**Imaging and quantifying homeostatic levels of intracellular silicon in diatoms**

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Diatoms are an abundant group of microalgae, known for their ability to form an intricate cell wall made of silica. Silicon levels in seawater are in the micromolar range, making it a challenge for diatoms to supply the rapid intracellular silicification process with the needed flux of soluble silicon. Here, we use three-dimensional cryo–electron microscopy and spectroscopy to quantitatively analyze, at submicrometer spatial resolution and sensitivity in the millimolar range, intracellular silicon in diatom cells. Our results show that the internal silicon concentration inside the cell is ~150 mM in average, three orders of magnitude higher than the external environment. The cellular silicon content is not compartmentalized, but rather unevenly distributed throughout the cell. Unexpectedly, under silicon starvation, the internal silicon pool is not depleted, reminiscent of a constitutive metabolite. Our spatially resolved approach to analyze intracellular silicon opens avenues to investigate this homeostatic trait of diatoms.

**RESULTS**

We chose to investigate the diatom model species *Thalassiosira pseudonana*, as the silicon pathways in this species are relatively well studied (12). The cell wall of *T. pseudonana* is barrel-shaped and is composed of two intricate circular valves and a more simple cylindrical structure made of several girdle bands (Fig. 1, A and B). To investigate the pristine intracellular environment within the cells, we used high-pressure freezing (HPF). This cryo-immobilization technique vitrifies cells in their native state, without any use of chemical fixation or staining. Next, we imaged the cellular organization within the cells with cryo–scanning electron microscopy (cryoSEM), which gives 2D images of a random fracture plane through the diatom cell wall occurs inside specialized organelles within the cytoplasm and is tightly linked to the cell cycle (9). Only after completion of the precipitation and morphogenesis process of the silicified element is it exocytosed to the cell exterior. A crucial requirement for this process is the transport of the silica building blocks from the environment, through the cell cytoplasm, into the silicifying organelle (10). These soluble intracellular silicon species, which are bioavailable and not yet incorporated into the insoluble silica phase, were termed silicon pools (11). It is established that diatoms can maintain a substantial intracellular pool of these silicon building blocks (12–15). Nevertheless, even after half a century of investigations into silicon metabolism in diatoms, it is still unclear how much silicon can be stored in the cell, in which cellular locations, in which chemical forms, and whether intracellular silicon concentrations fluctuate over time in response to physiological or environmental perturbations. The experimental techniques that have been used to study diatom silicon pools, namely, isotope labeling, nuclear magnetic resonance, and pulse chase of tracers, are limited in their ability to address such questions, since they probe bulk chemical information from specific subcellular volumes and some of them involve fractionation steps that may affect the chemistry of labile silicon species.

Electron microscopy has the needed sensitivity and spatial resolution to characterize silicon pools on the single-cell level. Nevertheless, previous electron microscopy reports could not directly detect silicon content in diatom samples (16–18). This limitation is mostly due to the inherent incompatibility of conventional sample preparation techniques with the preservation of soluble inorganic phases. In recent years, the development of cryo–electron microscopy modalities enables the investigation of biological samples vitrified in their native state, without any chemical fixation, modification, or staining (19). These techniques have facilitated the identification of new intracellular mineral pools in various organisms and, when coupled with spectroscopic analysis, have provided chemical information on the composition of metals and other inorganic compounds in the cell (20–22).

Here, we report the development of an experimental procedure to quantitatively analyze the chemical composition of intracellular Si pools in single diatom cells using cryo–electron microscopy. We used a three-dimensional (3D) imaging approach to reconstruct the cellular organization of diatoms. Simultaneously, we extracted chemical information from specific subcellular volumes and quantified the distribution and concentration of silicon within these volumes. Performing these experiments on cells grown under different environmental conditions allowed us to evaluate the response of the intracellular silicon concentration to the available silicon in the growth medium.
We obtained 3D datasets of diatom cells by using a cryo-focused ion beam scanning electron microscope (cryoFIB-SEM) slice-and-view approach. In this technique, the focused ion beam (FIB) removes a few-nanometer-thick layer from the sample and then the cryoSEM acquires an image of the newly exposed plane. Repeating these steps yields a 3D data stack of the entire cellular volume at a nanometer-scale resolution. Figure 1D shows a typical FIB-sliced plane in the cell, imaged with the cryoSEM detectors, while Fig. 1E shows a 3D volume rendering of the entire cell volume, demonstrating the closely packed organization of the organelles within the cell.

