Single Cell Environmental Microbiology with Nano Secondary Ion Mass Spectrometry

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Imaging Metabolic Interactions in Microbial Communities

- How much heterogeneity in C and N fixation at the single cell level? *(cyanobacteria cultures)*

- What emergent properties arise from interactions within microbial consortia? *(cyanobacteria & epibionts)*

- How is microbial community composition coupled to production and fate of energy? *(microbial mats)*

- What are the biogeochemical roles microorganisms play in the environment? *(Chip-SIP)*
SIMS: Secondary ion mass spectrometry
A surface sputtering technique

Primary Ion source

Mass spectrometer

Sputtering collision cascade

Mass specific detection

Data

TEM

$\delta^{13}C$
Advantages of NanoSIMS

- Primary beam scans sample surface to produce secondary ions
- Secondary ions detected to produce quantitative digital images
- Simultaneous detection of 5 species
- High sensitivity: MDL~200 atoms
Analysis of multiple stable isotopes in microbes

Incubate cells

Harvest 1  Harvest 2  Harvest 3

Cells fixed, dried on Si wafer

TEM

δ^{15}N


^{12}C, ^{13}C, ^{14}N, ^{15}N, P

^{92}Mo, Ni, Cu, Fe....
Elemental images are overlaid and corrected for known isotope ratio....

\[ \delta^{14N/15N} = \]

..to create a ratio image
Spatial heterogeneity in fixation

$\delta^{13}C$  $\delta^{15}N$

330  750
270  680
210  600
150  530
83  450
21  380
-42  300
-100  230
-170  150
-230  60

Anabaena oscillarioides filament after 4 hrs of isotope label incubation

Popa et al, ISME Journal (Nature), 2007
Isotope imaging in thin sections
Mo is an indicator of enzyme (nitrogenase) location.

- Heterocysts are consistently enriched in Mo, critical nitrogenase co-factor.
- Mo accumulation suggests active N fixation @ 8 hrs (not N storage).
Anabaena-Epibiont Association

Stevenson & Waterbury 2006

Epibiont
- *Rhizobium sp*. WH2K
  - axenic growth in rich medium
  - photosynthetic pigments?
  - *nifH*?

Anabaena sp.
SSM-00
- CO₂ fixation
- N₂ fixation
- axenic growth in minimal medium

Partitioning and distribution of C and N resources?
Nature of association?

Pearl, 1978, 1984, and others
Bulk Incorporation of C and N in Anabaena/Epibiont Association (IRMS)
NanoSIMS imaging of C and N partitioning

Elemental tag allows us to link metabolic interactions to phylogeny:
(EL-FISH)

Behrens et al. AEM 2008
Quantification of C and N enrichment in average cell by NanoSIMS

vegetative cell
epibiont
heterocyst

$\delta^{13}C$
$\delta^{15}N$

Time [Hours]

$\delta^{13}C$
$\delta^{15}N$
H$^{13}$CO$_3^-$ and $^{15}$N$_2$ incorporation in the presence and absence of the epibiont

Anabaena axenic
Anabaena/epibiont
Laminated microbial mats are found in coastal marine environments. Mats are dominated by filamentous cyanobacteria.
Net $\text{H}_2$ production in different layers of ES mat
Who is fixing C and N?

Incubated with $^{13}\text{CO}_2$ and $^{15}\text{N}_2$, analyzed by NanoSIMS

Lyngbya

Microcoleus chthonoplastes

SE

$\delta^{13}\text{C}$

$\delta^{15}\text{N}$

10 µm

20 µm

Lyngbya

Microcoleus
Stable isotope probing with NanoSIMS-effect of season

winter samples

summer samples

Microcoleus

Lyngbya

Unknown filamentous

Unknown filamentous

[Images of microscopy results for different samples]
Microorganisms are genetically diverse but difficult to tell apart

Major goal of environmental microbiology: to link biogeochemical activity to microbial identity (who is doing what)
Chip-SIP (chip = microarray, Stable Isotope Probing)

Incubate community in the presence of isotopically enriched substrate (e.g. $^{13}$C-cellulose), extract RNA

Array spotted with 16S probes specific to taxa

Array synthesized at LMAC on conductive surface

LMAC-NimbleGen Fluorescence Scan

rRNA profile of all community members

NanoSIMS: $^{13}$C:$^{12}$C analysis

Subset of active community that incorporated $^{13}$C-labeled substrate

- $^{12}$C rRNA
- $^{13}$C rRNA
**Proof of concept with pure bacterial cultures**

