CAMECA SCIENCE & METROLOGY SOLUTIONS / AMETEK

NanoSIMS

ENVIRONMENTAL MICROBIOLOGY

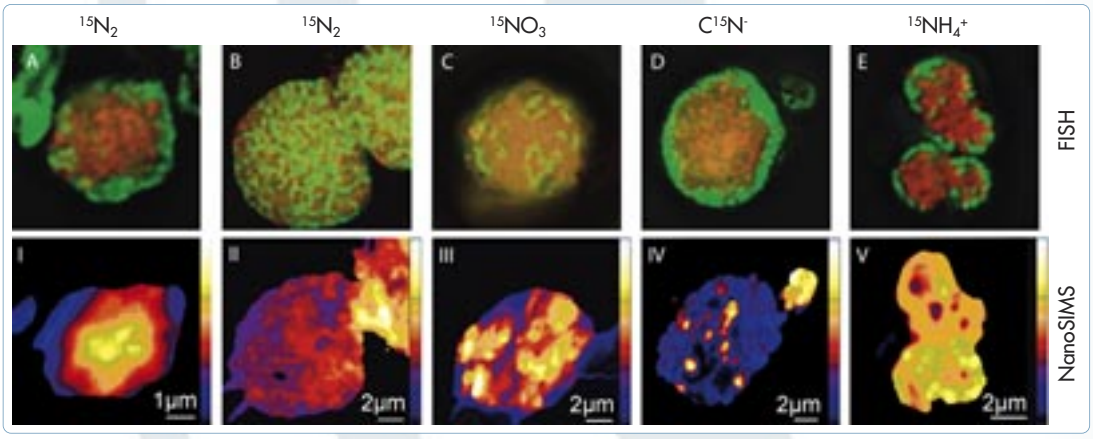
Quantitative Measurement of Metabolic Activity of Single Microbial Cells in Environmental Samples

Metabolic activity of individual cells & phylogenetic identity with ISH

In situ studies of uncultivated or rare microbial cells in complex ecosystems

Studies of symbioses & microbial interactions





A to E: FISH using probes targeting ANME-2 (Eel932) in red and Desulfobacteraceae (DSS658) in green.
I to V: Ion micrographs of $^{12}C^{15}N/^{12}C^{14}N$ ratios of the same microbial consortia imaged by FISH in (A) to (E), demonstrating the location of ^{15}N incorporation. The scale range varies between ion micrographs, with the min-imum consistently set to natural abundance $^{15}N/^{14}N$.

From: Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia, Anne E. Dekas et al., SCIENCE Vol. 326 (16 Oct. 2009).

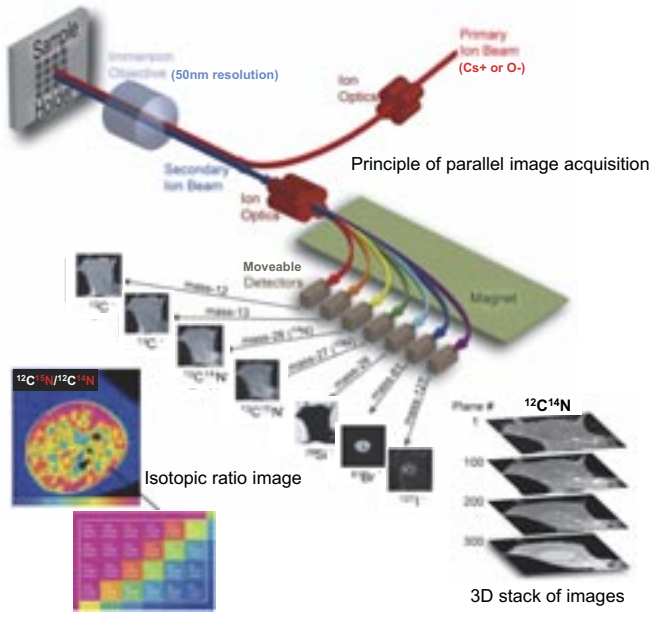
Deep-sea anaerobic methane-oxidizing archaea fix N_2, as well as structurally similar CN^-, and share the products with sulfate-reducing bacterial symbionts. Magnitude and distribution of ^{15}N incorporation in representative methanotrophic ANME-2/DSS consortia from sediments incubated with CH4 and different ^{15}N-labeled N sources, as indicated.