Energy-dispersive x-ray spectroscopy (EDS) is a sensitive analytical tool for the quantitative measurement of elemental composition. This is achieved by collecting the element-specific x-ray photons generated by the interaction of the electron beam and the sample. We combined EDS with cryoSEM to map silicon-rich locations inside *T. pseudonana* cells (Fig. 2, A and B). The EDS maps record an intense silicon signal from the cell circumference, arising from the mature cell wall. In several locations within the cells, the silicon signal was stronger than the background level (Fig. 2B), pointing to a possible concentrated silicon pool. However, since the photons detected by EDS are generated relatively deep within the sample, we could not rule out that this silicon signal is due to mature cell wall elements buried below the sample surface.

To preclude this possibility, we developed a dedicated cryoFIB-SEM-EDS approach that allows the collection of EDS data that are unequivocally intracellular. In this procedure (Fig. 2C), the cryoFIB-SEM starts collecting images from the top of a cell and is stopped when it reaches a plane of interest. The sample is then coated with a conductive layer, and an EDS map is generated to analyze the composition of the exposed plane. Subsequently, the cryoFIB-SEM acquisition is resumed until an additional volume of few micrometers below the analyzed plane is recorded. This methodology offers the advantage of being able to reconstruct the entire volume of the cell, including the specific subvolume that yields the EDS signal. The combined datasets enabled us to determine whether buried cell wall elements contribute to the intracellular silicon signal.

An EDS analysis of a diatom cell is presented in Fig. 2 (C to E). A cell was milled for about a micrometer and then EDS maps were collected for that plane within the cell (Fig. 2D), followed by an additional milling of 2 μm into the cell (fig. S1G). At first glance, the silicon in the cell wall appears to dominate the silicon distribution map, but careful examination of the pixels with lower silicon content shows that the cell’s interior has significantly more silicon counts than the extracellular background (Fig. 2E). The intracellular distribution, however, is not completely homogeneous; for example, the vacuole in Fig. 2 (D and E) gives a weaker silicon signal than the chloroplast. Although the EDS spatial resolution is lower than the SEM imaging owing to the large interaction volume, we discard the possibility that the intracellular silicon signal is simply a broadening of the cell wall signature. This is because such a broadening should be symmetric, and the extracellular medium does not transmit significant silicon signal from regions beyond 400 nm from the cell wall (see also fig. S2 for EDS simulations). Together, the combination of cryoFIB-SEM and EDS analysis allows the identification of intracellular silicon that is not associated with the mature silica cell wall.

We used this experimental approach to collect EDS data from several *T. pseudonana* cells and analyzed their intracellular silicon concentrations. Figure 3 (A to D) shows cryoFIB-SEM images and EDS maps of four different cells that were milled to expose their interior. Figure 3E shows the average silicon signal of each cell.
interior, namely, a silicon signal that does not arise from mature silica elements (see figs. S1 and S3 for complete spatial and spectral data). Different cells show different silicon contents, from a low amount, just above the noise level (Fig. 3A), to a distinct enrichment of the cell cytoplasm (Fig. 3, B to D; see also fig. S4 for further analysis). These data point to some heterogeneity within the cell population. As the unsynchronized culture contained cells at all stages of cell wall synthesis, we could not determine whether this variability is random or linked to specific metabolic cell states.

As our EDS measurements hold the possibility to quantify and compare silicon concentrations (as long as identical acquisition parameters are used and factors such as flat surfaces and measurement times are controlled), we developed the means to perform such quantitative analyses. As the first step, a sodium silicate solution containing 20 mM silicon was high-pressure frozen, and a flat plane was milled with the FIB within the vitrified homogeneous volume. We collected EDS spectra using several combinations of acceleration voltages and electron currents (Fig. 4A and fig. S5). These spectra

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**Fig. 2. Intracellular elemental mapping of T. pseudonana cells.** (A) A cryoSEM image of a freeze-fractured cell and (B) the corresponding EDS silicon map, which shows a silicon signal within the upper part of the cell. (C) The cryoFIB-SEM-EDS workflow. First, a cell is partially milled and then an EDS map of the exposed surface is collected, followed by additional milling and imaging of the remaining fraction of the cell. (D) Electron image and EDS elemental maps of the plane shown in (C). (E) Normalized intensity map of the Si map in (D), which offers better visualization of the low concentrations of silicon within the cell. The graph shows the silicon signal along the three white lines in the image, averaged from each corresponding rectangle. Note that intracellular silicon levels are higher in the cell relative to its environment. a.u., arbitrary units.

