

Fixation and fate of C and N in the cyanobacterium *Trichodesmium* using nanometer-scale secondary ion mass spectrometry

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The marine cyanobacterium *Trichodesmium* is ubiquitous in tropical and subtropical seas and is an important contributor to global N and C cycling. We sought to characterize metabolic uptake patterns in individual *Trichodesmium* IMS-101 cells by quantitatively imaging ¹³C and ¹⁵N uptake with high-resolution secondary ion mass spectrometry (NanoSIMS). *Trichodesmium* fix both CO₂ and N₂ concurrently during the day and are, thus, faced with a balancing act: the O₂ evolved during photosynthesis inhibits nitrogenase, the key enzyme in N₂ fixation. After performing correlated transmission electron microscopy (TEM) and NanoSIMS analysis on trichome thin-sections, we observed transient inclusion of ¹⁵N and ¹³C into discrete subcellular bodies identified as cyanophycin granules. We speculate that *Trichodesmium* uses these dynamic storage bodies to uncouple CO₂ and N₂ fixation from overall growth dynamics. We also directly quantified both CO₂ and N₂ fixation at the single cell level using NanoSIMS imaging of whole cells in multiple trichomes. Our results indicate maximal CO₂ fixation rates in the morning, compared with maximal N₂ fixation rates in the afternoon, bolstering the argument that segregation of CO₂ and N₂ fixation in *Trichodesmium* is regulated in part by temporal factors. Spatial separation of N₂ and CO₂ fixation may also have a role in metabolic segregation in *Trichodesmium*. Our approach in combining stable isotope labeling with NanoSIMS and TEM imaging can be extended to other physiologically relevant elements and processes in other important microbial systems.

NanoSIMS | stable isotope labeling | cyanophycin

The marine cyanobacterium *Trichodesmium* is ubiquitous in tropical and subtropical seas and is an important contributor to global N and C cycling (1). As a diazotrophic cyanobacterium, *Trichodesmium* is capable of both CO₂ and N₂ fixation. Studies have estimated that it may leak up to 30–50% of its newly fixed N (2), providing a valuable source of bioavailable N to other nondiazotrophic phytoplankton species cohabitating in the N-limited subtropical gyres. Capone et al. (3) estimate that it contributes ≈5.7 Tmol new N y⁻¹ in the North Atlantic Ocean, which is comparable with the rate of NO₃⁻ that diffuses from depth into oligotrophic upper ocean ecosystems (3, 4). Although *Trichodesmium* is not the sole diazotrophic cyanobacteria in the open ocean (5–7), it is the most conspicuous and well-studied. Also, it has been in culture since 1992 (8), allowing numerous studies of its physiology and response to different limiting and controlling factors. *Trichodesmium* is often included in ecosystem models that attempt to describe CO₂ and N₂ fixation in open ocean systems (9). A better understanding of CO₂ and N₂ fixation in *Trichodesmium* is critical, because this species has a large role in global C and N cycling in the open ocean.

Commonly referred to as “saw dust” on the surface of the sea (10), *Trichodesmium* grows in filaments (referred to as trichomes) that can have 100–200 cells (Fig. 1B). In the field, *Trichodesmium* may be found in its colony form, as either

puff-shaped or tuft-shaped colonies (Fig. 1A) (11). Colonies typically have 100–200 trichomes. In some systems, free trichomes may predominate (12). *Trichodesmium* are high-light adapted, and have gas vesicles that keep them near the surface of the ocean (Fig. 1C) (13). They often form thick blooms visible in satellite imagery (14). *Trichodesmium* grown in culture usually occurs as free trichomes of ≈80–100 cells per trichome during exponential phase, and aggregates during stationary phase.