Single Cell Environmental Microbiology wit

1) Description of the NanoSIMS instrument

A DC primary beam of Cs+ or O- primary ions is focused and rastered on the surface of the sample, down to 50nm beam size (= resolution). The collected secondary ion beam is shaped in order to be mass filtered by a magnetic sector mass analyzer. Up to seven mass selected images of different elements or isotopes can be simultaneously recorded, ensuring reliable isotopic ratio and perfect image registration. [1]

Each plane image is obtained through the consumption of typically a few nanometers of the sample. The scanning is repeated and a 3D image can be reconstructed from the stack of images.

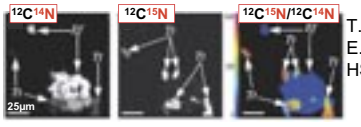


Isotope intensities and ratio are stored for each pixel. The isotopic ratio is displayed in false color.

Schematics from: M. Steinhauser et al., NATURE, Vol 481, 26 January 2012

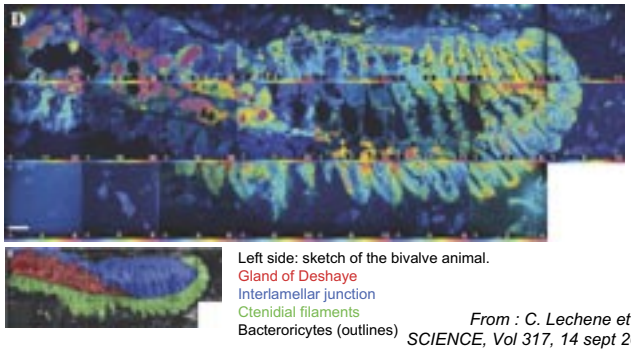
2) The general strategy of coupling stable isotope labeling with NanoSIMS: a bacterial symbiont fixes gaseous nitrogen for a shipworm

Culture of two bacteria in medium enriched with $15N_2$ gas. The bacteria fixing gaseous 15N are clearly quantitatively distinguished.



T. Turnarae bacteria (Tt): fixing 15N.
E. Faecalis (Ef): not fixing 15N.
HSI Color scale: Blue = terrestrial ratio

Below: Evidence of symbiosis between the marine shipworm L. pedicellatus and a bacterium fixing gaseous nitrogen. **The shipworm hosts the bacterium in its gills, that fixes gaseous nitrogen and delivers it under a useable form to the animal for its metabolism.** Experiment done by enriching sea water with $^{15}N_2$ during a given time and measuring quantitatively its fate in the bacteria and in the animal tissues through the localized measurement of 15N/14N ratio.

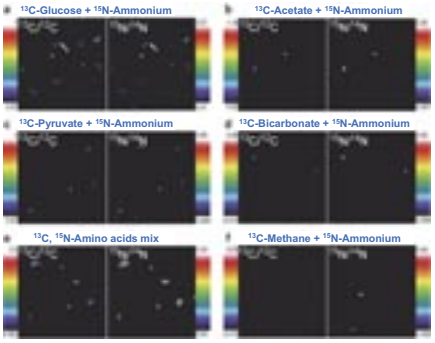


Left side: sketch of the bivalve animal.
Gland of Deshaye
Interlamellar junction
Ctenidial filaments
Bacteroricytes (outlines)

From : C. Lechene et al., SCIENCE, Vol 317, 14 sept 2007

3) Rare or uncultivable species: subseafloor microbes

Stable isotope-labeled substrate incorporation **into individual microbial cells** collected from **219-meter-deep subseafloor sediments** from the northwestern Pacific off the Shimokita Peninshula of Japan. **Incubations with 6 different carbon sources and 2 nitrogen sources.**



Field of view: 50 µm x 50 µm. Recorded on the NS50 of the University of Tokyo.