**Fig. 3. Intracellular silicon pools in T. pseudonana cells.** (A to D) FIB-milled planes within high-pressure frozen T. pseudonana cells. An electron image is shown with the accompanying EDS-normalized intensity silicon map (3D data of the volume milled before and after the EDS analysis can be found in fig. S1). (E) EDS spectra showing the silicon and phosphorus peaks, taken from the areas indicated by dashed lines in the EDS maps (full EDS spectra can be found in fig. S3). The two circles in cell D and their associated spectra demonstrate that contributions from specific pixels close to the cell wall, which might carry a signal from mature silica, do not significantly affect this analysis. The inhomogeneous Si signal from this cell wall is due to the contributions of both valves and girdle bands, which vary in their silica content (see also fig. S1).
show that EDS sensitivity increases with higher electron flux at higher energies. Therefore, all the analyses of T. pseudonana cells reported here (Figs. 2 and 3 and Table 1) were performed using an acceleration voltage of 8 kV and a current of 500 pA, to maximize detection sensitivity. Next, we measured a series of silicon-containing solutions with increasing silicon concentrations. Our results show a positive correlation between the silicon concentration and the emitted silicon signal (Fig. 4B). The integrated area of the silicon peak is a direct indicator of the number of silicon-related photons detected in each sample. This parameter, the cumulative silicon count, shows excellent correlation to the concentration of silicon in the solution (Fig. 4C), allowing us to establish a robust calibration curve and a minimal detection limit for silicon, which is ~20 mM.

In the following, we use this correlation to determine, in a direct and independent way, the silicon concentration in selected areas within the EDS maps.

We used the synthetic silicon-containing samples to further calibrate the EDS analysis. It should be noted that although the magnification should not affect the EDS analysis, we noticed that when the acquisition parameters were close to the sensitivity threshold (low acceleration voltage and electron current), higher magnifications yielded a stronger signal (Fig. 4D). We attribute this phenomenon to beam-induced water sublimation and took care to use only the 8 kV/500 pA settings to keep the linear relation between EDS signal and silicon concentration.

To determine the sample area in the EDS map that needs to be averaged to give a consistent silicon measurement, we compared the spectra of different areas of the same synthetic samples (Fig. 4E). We found that in a 200 mM silicon sample, a reliable reading can be obtained from an area of ~1 μm². In the more challenging 20 mM sample, an area of 10 μm² is needed, which is comparable to a cross section of a cell. Using computer simulations and our acquisition parameters, we calculated the depth within the sample from which most of the signal (~90% of the emitted photons) is generated (Fig. 4F) and found it to be the top micrometer of the cellular sample.

Applying the calibration curve of Fig. 4C to the EDS data of T. pseudonana cells yielded the average intracellular silicon concentration for each cell. For example, in the cells shown in Fig. 3 (A to D), the silicon concentrations are 63, 128, 208, and 254 mM, respectively (the silicon concentration of the cell in Fig. 3A is the lowest we found and that of the cell in Fig. 3D is the highest), and that in the cell of Fig. 2 (C to E) is 169 mM. Compiling the total silicon concentration results extracted from the analysis of 14 cells shows an average
intracellular silicon content of 140 ± 51 mM (Table 1; see also table S1 and figs. S6 and S7 for a detailed report). A more conservative analysis of intracellular areas that are ~1 μm away from the cell wall gave an average silicon concentration of 117 ± 44 mM (table S1 and figs. S6 and S7), putting the uncertainty of the quantification procedure around 15%. This is a narrow concentration range, which demonstrates a silicon concentration difference between the cytoplasm and the environment of at least three orders of magnitude.

We further used our approach to investigate whether silicon starvation, and the resulting synchronization of the cell cycle within the culture, will affect the intracellular silicon pools. It is established that transferring an exponentially growing culture to a silicon-deplete medium will cause cell cycle arrest at a preferred stage of the cell cycle (23, 24). Once silicon is added to the starved culture, the cells start to form new silica elements in a synchronized fashion (fig. S10). Our cryoFIB-SEM-EDS analysis of 26 silicon-starved cells gave an average intracellular silicon concentration of 187 ± 58 mM (Table 1), demonstrating that the cells did not lose their silicon pools as a result of the silicon starvation. Measurements of cells 1.5 hours after replenishing the starved culture with silicon (a time period when they synchronously resume silica formation) gave an average value of 146 ± 36 mM silicon pools. Overall, the intracellular silicon content of the cells rose marginally, but significantly, when the cells sensed a silicon limitation and returned gradually to the normal level once silicification and cell growth resumed.