Diazotrophy is a significant challenge for unicellular microorganisms, because the O₂ produced from CO₂ fixation is inhibitory to nitrogenase, the key enzyme in N₂ fixation. Therefore, diazotrophs have developed different behavioral, biochemical, and physical strategies to protect nitrogenase from the O₂ evolved during photosynthesis. Certain cyanobacteria, such as *Gloeotheca* spp., temporally segregate the processes over a diel cycle by fixing CO₂ during the day and fixing N₂ at night (15). Others, such as *Anabaena* spp., have terminally differentiated cells, termed heterocysts, with thickened cell walls, and reduced PS II and rubisco activity (16). These cells serve to spatially segregate the 2 processes, with N₂ fixation occurring in the heterocysts, whereas oxygenic photosynthesis and CO₂ fixation occurs in vegetative cells. *Trichodesmium* is unique in that it is a nonheterocystous cyanobacteria that fixes both CO₂ and N₂ concurrently during the day (1). There is considerable current debate among researchers as to how these processes co-occur. Current theories, and sometimes conflicting data, suggest increased O₂ consumption within cells protects nitrogenase [e.g., increased hydrogenase (17), Mehler (18, 19), and superoxide dismutase activity (20)], in addition to both spatial (21–23) and temporal segregation of CO₂ and N₂ fixation (19, 24, 25). Much of the current debate revolves around the metabolic potential of individual cells in a trichome, and how those capabilities may differ among cells in a trichome or within individual cells over time (26).

To investigate the dynamics of C and N metabolism at this level, we combined tracer-level additions of inorganic ¹³C and ¹⁵N with high-resolution secondary ion mass spectrometry (NanoSIMS; Cameca). NanoSIMS imaging allowed us to track C and N stable isotope incorporation rates and subsequent cellular fates by mapping distributions of isotopes (27) in multiple trichomes. The results of our NanoSIMS investigations are

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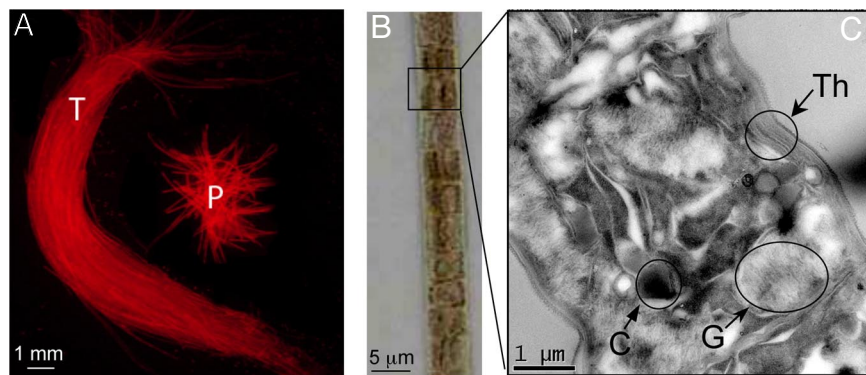


Fig. 1. Images of *Trichodesmium* filaments at 3 levels of magnification. (A) Image of *Trichodesmium* tuft (T) and puff (P) taken under green excitation (510–560 nm); (B) image of single *Trichodesmium* trichome by using light microscopy at 20× magnification; and (C) TEM image of individual *Trichodesmium* cell, demonstrating a cyanophycin granule (C), gas vesicles (G), and thylacoid membranes (Th).

compared with bulk isotopic analyses and related to existing models of *Trichodesmium* metabolism.

Results and Discussion

NanoSIMS imaging was performed on *Trichodesmium* trichomes spiked with $\text{NaH}^{13}\text{CO}_3$ and $^{15}\text{N}_2$, and harvested sequentially during 24 h (see *Materials and Methods*). Both whole- and thin-sectioned samples were imaged to quantify the distribution of newly fixed ^{13}C and ^{15}N within trichomes. The distribution of newly fixed ^{13}C and ^{15}N was determined by simultaneously imaging C and N isotopes and calculating quantitative $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratio images. Each $10\text{-}\mu\text{m}^2$ image includes 2 to 3 cells, and was scanned with 150-nm resolution to capture intercellular variability. Multiple adjacent cells along multiple trichomes were analyzed to quantify cell–cell variability. For thin-sectioned trichomes, we used transmission electron micros-

copy (TEM) imaging to morphologically map samples before NanoSIMS analysis.