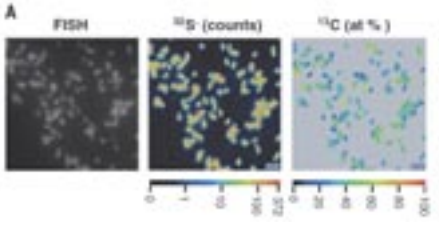
- The incorporation of substrates is detected even for subseafloor microbes (terrestrial: 13C/12C: 0.011; 15N/14N: 0.0037),
- A large variation of substrate incorporation is observed depending on the substrate.
- Subseafloor microbes preferentially require nitrogen assimilation for the recovery in vitro,
- Buried subseafloor microbes maintain potential for metabolic activities
- Growth is generally limited by energy but not by the availability of C and N compounds.

From : Yuki Morono et al. PNAS, Vol 108, 8 Nov 2011

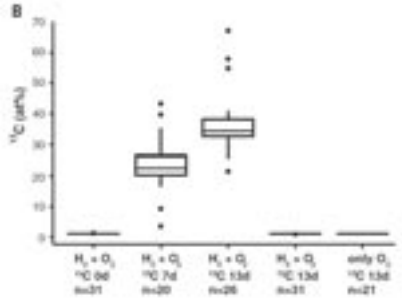
4) Combining FISH (fluorescent in-situ hybridization) and NanoSIMS: chemolithoautotrophic carbon fixation by N. moscoviensis with H_2 as sole energy source

FISH is based on the use of fluorescent-labeled probes specific to the organism of interest, binding to the intracellular 16S ribosomal RNA.

- **FISH**, measured in a dedicated optical microscope can identify the species **(WHO is there ?)**
- **NanoSIMS** can give the **metabolic activity of *single cells* (WHAT is it doing or eating and HOW MUCH ?)**



A: N. moscoviensis cells in the same field of view after **13 days of incubation** with **13C-labeled bicarbonate, H2, and O2**. Cells were detected by FISH with the Nitrospira lineage II–specific probe Ntspa1151 (control hybridizations with a nonsense probe gave no signals). The $32S^-$ illustrates the distribution of biomass. Carbon fixation is demonstrated by a high 13C content of cells.



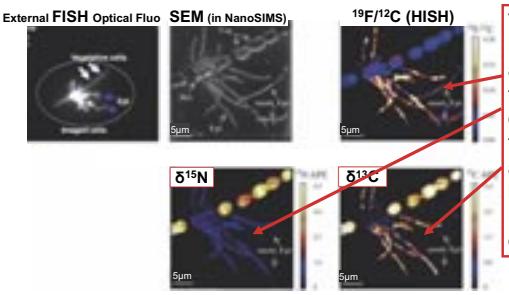
B: 13C labeling of N. moscoviensis quantified by NanoSIMS at the single-cell level at different time points during the same incubation as shown in (A) and control incubations. The label content is presented as the 13C/(12C + 13C) isotope fraction, given in atomic %. The box plots summarize the median and the quartiles of the 13C content of cells. Numbers of measured cells (n) are indicated.

"Our results demonstrate a chemolithoautotrophic lifestyle of nitrite-oxidizing bacteria outside the nitrogen cycle, suggesting greater ecological flexibility than previously assumed."

From : Hanna Koch et al., SCIENCE, 345, 29 Aug 2014

h NanoSIMS (secondary ion mass spectrometry)

5) Exchanges of nitrogen and carbon between single cells in a dual-species microbial consortium



The epibionts incorporate nitrogen and carbon through the heterocyst as evidenced by the two epibionts NOT attached to the heterocist cell, invisible in the δ13C δ15N images.

Microbial consortium consisting of **filamentous cyanobacteria** (*Anabaena* sp. strain SSM-00) and **heterotrophic alphaproteobacteria** (*Rhizobium* sp. strain WH2K) attached to heterocysts.

Enhanced **HISH-SIMS using an alphaproteobacteria probe** (ALF968).

