Quantitative 3D elemental microtomography of Cyclotella meneghiniana at 400-nm resolution

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X-ray fluorescence tomography promises to map elemental distributions in unstained and unfixed biological specimens in three dimensions at high resolution and sensitivity, offering unparalleled insight in medical, biological, and environmental sciences. X-ray fluorescence tomography of biological specimens has been viewed as impractical—and perhaps even impossible for routine application—due to the large time required for scanning tomography and significant radiation dose delivered to the specimen during the imaging process. Here, we demonstrate submicron resolution X-ray fluorescence tomography of a whole unstained biological specimen, quantifying three-dimensional distributions of the elements Si, P, Cl, K, Ca, Mn, Fe, Cu, and Zn in the freshwater diatom Cyclotella meneghiniana with 400-nm resolution, improving the spatial resolution by over an order of magnitude. The resulting maps faithfully reproduce cellular structure revealing unexpected patterns that may elucidate the role of metals in diatom biology and of diatoms in global element cycles. With anticipated improvements in data acquisition and detector sensitivity, such measurements could become routine in the near future.

Results and Discussion

Diatoms represent a useful test case for tomography at submicron length-scales because of their distinctive 3D structure. Figs. 1, 2, and 3 present reconstructions of fluorescence tomography measurements of C. meneghiniana. A short video of the reconstructed volume is available (Movie S1). The resolution of a tomographic reconstruction is not generally isotropic but depends strongly on the number and angular spacing of the measured projections. We estimate the resolution of our reconstruction in Fig. 4 by comparing the measured projections with an equivalent reprojection of the reconstructed data. These show differences of less than 2–3 pixels (of 150 nm size) across all projections, and so we estimate the resolution to be better than ~400 nm.

This resolution is sufficient to reveal several structures typically associated with the opaline silica frustule of this species: two valves that form the ends of the cylindrical cell, highly silicified ribs that run from the edge toward the center of each valve face, and the sinuous topology of the central valve face. Girdle bands—overlapping rings of silica that encase the region between the valves—are less obvious in the silicon map but show up clearly in iron. Sulphur—a proxy for protein—indicates the cytoplasm is distributed as a thin layer beneath the frustule and in a pillar parallel to the cell’s longitudinal axis. The remaining interior consists of a large central vacuole. Associated with the cytoplasmic pillar are two bodies enriched in P, K, and Ca, with a third similar body located along one of the valve faces. These may represent aggregations of Ca and K salts of either P-rich nuclear material.
or polyphosphates. Elevated Zn concentrations within the cytoplasmic pillar may indicate the presence of Zn finger proteins. While such proteins are typically closely associated with nuclear material and are often found in association with nuclei in freshwater diatoms (6), they dissociate from the DNA prior to and during mitosis (13). Multiple smaller P-rich bodies, which possibly represent polyphosphate bodies or local aggregations of ribosomes containing nucleic acids, are apparent when the isosurface threshold is reduced (also visible in Fig. 4).

Fig. 3. Detailed exploration of the 3D structure of C. meneghiniana. (A–C) Longitudinal cross sections of C. meneghiniana, showing the distributions of Si (gray), Fe (orange) and Mn (red) on a fine scale. A shows all three elements, showing that Mn and Fe are contained within the Si frustule. This is particularly apparent at the cross-sectional cut of the frustule. Mn is associated with a ridge of Si inside the cell that demarks the boundary between the valve face and the girdle bands. B presents only Si and Mn, showing quite clearly that one of the Fe bands in the lower half of the cell coincides with a region of light silicification. The location is consistent with the a region of cell expansion, which should occur near the junction between the epithea—the older, static part of the frustule—and the hypotheca—the new daughter half of the frustule that is smaller and expanding in the girdle-band region. We argue in the text that Fe-containing proteins associated with silicification may become embedded within the frustule. The Fe band in this region may be associated with the initiation of silicification prior to the Fe being embedded. C presents only Mn and Fe, showing the Fe banding more clearly and the colocalization of Mn and Fe at the junction of the valves and the girdle bands. (D–F) Longitudinal slice of C. meneghiniana, showing the distributions of P (blue), S (yellow), Cl (yellow-green), and Zn (purple), and their detailed associations. The images clearly show two of the major P-bodies, the fact that S and Cl delineate the central isolated pillar and patches of cytoplasm just beneath the frustule, the association of the P-bodies with the pillar, the presence of a large empty vacuole, and the dense concentration of Zn in the pillar. Zn is seen associated with S and Cl in other areas and periodically in the frustule. (G) Transverse slice of C. meneghiniana, showing the cytoplasmic pillar and the vacuole and the association of two of the P-bodies with the pillar.
concentrated within the silica frustules in two distinct rings located near the convergence of the girdle bands and the frustule valves (see Fig. 3). Manganese in diatoms is generally believed to be primarily contained within the oxygen-evolving complex of Photosystem II and in superoxide dismutases (14), neither of which is believed to be associated with the frustule or involved in silica deposition. In the 3D maps presented here, Fe is also incorporated into the siliceous shell but only in conjunction with the girdle bands. Iron is presumed to be utilized primarily for photosynthetic and respiratory electron transport proteins as well as nitrate reductase and other redox enzymes (15). However, at high concentrations Fe can become incorporated into diatom frustules (16).

