two steps: (i) phase retrieval calculation using an inhouse developed octave script and (ii) filtered backprojection reconstruction using ESRF software PyHST2 [9].

3. Results and discussion

A typical 3D Holotomography image of the frustule is shown in Fig. 1. With the 3D image of the diatom one can immediately appreciate the magnificence of this centric diatom. The inner layer exhibits a honeycomb-like structure called areolae in which large holes known as foramen are observed. The full size of the diatom is $75 \mu\text{m}$ and its thickness varies in the range 1.35–1.5 μm . The size of each foramen varies from one to one another. As a result, the foramen pattern does not exhibit a perfect honeycomb structure.

An evaluation of the foramen radius was made after thresholding the axial section of the diatom (see Fig. 2a and b). All the analysis of the pore size distribution was made with the FIJI software [12]. It was found that the distribution of foramen radii was mainly bimodal with a main mode located at $R_1 = 0.68 \pm 0.06 \mu\text{m}$ and a minor one located at $R_2 = 0.57 \pm 0.12 \mu\text{m}$ (see Fig. 2c). The analysis was carried out over 871 holes thus providing a reasonable statistic.

When moving from the inside of the diatom to the outside, one then find the cribrum which is the central part of the diatom. As shown in Fig. 3 the cribrum is made of an array of ill formed hexagons. It can be seen that the organization of hexagons can vary significantly from one place to one another. For instance, in the left part of Fig. 3 one can see

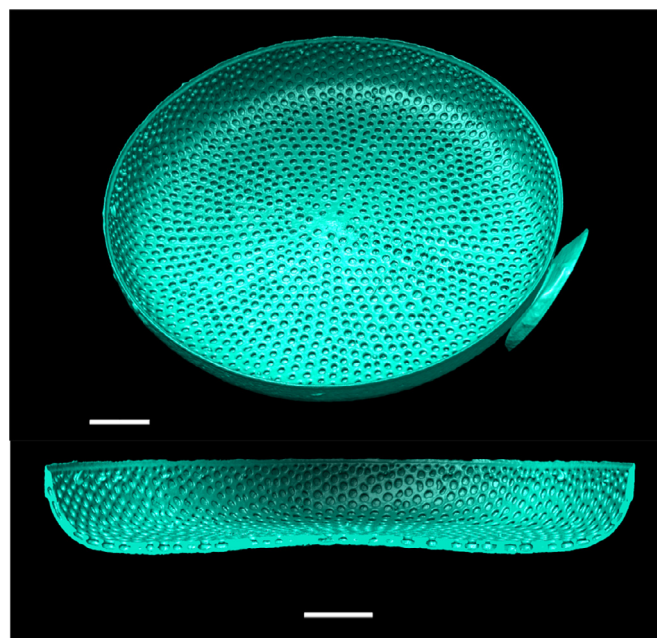


Fig. 1. A representation of the diatom frustule showing the nice architecture made of a large number of foramens. Scale bar = 10 μm . The size of this image was corresponding to a pixel size of 160 nm after reduction of the initial 3D matrix by a 3D factor of 64.

that the array forms a well ordered 2D hexagonal structure with a nice pentagonal defect located in the top part. The presence of such a defect is not fortuitous as such defects are necessary to pave the space in 3D. On the right part of the figure, a neighboring area exhibits a much more disordered structure in which hexagons are irregular and with a bigger number of pentagons. Every hexagon is made of biosilica walls separating the cavities located between the inner part of the frustule and the cribrillum.

In order to understand the shape of the cribrillum it is necessary to zoom at high resolution the 3D image of the diatom. This is shown in Fig. 4.

The top part of Fig. 4 shows a small part of the inner frustule on the left panel and the cribrillum on the right panel. Below we have selected a few images taken as top views at different altitudes in the frustule. One can clearly see on these images the richness of the structure with big foramens followed by biosilica walls and finishing with very small pores located in the cribrillum. The analysis of the 20 foramens in the image (without taking in account the ones at the edges gives an average radius of 0.63 μm . This value is in reasonable agreement with the one (0.68 μm) found on a much larger series of Fig. 2. The biosilica walls have a width of about 200 nm in the middle of each segment. Their width slightly increases when one moves towards a node. The small holes seen in the cribrillum do not really exhibit a 2D hexagonal ordering except locally. Their diameter is of the order of about $125 \pm 20 \text{ nm}$ (see Fig. 5).

With a voxel size of 40 nm, it is clear that it was nevertheless impossible to identify any features below this value. This is why we could not resolve the hierarchical structure contained in the cribrillum. In particular very small pores ($45 \pm 9 \text{ nm}$) as evidenced by Losic et al [6] by Atomic force microscopy in high resolution mode were not identified. Our results are on the other hand perfectly in line with ones found by FIB-SEM analysis of the same specie [15] with the great advantage that X-ray tomography is non destructive compared to FIB-SEM.