*Pseudomonas putzeri* grown on $^{13}$C-glucose as sole carbon source

- Fluorescence by microarray scanner
- $^{13}$C enrichment by NanoSIMS

- Fluorescence (measures hybridized RNA) and $^{13}$C enrichment are positively correlated, demonstrating successful detection of labeled RNA by NanoSIMS
- 25% ($^{15}$N) labeled taxon can be differentiated from 100%, 0.5% and unlabeled taxon

![Graph showing correlation between fluorescence and $^{13}$C enrichment](image)

$$R^2 = 0.68557$$
Environmental application of Chip-SIP

San Francisco Bay water collected at Berkeley pier, incubated with 200 uM $^{15}$NH$_4$ for 24 hours

taxa

alpha1 (Alpha Proteobacteria; *Roseobacter*)
bacter7 (Bacteroidetes; Flavobacter)
betaOM43 (Beta Proteo; OM43)
gamma7 (Gamma Proteo)
eurygrpII3 (Archaea; Euryarchaeota)
alveo4 (Eukarya; Alveolata)
Ammonium addition, $T = 6$ hrs

![Graph showing delta 15N vs array fluorescence with different species represented by various symbols and error bars.](image)
Ammonium addition, $T = 24$ hrs

Taxa become differentially enriched over 24hrs, especially the eukaryote
Chip-SIP environmental application, Part 2

San Francisco Bay water collected at Berkeley pier, incubated with 50 μM $^{13}$C amino acids (98% $^{13}$C), 30 μM fatty acids (98% $^{13}$C), or 10 mg L$^{-1}$ starch (10% $^{13}$C) for 12 hours.

![Graph showing isotopic enrichment (d$^{13}$C or d$^{15}$N) for different taxa.]

- **Taxa:** acid0, alpha1, alve04, bacter7, bacteria, betaOM43, gamma7, marga7, planco, rhodo
- **Lines:**
  - Light blue: amino acids
  - Red: fatty acids
  - Green: starch
  - Purple: NH4
- **Legend:**
  - Crosses: no enrichment detected

Isotopic enrichment (d$^{13}$C or d$^{15}$N)
Imaging organic residues in soil aggregates

- **STXM**: Scanning Transmission X-ray Microscopy
- Can indicate location of classes of organic matter (lipids, proteins, chitin) or C in various functional groups (quinonic, aromatic, phenolic, aliphatic, peptidic, carboxylic, and carbonate/carbonyl)
$^{13}$C and $^{15}$N yeast/bacteria mixed with smectite minerals

RGB composites:
- Lipid
- Albumin
- NAG
Combining STXM and SIMS results

Features #1, 2, and 3 show identical proteinaceous chemistry → membrane

Feature #4: membrane, more polysaccharides

Feature #5: core, mostly carboxylic and aromatic C signal → nucleic acids

Combining STXM and SIMS results
**In situ imaging and isotope tracing**

What do we gain?

- Identify keystone taxa (e.g. N-fixers)
- Link nutrient flow to gene expression
- Model C, N, and H in “microbial food webs”
- Nature of symbiotic interactions
- Link community phylogeny to metabolic function
- Soil OM—what organic fractions end up on which mineral surface types?
- Chemical-physical-biological interactions in soil aggregates
NanoSIMS Approach Summary

- NanoSIMS can map trace elements and isotopes at sub-micron scale
- Chemical fate can be localized down to nanomole/gram (ppb) level, precision is ~ 1‰
- Isotope/elemental labeling is key ("tracer experiments")
- Best results when integrated with other analytical techniques (SEM, TEM, AFM, FISH, antibody labeling, STXM/NEXAFS)
- Issues: number of atoms, flatness, element-specific sensitivity

Soil aggregates  Microbial biofilms  RNA microarrays  Hyphal fusion
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STXM images of soil aggregates

• STXM: Scanning Transmission X-ray Microscopy

• Can indicate location of classes of organic matter (lipids, proteins, chitin) or C in various functional groups

• (a) quinonic, (b) aromatic, (c) phenolic, (d) aliphatic, (e) peptidic, (f) carboxylic, and (g) carbonate/carbonyl