NanoSIMS analysis showed substantial subcellular spatial variability in ^{15}N and ^{13}C enrichment along *Trichodesmium* trichomes and with depth through individual cells (Figs. 2, 3, and 4); $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratio images generated from sectioned trichomes, along with correlated TEM maps, provide direct evidence of subcellular uptake localization within cells ≈ 8 h, and the redistribution of that enrichment after 24-h incubation (Fig. 3). In both sectioned and whole cells, we observed discrete hotspots enriched in ^{15}N and ^{13}C at 4 h, with increased density at 8 h (Figs. 2 I and J, and 3 B and C). These features are likely cyanophycin granules, a type of N-rich storage vacuole comprised of an asparagine and aspartate polymer found in many cyanobacteria (28–30), including *Trichodesmium* (21, 31). NanoSIMS analyses of *Trichodesmium* sections from 8 h indicate that

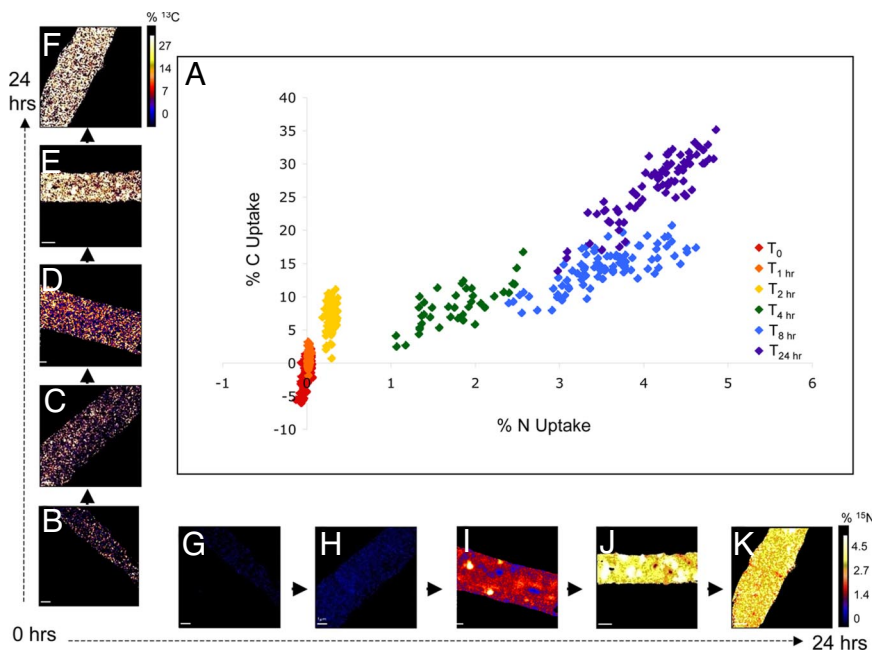


Fig. 2. Percentage ^{13}C and ^{15}N uptake by *Trichodesmium* trichomes at 6 time points and corresponding NanoSIMS images. (A) Percentage ^{13}C and ^{15}N uptake at 5 time points in *Trichodesmium* cells analyzed by NanoSIMS. Along the x axis are representative NanoSIMS images of ^{15}N enrichment in ≈ 2.5 cells at (B) 0 h, (C) 2 h, (D) 4 h, (E) 8 h, and (F) 24 h. Along the y axis are corresponding images of ^{13}C enrichment at (G) 0 h, (H) 2 h, (I) 4 h, (J) 8 h, and (K) 24 h. Data points represent average values for individual cells measured along a single trichome by NanoSIMS. [Scale bar, $1\ \mu\text{m}$; image enrichment scale ranges from 0 to 5% uptake (N), and 0 to 30% uptake (C), moving from black-blue-red-yellow-white.]