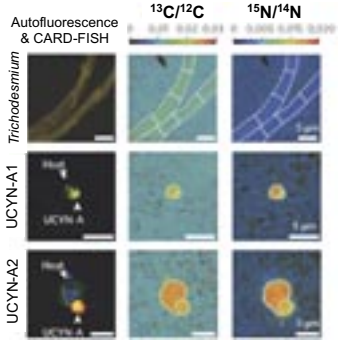
Images taken after a 24-h incubation with 13C-bicarbonate and $^{15}N_2$.

The Heterocysts fix nitrogen and export it to the vegetative cells and the *attached* epibiont. The interaction between the communities is evidenced by absence of 13C or in 15N enrichment in the *unattached,* phylogenetically identified epibionts.

From: Sebastian Behrens et al., Applied And Environmental Microbiology, May 2008,

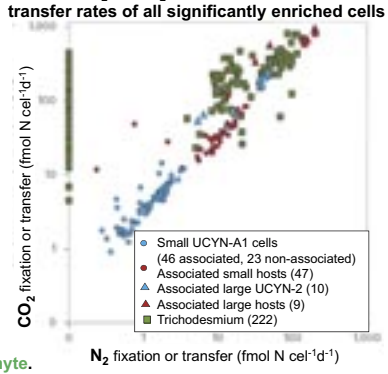
6) Quantification of the nitrogen fixation of an unicellular cyanobacteria in association with an algua.

In the tropical North Atlantic the symbiotic cyanobacterium UCYN-A contributes to N2 fixation similarly to Trichodesmium, major oceanic N2 fixer. Two types of UCY in **symbioses with speci** w that both algae-UCYN-A J2 fixation in the tropical N es five to ten times higher t



Autofluorescence: *Trichodesmium spp.*
CARD-FISH: UCYN-A & associated haptophyte.
1rst row: *Trichodesmium* cells with low to non-detectable N2 fixation (~55% of all investigated cells).
2nd & 3rd row: UCYN-A1 and UCYN-A2 associations (~75% of all investigated cells)

Cellular CO_2 and N_2 fixation or C and N transfer rates of all significantly enriched cells



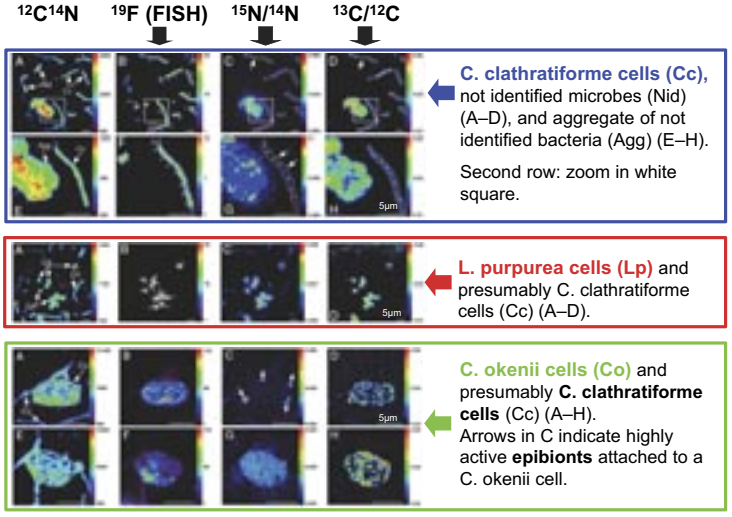
Surprisingly only 46% of Trichodesmium are actively fixing N2 almost all are fixing CO2.

From: The small unicellular diazotrophic symbiont, UCYN-A, is a key player in the marine nitrogen cycle. C. Martinez-Perez et al., Nature Microbiology, 12 Sept 2016.

7) Ecophysiology of anaerobic phototrophic bacteria: ammonium and inorganic carbon assimilation by green and purple sulfur bacteria in Lake Cadagno

Individual cells of the anaerobic phototropic bacteria **Chromatium okenii**, **Lamprocystis purpurea** and **Chlorobium clathratiforme** inhabiting the oligotrophic meromictic Lake Cadagno, stratified alpine lake in Switzerland, have been analyzed with respect to H13CO3- and 15NH4+ assimilation. Water samples from 11.5 m depth were incubated after the addition of **15N-labeled** ammonium and **13C-labeled** bicarbonate at in situ light and temperature conditions. After incubation the samples were hybridized with HRP-labeled oligonucleotide probes targeting specific regions of the 16S rRNA of the three species. After the deposition of **fluorine**-containing tyramides [2], filters were immediately analyzed using NanoSIMS.