**DISCUSSION**

The intracellular silicon content of diatoms has been viewed as a dynamic reservoir that is regulated by the needs of the cell for the silicification process (4, 25, 26). According to this prevailing view, the diatom cell concentrates silicon from its dilute environment into specific compartments or by specialized macromolecular carriers inside the cell, which, in turn, supply the silicification organelle with the silicon needed for silica formation (27). Our results point to a different mechanism, in which intracellular silicon levels, throughout the cell cycle, are kept almost constant, at around 150 mM, a concentration much higher than in the external environment. This resembles the homeostatic ability of cells to regulate the intracellular concentration of other inorganic ions, such as sodium, potassium, and calcium, which are also inhomogeneously stored in subcellular locations such as the ER or vacuoles. The high millimolar silicon concentration values are similar to those of other prevalent ions within the cell (see the relevant peaks in fig. S3), placing the regulation mechanisms of silicon in line with other ion-transport cellular processes. A rough calculation of the number of silicon atoms in the intracellular pool yields an amount equivalent to that needed to form a single new valve (see Materials and Methods).

The fact that the silicon concentration is constant in the cell is in agreement with recent reports that show that silicon transport proteins in diatoms do not function solely in silicon uptake but rather also serve a regulatory role in silicon homeostasis (28, 29). It should be noted that many of the earlier studies that reported dynamic silicon pools had to use extraction protocols that can easily dissolve labile silica elements and by that affect the overall reading (25, 30). Hence, the inconsistent values and large data ranges in previous quantitative silicon measurements, which varied from undetectable amounts to hundreds of millimolar, depending on the species, growth conditions, and species, may be the result of experimental limitations, ones that are mitigated by our spatially resolved, in situ approach.

As silicate ions in a dilute aqueous solution polymerize at low millimolar concentrations, the high intracellular silicon concentration raises the question of silicon stabilization (31). Several hypotheses have been raised: that silicon is compartmentalized in specialized organelles, that it is bound by soluble macromolecules in the cell, or that it is directly transported into the silicification organelle (4, 25). Our data preclude the option that dense silicon phases are stabilized in specialized compartments, as was observed for calcium and phosphate in other microalgae (20, 21). Conversely, large amounts of silicon are distributed within the cell cytoplasm and organelles, although not necessarily in a homogeneous manner. This supports the notion of chemical stabilization of the reactive silicon species by some reversible binding to unknown functional molecules within the cell. It remains to be determined in which chemical forms the intracellular silicon is present and what their solubility is.

Quantifying the cellular fluxes of inorganic components in marine organisms is crucial for understanding several biogeochemical cycles. The cryoFIB-SEM-EDS pipeline circumvents many of the limitations of conventional techniques and offers new possibilities to image and quantify, with 3D microscale resolution, various elements within distinct intracellular locations. Here, we show how this workflow can be calibrated for silicon to maximize the spatial and elemental information extracted from the sample. Previous work in other protists showed similar sensitivities with respect to other elements (22). The major obstacle for measuring concentrations below the millimolar range is the electron beam damaging the vitrified sample, which limits acquisition times. In the future, with
better EDS detectors positioned closer to the sample, it might be feasible to acquire higher number of photons to generate the EDS signal and break the millimolar detection barrier.

To conclude, we demonstrate an experimental strategy to measure intracellular silicon concentrations in single diatom cells. The results show that diatoms maintain a constant silicon pool, in a manner similar to the regulation of other inorganic ions. We observed an increase in silicon content upon silicon starvation, which is counterintuitive to the effect of such stress on diatoms. This in situ, spatially resolved, chemical analysis approach can be applied to elucidate cellular responses to genetic and physiological perturbations of the silicification process in various diatom species, thereby unraveling the cellular mechanisms that allow diatoms to maintain their remarkable ability to silify.