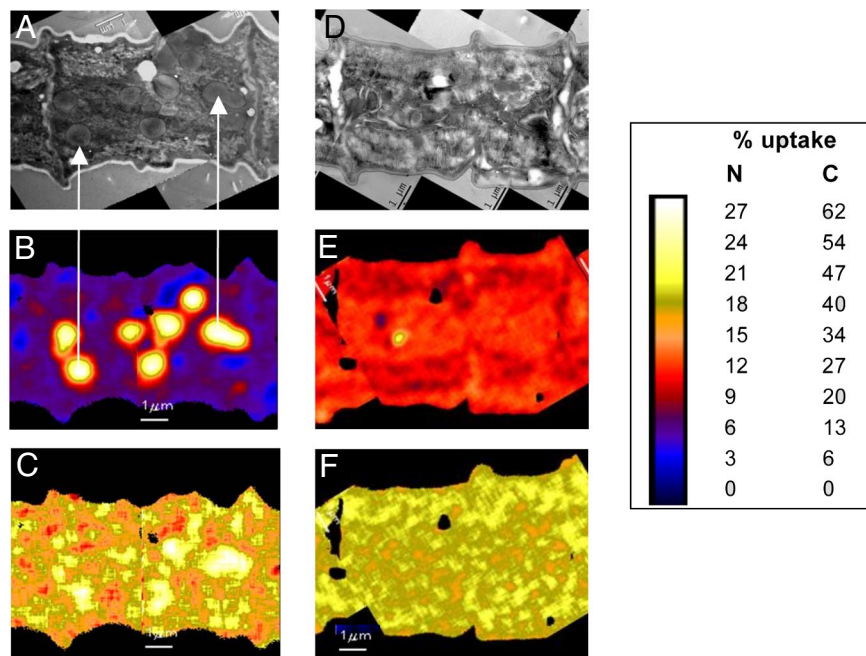


Fig. 3. TEM images of ≈ 2 cells from a *Trichodesmium* filament after (A) 8-h and (D) 24-h incubation with H^{13}CO_3 and $^{15}\text{N}_2$. Correlated NanoSIMS images demonstrate percentage fixed ^{15}N after (B) 8 h and (E) 24 h, percentage fixed ^{13}C after (C) 8 h and (F) 24 h. Arrows indicate correlation between cyanophycin granules identified by TEM and ^{15}N enriched hotspots evident in NanoSIMS image. (Scale bar, $1\ \mu\text{m}$.) Because NanoSIMS analysis is a destructive process, distinct cells were imaged for the 2 different time points.

the $^{13}\text{C}:^{15}\text{N}$ ratio of the cyanophycin granules is consistent with a mixture of asparagine (2:1) and aspartate (4:1), providing chemical evidence to identify these granules as cyanophycin. Also, these ^{15}N hotspots correspond directly to vacuole-like structures identified as cyanophycin granules in corresponding TEM images (Fig. 3 A and B). The hotspots were no longer evident after 24-h incubation, at which point cells were uniformly enriched with newly fixed ^{15}N (Figs. 2K and 3E). These results demonstrate the dynamic nature of these subcellular features, where it appears that newly fixed ^{15}N is stored during the daytime

(when N_2 fixation is active), and is subsequently used at night for cellular biosynthesis (when N_2 fixation has ceased). As might be expected, elevated levels of ^{13}C are also associated with these cyanophycin granules (Fig. 3C).

NanoSIMS analysis also allowed us to examine subcellular isotope enrichment in the vertical dimension of cells. Based on analyses of multiple cells examined from multiple trichomes and time points, we determined that cyanophycin granules can comprise up to 6.4% ($\pm 0.7\%$) of cell area/volume, and are evenly distributed along trichomes. In depth profile analyses of whole cells, as the NanoSIMS analyzed deeper and deeper layers, individual cyanophycin granules became apparent, and then disappeared as the ion beam sputtered through them (Fig. 4), suggesting that the granules are randomly distributed throughout the cell volume. It has been hypothesized that certain nondifferentiated cells, called “diazocytes”, along a trichome contain nitrogenase and are the sole sites of N_2 fixation (21, 22, 32). Fredriksson and Bergman (21) found that only 24% of these diazocyte cells contained cyanophycin granules, compared with 61% of non- N_2 -fixing cells. We did not find evidence for this theory in our samples. When cells were examined in TEM thin sections (0-, 8-, and 24-h incubations), and with NanoSIMS depth profiling (4- and 8-h incubations), cyanophycin granules were observed at similar densities in all cells.

Previous studies have examined the transient nature of cyanophycin granules in single-celled and heterocystous diazotrophs. Sherman et al. (30) used immunocytochemical analysis to demonstrate the localization of cyanophycin at the polar plugs of mature heterocysts in *Anabaena* sp. PCC7120. Mackerras et al. (33) used cultures of *Anabaena cylindrica* (a heterocystous cyanobacteria) and *Synechocystis* 6308 (a unicellular cyanobacteria that temporally segregates CO_2 and N_2 fixation) grown in media with limited ammonium concentration ($[\text{NH}_4^+]$). They demonstrate that synthesis and subsequent degradation of cyanophycin granules in these 2 organisms as $[\text{NH}_4^+]$ in the media is drawn down, indicating that the 2 cyanobacteria produce cyanophycin granules in N-replete conditions and use the stores