Thus ***F-* elemental-FISH** signal gives ***phylogenic identity*** and ***15N/14N*** and ***13C/12C*** give ***metabolic activities*** at ***single cell*** level.



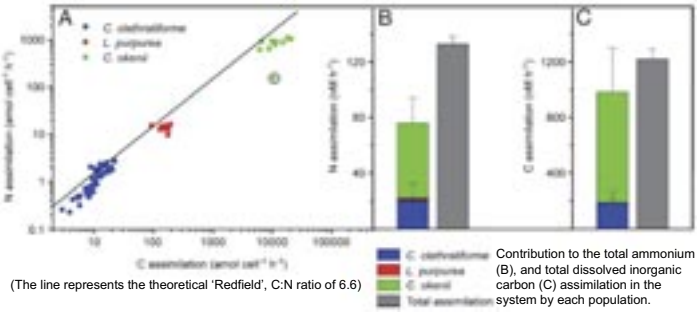
C. clathratiforme cells (Cc), not identified microbes (Nid) (A–D), and aggregate of not identified bacteria (Agg) (E–H).

Second row: zoom in white square.

L. purpurea cells (Lp) and presumably C. clathratiforme cells (Cc) (A–D).

C. okenii cells (Co) and presumably **C. clathratiforme cells** (Cc) (A–H).
Arrows in C indicate highly active **epibionts** attached to a C. okenii cell.

***Individual isotopic ratios* are measured for each ROI *(=individual cell)* can be plotted in charts as below:**



(The line represents the theoretical 'Redfield', C:N ratio of 6.6)

Contribution to the total ammonium (B), and total dissolved inorganic carbon (C) assimilation in the system by each population.

For the calculation of total nitrogen and carbon uptake, the absolute abundance of each species as determined by CARD-FISH was taken into account.

➔ **A large variability** in metabolic rates between individual cells of the same species is observed (note the LOG scale). The microbial populations in the environment are heterogeneous, comprising physiologically distinct individuals.

➔ C. okenii, the least abundant species representing about 0.3% of the total cell number contributed more than 50% of the total ammonium and carbon uptake in the system. This reveals the **significance of inconspicuous microbes** to the N and C cycles in the environment.

From: A single cell view on the ecophysiology of anaerobic phototrophic bacteria. N. Musat et al., PNAS, Nov. 18, 2008, vol. 105, no. 46.

8) Conclusion

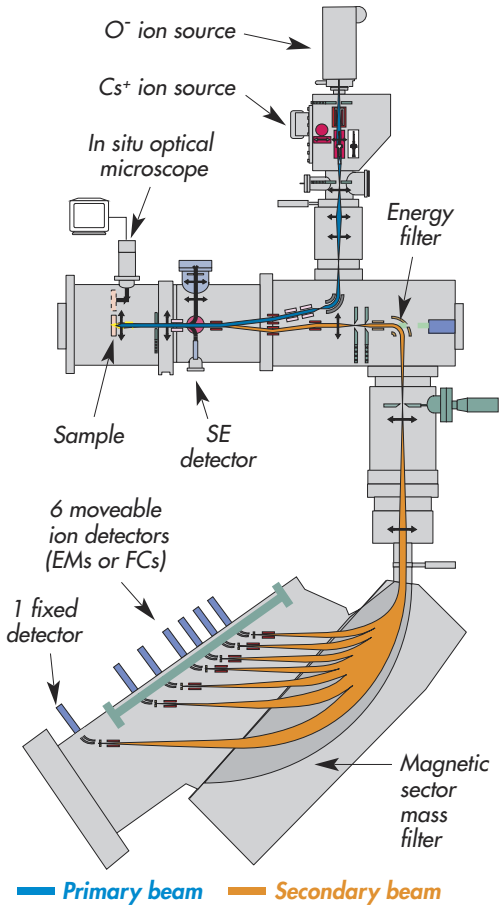
- A new Multi Isotope Imaging technique is available allowing to measure quantitatively in-situ the metabolic activity of single cells.
- It is based on the incubation of cells with stable isotope-labelled substrates. Kinetic studies are possible by harvesting cells at different incubation times.
- It can be applied to uncultivated or rare cells.
- Coupling isotopic measurements with FISH or elemental-ISH allows to link the phylogenetic identity of uncultivated single cells to their metabolism activity.
- The shown examples were obtained for nitrogen and carbon but the same method can be used for phosphorous, sulphur and other biologically active elements within microbial communities.