The high metal conditions in the culture medium could have caused metal ions to become associated with cell surfaces directly via adsorption or indirectly via association with adsorbed oxides and colloids. It is possible that such mechanisms could produce metal distributions that would not be observed for this species in nature. However, the clear localization of Fe and Mn within the specific regions of the frustule indicates codeposition of metals within silica via a process unique to these locations in the cell, whereas adsorption should have produced a more uniform distribution. Moreover, the cellular concentrations of Zn and Fe in the imaged cell are comparable to those measured on single diatom cells collected from two pristine regions of the ocean (17–19) (Table S1). While cellular Mn concentration is fourfold greater in the imaged cell than in oceanic diatom cells, it is also 20-fold less than reported for another freshwater diatom grown in culture at environmentally realistic Mn$^{2+}$ and Zn$^{2+}$ concentrations (20). Because C. meneghiniana is common to rivers and lakes in which trace elements are far more abundant than in the open ocean, our findings are likely relevant to these natural conditions.

A recent transcriptome analysis of a model marine diatom suggests that Fe may serve as a cofactor for some processes involved in silica deposition or silicon metabolism (21). The presence of Fe and Mn within the frustules may reflect incorporation of metallo-proteins within deposited silica. Previous studies have noted the colocalization of certain proteins within these structures (22). Close inspection of the distributions in Fig. 3 reveal a band of Fe in a region of low Si that corresponds to the location of new silica deposition associated with a region of cell expansion. This pattern is consistent with the hypothesis that the Fe contained in the frustules reflects the presence of metallo-proteins involved in initiating silica polymerization. However, a role for Fe$^{3+}$ adsorption to that specific region of the cell followed by uptake and incidental incorporation into the frustule cannot be ruled out. Differences in the composition of the girdle bands and the valves may result from the valves being formed within the cell during cell division inside specialized silica deposition vesicles while the girdle bands form at the periphery of the cell as it expands during interphase (23).

The 3D information provided by tomography allows unambiguous assessments of elemental colocalization within cells. Scatter plots, such as shown in Fig. 5, are often used to examine elemental colocalizations and to elucidate causal relationships (24). However, scatter plots derived from various 2D projections show different signatures, due to the changing influence of elemental overlay, as observed here for Si and Ca. On the other hand, scatter plots derived from the tomographic reconstruction unambiguously show that there is absolutely no colocalization of Si and Ca (upper) while there is a very clear correspondence of Ca and P (lower). Further, quantitative 3D scatter plots provide insight into chemical composition through molecular ratios: in this case the P:Ca mass ratio indicating a well-defined molar ratio of 2.00 (±0.03):1. It does not appear that distinct P:Ca signatures exist that can be used to distinguish separate inorganic polyphosphates and nucleic phosphate moieties within the cell.
7 h of actual data acquisition were required for the present measurements, and this is the time that will be needed for an identical measurement using such an advanced detector system. Additional time savings resulting from increased detector sensitivity should reduce the measurement time (for an equivalent dataset) to around 2.5 h.

**Materials and Methods**

The measurements were made using the X-ray fluorescence microprobe at beamline 2-ID-E of the Advanced Photon Source (Fig. 6). A pair of X-ray zone-plates (160-μm diameter, 70 nm outermost zone, 300 nm to 1 μm Au) were “stacked” by aligning them in the near-field and used to focus X-rays. Due to the finite source size and the 2-ID-E beamline optics the zone-plate illumination is not completely coherent, and so the focal spot does not reach to the theoretical minimum size. A Cr knife-edge scan was used to determine the size of the focus to be 270 nm (Gaussian FWHM of intensity derivative). X-ray fluorescence in the specimen was measured using a silicon drift diode detector.

Cells of *C. meneghiniana* were grown in a modified WC-L1 media that consisted of Milli-Q (>18.2 MΩ) water to which were added the following salts (final concentrations in parentheses): NaHCO$_3$ (0.15 mM), CaCl$_2$ · 2H$_2$O (0.25 mM), Na$_2$HPO$_4$ · 7H$_2$O (0.05 mM), KCl (0.1 mM), MgSO$_4$ · 7H$_2$O (0.15 mM), NH$_4$Cl (0.05 mM), NaNO$_3$ (221 μM), NaH$_2$PO$_4$ · H$_2$O (42 μM) and Na$_2$SiO$_3$ · 9H$_2$O (106 μM) (31). A trace metal solution containing the chelator ethylene diamine tetracetic acid (EDTA) was added to attain the following concentrations: Na$_2$EDTA · 2H$_2$O (11.7 μM), FeCl$_2$ (11.7 μM), CuSO$_4$ · 5H$_2$O (0.04 μM), NaMoO$_4$ · 2H$_2$O (0.026 μM), ZnSO$_4$ · 7H$_2$O (0.076 μM), CoCl$_2$ · 6H$_2$O (0.042 μM), MnCl$_2$ · 4H$_2$O (0.9 μM). Cultures were inoculated with ~1 × 10$^5$ cells ml$^{-1}$ and allowed to grow 4 d at 16°C under cool fluorescent lighting at an irradiance 120 μEin cm$^{-2}$ sec$^{-1}$ and a light:dark cycle of 14 h:10 h before reaching early stationary phase (9 × 10$^5$ cells ml$^{-1}$).