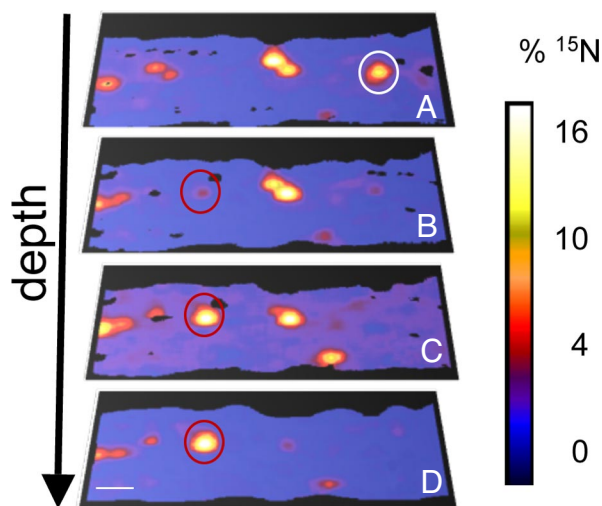


Fig. 4. Images of cyanophycin granules, as evidenced by hotspots of $^{15}\text{N}/^{14}\text{N}$ accumulation (percentage ^{15}N uptake), with depth through ≈ 2 individual cells. The white circle in A represents a cyanophycin granule that does not appear in the subsequent images. The red circles in B–D indicate a cyanophycin granule that becomes more prominent with depth through the cell. (Scale bar, $1\ \mu\text{m}$.)

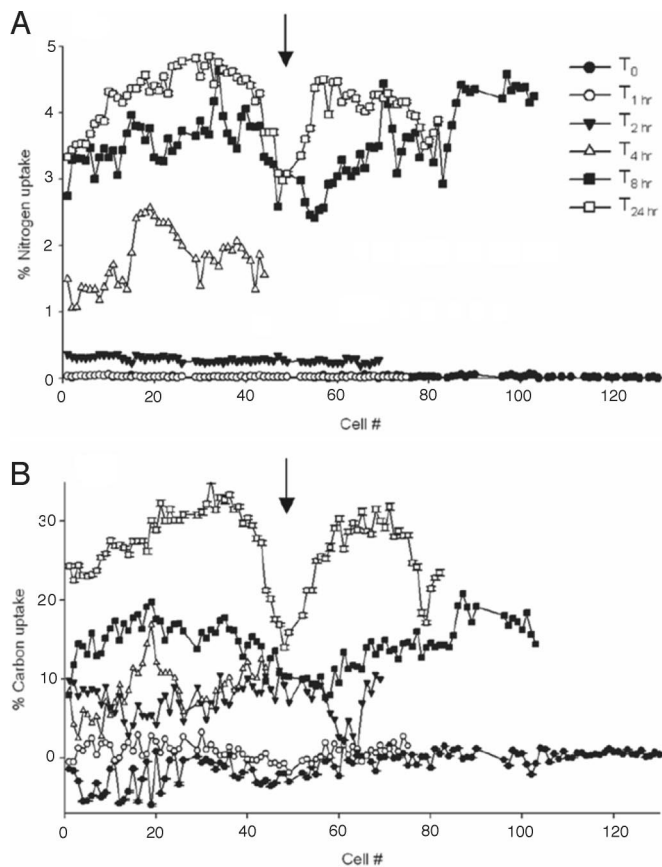


Fig. 5. Percentage isotope uptake in individual cells along the full length of *Trichodesmium* trichomes (filaments) at increasing time intervals for ^{15}N (A) and ^{13}C (B). Each data point represents average enrichment (measured by NanoSIMS) in a single cell, and data are plotted in the order found in the analyzed trichome. For example, the midpoint of the T_{24} trichome is found at approximately cell 50. Different symbols indicate trichomes from successive harvest points after addition of stable isotope labels. An arrow marks the point of proposed trichome division. Error bars represent 2 sigma error, but are small enough as to be obscured by the data symbols.

during N-deplete conditions. Our study demonstrates the dynamic nature of the cyanophycin pool in *Trichodesmium* grown under diazotrophic conditions. Transient accumulation of cyanophycin indicates an uncoupling of CO_2 and N_2 fixation with cell growth. Interestingly, recent modeling of *Trichodesmium* metabolism (34) suggested that storage pools of C and N are dynamic over the diel cycle, accumulating during the day and consumed during the dark period. Our results provide direct evidence for this prediction.