MATERIALS AND METHODS
Experimental design of algal cultivation, silicon starvation, and cell synchronization
T. pseudonana (strain CCMP1335) was grown in artificial seawater (ASW) supplemented with 300 μM silicic acid (ASW + Si) at 18°C and 24-hour light, under gentle shaking conditions in plastic cell culture flasks. The cultures were maintained at exponential growth phase by diluting 5 ml of the parent culture into 50 ml of fresh ASW + Si medium every other day. Twelve hours before subjecting the culture to silicon starvation, the shaking of the culture flasks was stopped, according to established protocols (24). To achieve silicon starvation, cells from a 50-ml culture were collected by centrifugation at 4000 rpm for 5 min at 18°C. Cell pellets were resuspended and washed twice with 25 ml of ASW without silicon supplementation (ASW − Si). The cell pellets were then resuspended in ASW − Si at a concentration of about 10⁶ cells ml⁻¹. The silicon-starved culture was kept under gentle shaking at 18°C for 12 hours. To track cell cycle synchronization, 1 μM of PDMPO, 2-(4-pyridyl)-5-((2-dimethylaminoethyl-amino-carbamoyl)methoxy)phenyl oxazole (Thermo Fisher Scientific, USA), a fluorophore that stains newly formed silica, was added 5 min before silicon repletion. For silicon repletion, silicic acid was added to a final concentration of 330 μM, and the culture was returned to shaking conditions.

Preparation of synthetic sodium silicate samples
Five hundred microliters of commercially available Na₂SiO₃ solution (about 6 M) was added to 9.5 ml of double-distilled water. The resulting stock solution of 300 mM silicic acid was further diluted to 200, 100, 50, 20, and 10 mM silicic acid. The silicon content in the 10 mM silicic acid solution was verified using a silicate test kit (Merck Millipore, USA).

HPF of cells and synthetic samples
Fifty milliliters of a T. pseudonana culture at the appropriate conditions was pelleted by centrifugation at 4000 rpm for 5 min at 18°C. Unsynchronized and silicon-replete cell pellets were resuspended in 50 μl of freshly prepared 10% dextran solution in ASW + Si, while silicon-starved cell pellets were resuspended in 50 μl of freshly prepared 10% dextran solution in ASW − Si. The cell sample was placed between two metal discs (3 mm diameter; cavity, 0.05 or 0.1 mm) and cryo-immobilized using a Leica LM ICE HPF device (Leica Microsystems, Germany). To high-pressure freeze the synthetic samples, 100 mg of dextran powder was dissolved in 1 ml of silicate solution. The resulting synthetic samples were high-pressure frozen without any centrifugation step. High-pressure frozen biological or synthetic samples were stored in liquid nitrogen until further use.

Freeze fracture, cryoSEM, and EDS measurements
The high-pressure frozen samples were shuttled using a vacuum cryo-transfer device (VCT 100, Leica Microsystems, Germany). The sample was transferred into a freeze-etching/freeze-fracture device (BAF 60, Bal-Tec, Germany), the stage of which was maintained at −120°C and a vacuum of about 5 × 10⁻⁷ millibar. After fracturing, the disc remaining in the sample holder was coated with 10 nm of carbon. The coated sample was transferred to the SEM (Ultra 55, Zeiss, Germany) and observed at −120°C and a vacuum of about 5 × 10⁻⁷ millibar, using an acceleration voltage of 1.5 to 4.0 kV, an aperture size of 10 to 30 μm, and a working distance of 2 to 4 mm.

cryoEDS of the freeze-fractured cells was recorded using a Bruker Quantax microanalysis system equipped with an XFlash6 60-mm detector. To acquire EDS maps, the acceleration voltage was increased to 5 kV, the working distance was kept between 6 and 7 mm, and the aperture size was 30 μm with a high current, for a higher signal-to-noise ratio. EDS spectra were recorded for at least 8 min.