So far the main limitation of the X-ray holotomography compared to TEM or AFM is a coarser resolution. Indeed it is difficult to image materials with a resolution better than 30 nm with X-ray holotomography while SEM (1–20 nm), TEM (0.1 nm) and AFM (1 nm) lateral

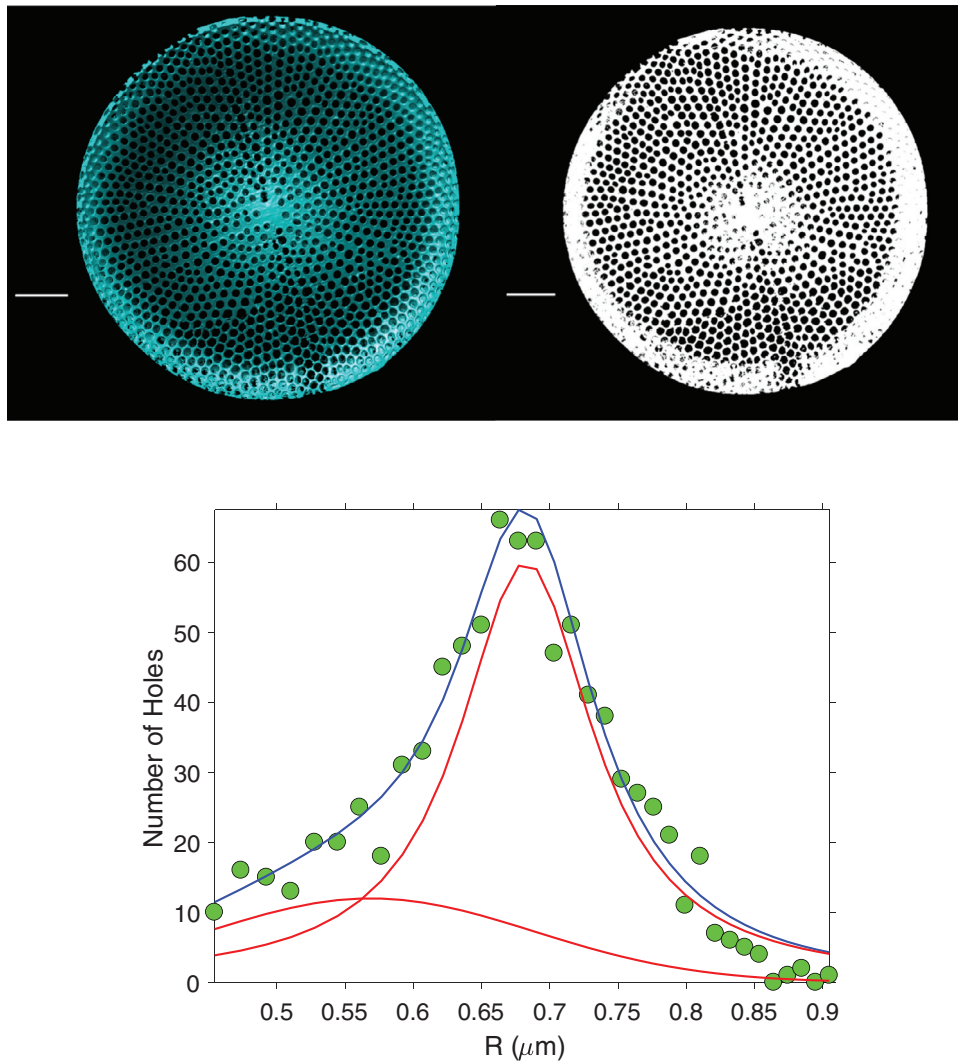


Fig. 2. Cross section of the diatom before and after binarisation of the image. The bottom panel shows the distribution of the foramen radii found in the frustule. Scale bar = 10 μm and voxel size 160 nm.