The diatoms were prepared for X-ray imaging by dropping a small volume of cell suspension onto an electron microscopy grid and allowing it to air dry. The diatom was scanned through the focal plane in 150 nm steps in two axes orthogonal to the beam and was rotated in 6° intervals to obtain projections over as great a range as possible. Ultimately the specimen substrate and aligning factors affecting growth, silicification, and trace elemental content of diatoms at the subcellular level, future tomographic analyses using X-ray fluorescence microprobes can improve our ability to reconstruct past environmental conditions and to predict future outcomes. The trace element content of diatomaceous biogenic silica in sediments has been used to reconstruct past climate and ocean conditions (25, 26). An improved understanding of cellular metal-silica associations should help constrain interpretations of paleoceanographic data. Moreover diatoms account for approximately 20% of global carbon fixation (27) and facilitate the transport of photosynthetically fixed CO$_2$ from the surface ocean to deep water (28). Therefore, understanding the bases of variations in the elemental content should help us determine how the supply of Fe and other elements via dust or upwelled deepwater may cause changes in atmospheric CO$_2$ over interglacial time scales (29).

The total time for the measurement was about 36 h, which presently limits routine application of the method. However, this time was dominated by user intervention (18 h), resulting from the manual nature of this prototype setup. Approximately 240,000 single-point fluorescence spectra were recorded to form the 24 projections, and so significant further savings can be obtained by reducing per-pixel measurement times. In particular, we note that the next most significant time cost (11 h) resulted directly from the step-and-dwell operation of the scan, which is strongly linked to the architecture of present generation fluorescence detectors. Next-generation detectors featuring event-mode readout (30) will completely eliminate this time cost.
rotation axis was defined near to the center of the diatom by applying a simple linear transform to each projection based on its rotation angle. Tomographic reconstruction used the algebraic reconstruction technique (33, 34) to obtain quantitative volumetric maps for each detected elemental species. The voxel size is determined by the 150 nm sampling interval used to obtain the projections; however, the resolution is limited by the X-ray resolution and the alignment of the rotation series and is estimated to be better than about 400 nm, as described in Fig. 4.

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Movie S1. The movie shows the 3D elemental distribution of *C. meneghiniana* with a resolution of approximately 400 nm. Several moments within the animation serve to highlight various aspects of the structure, and these are detailed below.

1. Initially the entire structure is presented, but only the external siliceous shell is evident.
2. The shell is stripped off to reveal the internal organization. Three main structures are evident:
   a. bands of Fe and Mn residing with the siliceous frustules;
   b. three spheroidal organelles, dominated by K, P, and Ca;
   c. the cytoplasmic pillar, dominated by Cl, Cu, and Zn.
3. K, P, and Ca are revealed in sequence, showing that these elements are strongly associated within the organelles.
4. Cl, Zn, and Cu are shown in sequence, showing strong association with the cytoplasmic pillar, but also that Cu has further associations with some structures coating the frustule.
5. The Mn and Fe rings are revealed (completing the sequence of internally distributed metals), and the diatom is rotated for further visualization of the internal organs.
6. The cytoplasm (Cl, Zn, and Cu) is hidden, and half of the siliceous frustules is revealed. Rotation of the diatom shows the exact location of the Mn, and Fe rings, buried within the inner part of the frustule.

*Movie S1 (MOV)*
Table S1. Comparison of cellular Mn, Fe, and Zn concentrations (mmol/L)

<table>
<thead>
<tr>
<th>Element</th>
<th>Cyclotella meneghiniana</th>
<th>Equatorial Pacific</th>
<th>Southern Ocean</th>
<th>Stephanodiscus hantzschii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Fe</td>
<td>High Fe</td>
<td>Low Fe</td>
<td>High Fe</td>
</tr>
<tr>
<td>Mn</td>
<td>0.25</td>
<td>0.06</td>
<td>n.d.</td>
<td>0.03</td>
</tr>
<tr>
<td>Fe</td>
<td>3.5</td>
<td>0.17</td>
<td>2.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Zn</td>
<td>0.71</td>
<td>0.18</td>
<td>n.d.</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Comparison of concentrations in the diatom imaged in this paper with single cell measurements (using 2D SXRF) in diatom cells collected from the open ocean and freshwater diatoms grown under defined, environmentally realistic concentrations. "Low Fe" refers to concentrations measured for cells collected under ambient conditions in the Equatorial Pacific (1) and the Southern Ocean (2). "High Fe" refers to concentrations measured 6 d after a mesoscale iron addition in the Southern Ocean (2), or 48 h after an iron addition to 20 L carboys during experiments in the equatorial Pacific (3). Elemental composition of *Stephanodiscus hantzschii* was determined by ICP-MS analysis of cells grown at calculated Mn$^{2+}$ and Zn$^{2+}$ concentrations similar to those present in this study (4). n.d. = no data.