In more than half of the trichomes analyzed from the 8- and 24-h incubations, we observed a marked reduction in ^{13}C and ^{15}N enrichment in cells located near the middle of trichomes (Fig. 5); this pattern may be related to programmed cell death (PCD) and/or trichome splitting. In *Trichodesmium*, as cells divide and the trichome increases in length, it is hypothesized that cells at the center of the trichome begin to have decreased metabolic activity and CO_2 and N_2 fixation. Eventually, the trichome splits at this point, yielding 2 shorter trichomes. Although PCD has been described for *Trichodesmium* (24, 35), we cannot confirm whether zones of reduced ^{13}C and ^{15}N uptake we observed are undergoing PCD, or whether the reduction in activity is due to other factors. However, secondary electron images we obtained before SIMS analysis provide visual evidence of thinner cells at these points (Fig. S1), suggesting that the trichome is stressed and about to break.

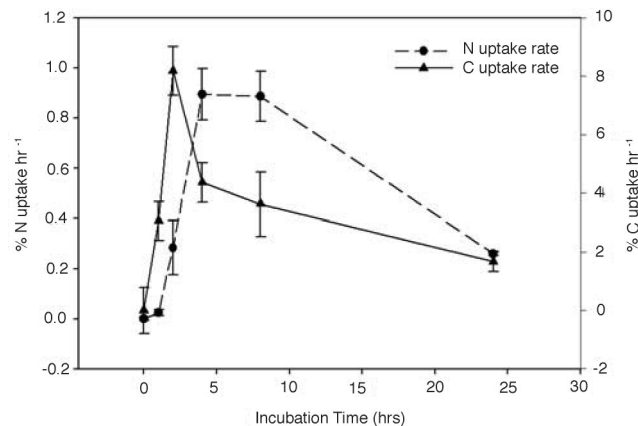


Fig. 6. ^{15}N and ^{13}C fixation rates (percentage uptake hour^{-1}) in *Trichodesmium* cells during a 24-h isotope labeling experiment. Data are generated from single cell NanoSIMS analysis of multiple filaments, and are averaged by time point (average $n = 73$ cells per time point). Error bars represent 2 sigma error.

We used NanoSIMS analysis of whole cells to calculate uptake rates of CO_2 and N_2 fixation at the single cell level. These data were averaged for 40–120 contiguous cells located in multiple trichomes from each time point (Fig. 6), and were comparable to rates calculated from samples harvested in parallel, and bulk-analyzed by isotope ratio mass spectrometry (Fig. S2). Results from both approaches indicate that CO_2 fixation rates were highest in the morning (≈ 10 AM), whereas N_2 fixation rates were highest in the afternoon (≈ 12 PM; Fig. 6 and Fig. S2). These results provide evidence for a temporal decoupling of CO_2 and N_2 fixation in *Trichodesmium*, and corroborate earlier findings where Berman-Frank et al. (24) used fast repetition rate fluorescence (FRRF), O_2 production, $^{14}\text{CO}_2$ uptake, and acetylene reduction, to show maximal CO_2 fixation rates in the morning (≈ 11 AM) and afternoon (≈ 3 PM). Maximal N_2 fixation rates were measured at midday (≈ 12 PM), when CO_2 fixation was down-regulated. Küpper et al. (25), using fluorescence kinetic microscopy, provided further evidence for temporal segregation within individual cells, and suggest that there is a finely regulated shift between photosynthetic activity states throughout the course of the day that contributes to the protection of nitrogenase from O_2 evolved during photosynthesis. Together, our findings and these aforementioned studies build on the theory that increased O_2 -consumptive activities within individual cells (17–20) enable them to temporally segregate the incompatible processes of CO_2 and N_2 fixation.