CryoFIB-SEM and EDS of T. pseudonana samples
High-pressure frozen T. pseudonana samples were transferred to the precooled stage of a cryoFIB-SEM using a VCT 100. At all times, the temperature of the cryo-stage was below −150°C, and the vacuum inside the chamber of the cryoFIB-SEM (Crossbeam 550, Zeiss, Germany) was around 5 × 10⁻⁷ millibar. Rough milling (which does not involve imaging) was done using the following parameters: 80 μm by 30 μm milling area, 5.0-mm working distance, 30-kV FIB acceleration voltage, 3- or 7-nA FIB probe current, and 70- or 121-μs dwell time. Fine milling and imaging were done with the following milling parameters: 5.0-mm working distance, 30-kV FIB acceleration voltage, 1.5- or 3-nA FIB probe current, and 70- or 70-μs dwell time. The lateral image pixel size was between 12.0 and 18.0 nm, and the slice thickness was 12.0 or 16.25 nm. To acquire EDS data, the milled sample was brought to the BAF 60 (stage temperature of −150°C and a vacuum of about 5 × 10⁻⁷ millibar) and coated with 10 nm of carbon. The sample was returned to the Crossbeam 550, and EDS was performed using an acceleration voltage of 8 kV and a probe current of 500 pA at a working distance of 5 mm for 10 min. The EDS signal from the milled sample was recorded on the Crossbeam 550, using a Bruker Quantax microanalysis system equipped with an XFlash6 60-mm EDS detector.

Image processing and segmentation of 3D data were performed as follows: vertical curtains arising from the FIB milling were removed using Fourier filter plugin of ImageJ. Image slices from a stack were aligned, and segmentation of the cell components was performed using Amira 3D (Thermo Fisher Scientific, USA).

Calibration of quantitative cryoFIB-SEM-EDS data
Synthetic samples were milled and coated with 10 nm of carbon. EDS data were acquired at three different magnifications (namely, 12k×, 18k×, and 24k×) for 10 min under four combinations of electron acceleration voltages and probe currents: (i) 5 kV and 300 pA, (ii) 5 kV and 500 pA, (iii) 8 kV and 300 pA, and (iv) 8 kV and...
500 pA. Integration of the silicon peak area from the EDS spectra was done using peak fitting in OriginPro. A single exponential baseline was fitted and subtracted from the Si peak. The background-removed peak was fitted to a Lorentzian function, and its area is reported. To evaluate the horizontal resolution of EDS mapping, silicon spectra from random 1-, 10-, and 100-μm² areas in the raw data were compared.

**EDS simulations**

EDS simulations for depth profiles were performed using Win X-ray (Copyright © 2002–2004: H. Demers, P. Horny, R. Gauvin, and E. Lifshin. McGill University, Montreal, Quebec, Canada and University at Albany, Albany, New York, USA). The simulations were performed at 8 kV with 1000 electrons. The lateral distribution was simulated using the Casino (monte CARlo Simulation of electronN trajectory in sOlids) Version 2.24 simulator (CASINO Copyright © 2001: D. Drouin, A. Réal Couture, R. Gauvin, P. Hovongton, P. Horny, and H. Demers). The simulations were performed at 8 kV, with 1-nm probe size and 200 electron trajectories.

**Quantification of intracellular silicon pools and statistical analysis**

To quantify the internal silicon pool from unsynchronized, 12-hour silicon-starved, and 1.5-hour post–silicon-repletion samples, the average EDS spectra were taken from the internal area of the cells, the silicon peak was fitted and integrated, and the silicon concentration was calculated using our calibration curve. The average internal silicon pool and SD were calculated for 14 unsynchronized cells, 26 silicon-starved cells, and 19 silicon-replete cells. A statistical significance test (MS Excel) was carried out for each pair using a t test: two-sample assuming equal variances (P ≤ 0.05).

For estimating the relative size of the intracellular pool, we considered the concentration of Si in condensed silica, which is ~15 M, thus ~100× the concentration of the intracellular pool. The volume of the cell is ~100× higher than the volume of a single valve (both are estimated as cylinders with an identical base area, but a valve is ~40 nm thick and a cell is ~4 μm thick). Under these approximations, the amount of silicon in the intracellular pool is equivalent to the amount needed to construct a single valve.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/42/eaaz7554/DC1

View request a protocol for this paper from Bio-protocol.

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Acknowledgments

Funding: This project has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement no. 848339). Author contributions: S.K. and A.G. conceived the idea and planned and
designed the experiments. S.K., K.R., and I.K.-A. performed the experiments. All the authors analyzed and interpreted the data. A.G. prepared the initial draft. All the authors added, commented, and revised the manuscript and approved the final version for publication.

**Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

**Citation:** S. Kumar, K. Rechav, I. Kaplan-Ashiri, A. Gal, Imaging and quantifying homeostatic levels of intracellular silicon in diatoms. *Sci. Adv.* 6, eaaz7554 (2020).