REFERENCE

1. G. Slodzian, B. Daigne, F. Girard, F. Boust, High Sensitivity and High Spatial Resolution Ion Probe Instrument, Proceedings of the 6th SIMS Conference, Versailles sept. 87.

NanoSIMS:
A revolution in single cell microbiology

Synopsis of a CAMECA NanoSIMS 50L

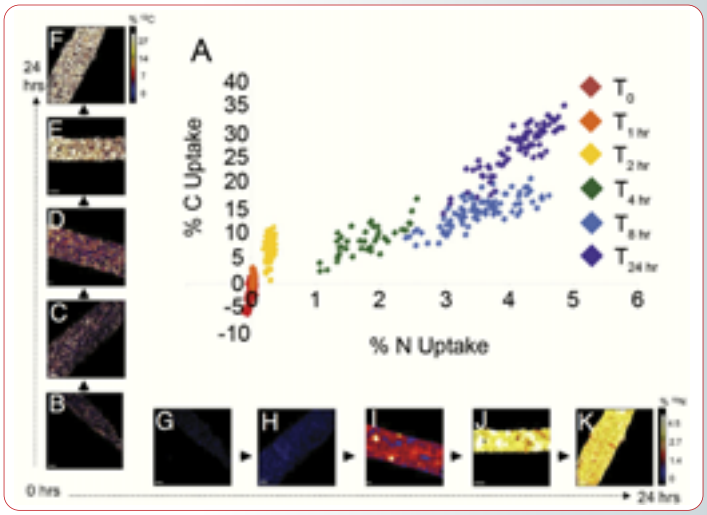


The CAMECA NanoSIMS is an original high resolution secondary ion mass spectrometer (SIMS). A tightly focused beam of reactive primary ions (Cs^+ or O^-) is rastered on the surface of the sample, at normal incidence.

The secondary ions sputtered by this primary beam are collected and mass analyzed in a magnetic sector with a **parallel detection of up to seven masses**. The short working distance of the probe forming lens ensures **50 nm spatial resolution**.

The mass analyzer ensures high transmission at high mass resolution required by the many interfering peaks (ex: $^{12}CH^-$ and $^{13}C^-$, or $^{12}C^{15}N^-$ and $^{13}C^{14}N^-$, etc…).

Applying the NanoSIMS to environmental microbiology is generally based on a **strategy of stable isotope labeling** (ex: ^{13}C, ^{15}N, …) with variable incubation times. The **metabolic activity of single cells** and their interactions can be measured quantitatively, **in situ**, even on **rare or uncultivable cells**.



Percentage ^{13}C and ^{15}N uptake by Trichodesmium trichomes at 6 time points and corresponding NanoSIMS 50 images (scale bar = 1µm). (A) Percentage ^{13}C and ^{15}N uptake at 5 time points in Trichodesmium cells analyzed by NanoSIMS 50. Data points represent average values for individual cells measured along a single trichome by NanoSIMS. Along the x axis: 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. Color scale: from 0 to 5% nitrogen uptake. Along the y axis: images of ^{13}C enrichment at (G) 0 h, (H) 2 h, (I) 4 h, (J) 8 h, and (K) 24 h. Color scale: from 0 to 30% carbon uptake.

From: Finzi-Hart et al., PNAS, vol. 106, no. 15, April 14, 2009

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