A controversial and evolving area of debate in the field of *Trichodesmium* physiology has been the suggestion that CO_2 and N_2 fixation within *Trichodesmium* are spatially segregated between different cells. The earliest microautoradiography studies suggested that spatial segregation happens at the level of the colony, with anoxic microzones occurring near the center of aggregates, where N_2 fixation was localized and photosynthesis down-regulated (36, 37). This theory was later questioned, when it was demonstrated that individual trichomes from disrupted colonies still fix both N_2 and CO_2 (38). More recently, conflicting immunochemical studies have examined the distribution of nitrogenase proteins along trichome cells. Some studies (23, 39) indicate that nitrogenase is dispersed throughout all cells in colonies, whereas other research suggests that only $\approx 15\%$ of cells along a trichome contain nitrogenase (21, 22), and that these cells occur in clusters along the trichome (21, 32). These clusters, referred to as diazocytes, suggest a spatial segregation of N_2 fixation and CO_2 fixation. It should be noted that cells

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Corrections

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Correction for “Fixation and fate of C and N in the cyanobacterium *Trichodesmium* using nanometer-scale secondary ion mass spectrometry,” by Juliette A. Finzi-Hart, Jennifer Pett-Ridge, Peter K. Weber, Radu Popa, Stewart J. Fallon, Troy Gunderson, Ian D. Hutcheon, Kenneth H. Nealson, and Douglas G. Capone, which appeared in issue 15, April 14, 2009, of *Proc*

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The authors note that due to a printer’s error, on page 6346, the following text was not included in the legend for Fig. 1A: “Photo credit: R. Foster (University of California, Santa Cruz, CA).” The figure and its corrected legend appear below.

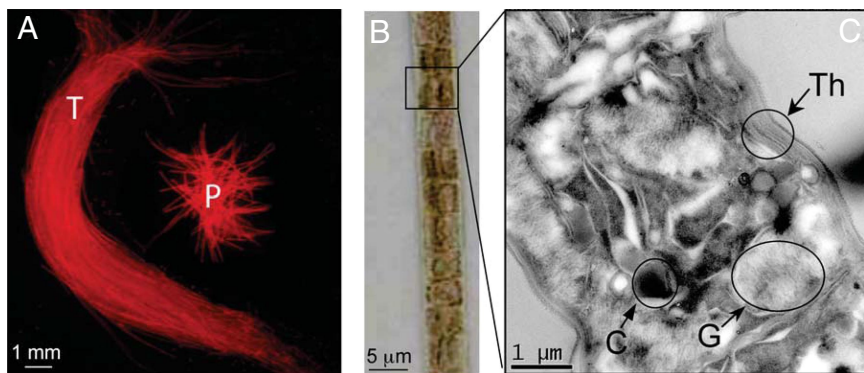


Fig. 1. Images of *Trichodesmium* filaments at 3 levels of magnification. (A) Image of *Trichodesmium* tuft (T) and puff (P) taken under green excitation (510–560 nm). Photo credit: R. Foster (University of California, Santa Cruz, CA). (B) Image of single *Trichodesmium* trichome by using light microscopy at 20 \times magnification. (C) TEM image of individual *Trichodesmium* cell, demonstrating a cyanophycin granule (C), gas vesicles (G), and thylacoid membranes (Th).

www.pnas.org/cgi/doi/10.1073/pnas.0904281106

NEUROSCIENCE

Correction for “Evidence on the emergence of the brain’s default network from 2-week-old to 2-year-old healthy pediatric subjects,” by Wei Gao, Hongtu Zhu, Kelly S. Giovanello, J. Keith Smith, Dinggang Shen, John H. Gilmore, and Weili Lin, which appeared in issue 16, April 21, 2009, of *Proc Natl Acad Sci USA* (106:6790–6795; first published April 7, 2009; 10.1073/pnas.0811221106).

The authors note that on page 6790, right column, the eighth line of the first full paragraph, “Uidden et al. (13) investigated the 2 hubs of the default network and found that the interaction patterns with other networks are significantly different for these 2 hubs, suggesting functional differentiation within the default network,” should instead read “**Uddin** et al. (13) investigated the 2 hubs of the default network and found that the interaction patterns with other networks are significantly different for these 2 hubs, suggesting functional differentiation within the default network.”

www.pnas.org/cgi/doi/10.1073/pnas.0905295106

BIOCHEMISTRY

Correction for “Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities,” by Gongshe Han, Sita D. Gupta, Kenneth Gable, Somashekarappa Niranjanakumari, Prasun Moitra, Florian Eichler, Robert H. Brown, Jr., Jeffrey M. Harmon, and Teresa M. Dunn, which appeared in issue 20, May 19, 2009, of *Proc Natl Acad Sci USA* (106:8186–8191; first published May 5, 2009; 10.1073/pnas.0811269106).