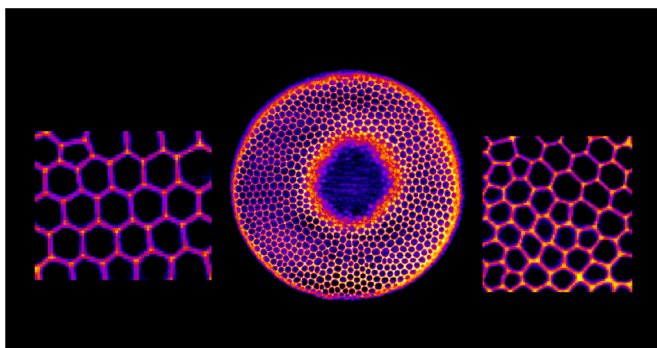


Fig. 3. Magnified view of the areolae of the frustule showing the presence of biosilica walls with either a nearly perfect 2D hexagonal structure or with a disordered one.

can go down to much higher resolution [5]. Yet with the advance of Extremely Bright Sources (as what will be available after the E.S.R.F. upgrade) one should achieve a better resolution (< 10 nm) in the near future. The main advantages of holotomography over these techniques is that it does not require the sample to be under vacuum as is the case for SEM, and TEM and it allows the collection of a 3D image. The 3D image is of crucial interest for having an overview of the inner and

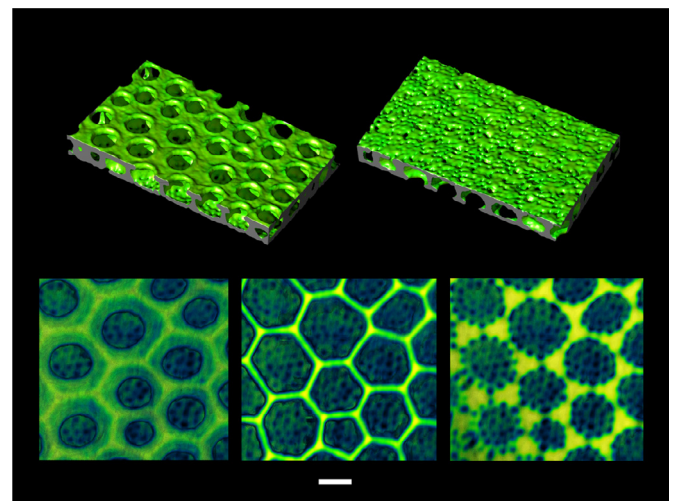


Fig. 4. Top part 3D representation of the areola and of the cribellum showing the large difference in size between the pores contained in both areas. All images are obtained with the original voxel size of 40 nm. The very small holes present in the cribellum can be very well identified. Scale bar 1.4 μm .

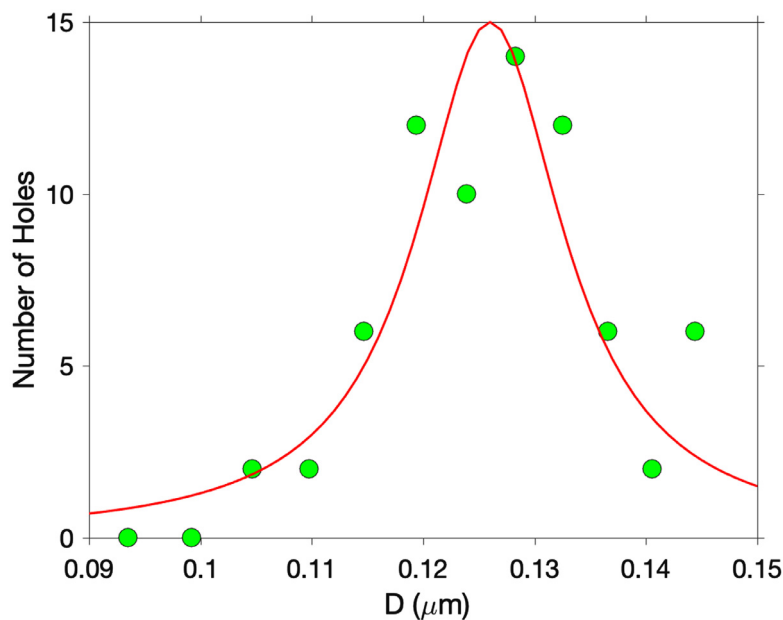


Fig. 5. Distribution of the diameters of holes in the cribellum.

outer part of a material. Note that a 3D image is also the only way to observe the inner morphology of a material which is not feasible using SEM or TEM. This also provides the possibility to extract the mechanical properties of such tiny materials as the 3D array can be processed using finite elements analysis.

4. Conclusion

In this paper we have shown that 3D holotomography is a useful technique to image in 3D the magnificent structure of diatoms. In particular, one can determine by this technique the pore size distribution of the areola and cribellum and evidence the arrangement of the silica walls (cribrum) inside the frustule. As the typical voxel size that we could achieve was 40 nm, it was impossible to probe the very fine structure that was previously observed in the cribellum by high resolution AFM [6]. In this respect holotomography provides similar information as the one obtained by FIB-SEM [15]. This first study of diatoms by this technique is of great interest to foster further studies of these materials by finite elements thus providing useful information about their mechanical properties.

Conflict of interest

None.

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