The authors note that the reference numbers throughout the article appeared incorrectly and that four references were inadvertently omitted from the reference list. The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.0905301106

Supporting Information

Finzi-Hart et al. 10.1073/pnas.0810547106

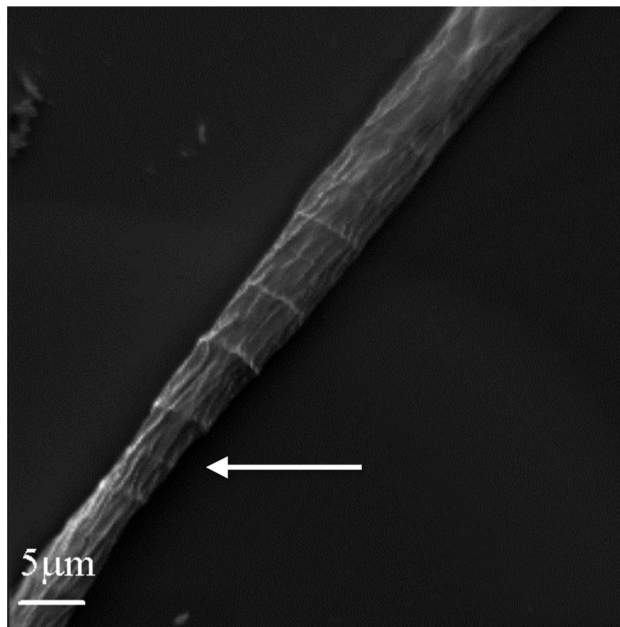


Fig. S1. Nanometer-scale secondary ion mass spectrometry (NanoSIMS) secondary electron image ($50 \mu\text{m}^2$) of a *Trichodesmium* trichome. The arrow indicates a potential "break point," a region of thinning cells with relatively low uptake activity.

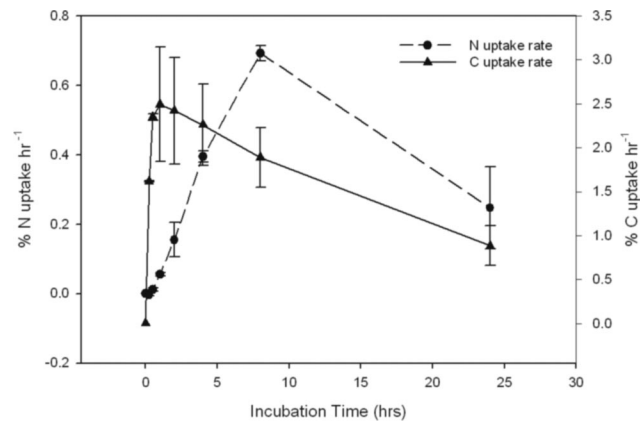


Fig. S2. ^{15}N and ^{13}C fixation rates (percentage uptake hour^{-1}) in *Trichodesmium* cells during a 24-h isotope labeling experiment. Data are generated from bulk IRMS analysis of pelleted cells sampled at 8 time points. Error bars represent 2 sigma error.

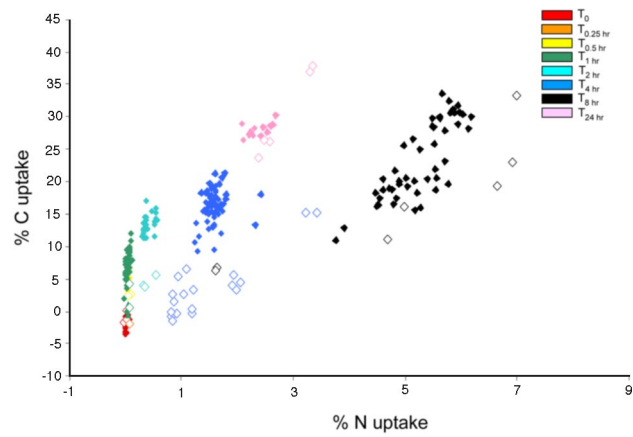


Fig. S3. Percentage ^{13}C and ^{15}N uptake at 8 time points in *Anabaena oscillarioides* cells analyzed by NanoSIMS, following stable isotope labeling with H^{13}CO_3 and $^{15}\text{N}_2$. Data points represent average values for individual cells measured along a single trichome by NanoSIMS. Time points are shown for both vegetative cells (filled diamonds) and heterocysts (open diamonds).