Tracking microbial interactions with NanoSIMS

Niculina Musat^{1*}, Florin Musat¹, Peter K. Weber², & Jennifer Pett-Ridge²

Key words: NanoSIMS, nanoSIP, FISH, single cell, stable isotope labelling, chemical microscopy, nutrient transfer

¹ Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany

² Physical and Life Science Directorate, Lawrence Livermore National Laboratory, Livermore, CA, USA

* Corresponding author: Niculina Musat, Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstraße 15, D-04318 Leipzig, Germany

Email: niculina.musat@ufz.de

Graphical abstract

ABSTRACT

The combination of stable isotope probing (SIP), NanoSIMS imaging and microbe identification via fluorescence in situ hybridization (FISH) is often used to link identity to function at the cellular level in microbial communities. Many opportunities remain for nanoSIP to identify metabolic interactions and nutrient fluxes within syntrophic associations and obligate symbioses where exchanges can be extremely rapid. However, additional data, such as genomic potential, gene expression or other imaging modalities are often critical to deciphering the mechanisms underlying specific interactions, and researchers must keep sample preparation artefacts in mind. Here we focus on recent applications of nanoSIP, particularly where used to track exchanges of isotopically labelled molecules between organisms. We highlight metabolic interactions within syntrophic consortia, carbon/nitrogen fluxes between phototrophs and their heterotrophic partners, and symbiont-host nutrient sharing.

Introduction

Understanding the functional roles and interactions of individual microorganisms within complex communities is a major goal of environmental microbiology. Significant strides have been made to identify the *in situ* function of individual microbes [1-8]. Yet interactions among cells in environmental communities remain difficult to disentangle. Many factors obscure microbe-microbe interactions, including functional redundancy, transient metabolites, proteins with unknown function, and disconnects between genomic potential and actual function. Researchers are increasingly overcoming these challenges with multi-prong approaches—"community system biology"—where next generation sequencing methods ('-omics') are used in combination with direct process measurements (e.g. stable isotope probing 'SIP') to map functions, energy flows, and biotic relationships in previously intractable complex communities.

Here, we review recent studies of microbial interactions where high-resolution imaging secondary ion mass spectrometry (SIMS) was combined with stable isotope probing ('nanoSIP' [9]) to allow tracing of stable isotope assimilation into specific microbial cells. SIMS is a type of imaging mass spectrometry where a primary ion beam is used to generate secondary ions from the surface of a solid sample. The Cameca NanoSIMS enables 'nanoSIP' because of its particularly high spatial resolution (50 nm), high sensitivity, and high mass specificity [9,10]. NanoSIP usually requires targeted microbes to be phylogenetically identified, therefore we also emphasize the *in situ* hybridization methods (e.g., FISH, CARD-FISH, SIMS-ISH, EL-FISH, HISH) which help link identity to isotopic enrichment [11-14]. Many studies have shown these *in situ* hybridization methods are crucial to characterizing

microbial activity in the context of spatial relationships [6,15] that range from microbe-host and microbe-microbe interactions, to cell-cell nutrient or metabolite exchanges, and interactions between cells and their organic/inorganic matrix.

NanoSIP in microbial ecology

In the past decade, microbial ecology has benefitted greatly from the use of stable isotope tracing (SIP) techniques (DNA-SIP, RNA-SIP, Protein-SIP, PLFA-SIP, metabolite-SIP) to link identity and function within interacting microbial populations, via detection of specifically labeled cellular components [16-19]. In these approaches, isotope enrichment of specific DNA sequences, proteins or lipids extracted from bulk samples is measured, yet it is not possible to resolve the contributions of individual cells or cell compartments to a particular process [20]. Many critical questions in microbe-microbe and host-microbe interactions require micro-scale spatial information that is lost during bulk sample processing. This is where the NanoSIP approach plays an important role, as the high resolution of NanoSIMS elemental and isotopic images can document cellular metabolism and transfers between individual, closely associated cells. For many researchers, direct measurements of how microbial partners function, and cell-specific rates of metabolic activities are necessary to link symbiont activity, host ecological processes and ecosystem-level biogeochemistry. As access to NanoSIMS instruments becomes more and more available, particularly via user facilities in the USA and Europe, researchers will increasingly be able to balance the availability, cost, and appropriateness of the NanoSIP approach to their individual scientific questions.

The nanoSIP combination has been broadly used in cell biology [10,21-27], microbiology [2,28-30] and phytoecology [31-34] (Fig. 1). NanoSIP is particularly valuable for microbial ecology because it provides spatially resolved information about uptake and transfer of isotopically-labelled compounds in environmental samples where the component taxa cannot be cultured individually. Moreover, by imaging the intracellular localization of stable isotope-labelled molecules, nanoSIP can provide direct evidence of cellular metabolism, distribution of labelled molecules and even quantitative tracking of molecules as they are assimilated into cell biomass or exchanged with other cells [20,21,35]. The most commonly used metabolic tracers in labelling experiments are ^{13}C and ^{15}N [9,35]; additional studies have used ^{34}S and ^2H [6,36].

Phylogenetic labelling for nanoSIP

In nanoSIP studies, cells or subcellular structures (ribosomes or proteins) of target organisms are labelled with oligonucleotide probes or antibodies to provide phylogenetic identification. This procedure takes place immediately after a SIP experiment and typically includes chemical fixation and hybridization steps prior to NanoSIMS analysis [9,35]. Label detection can be done by either (a) sequential imaging by fluorescence microscopy followed by NanoSIMS (FISH-NanoSIMS) e.g. [7,8] or (b) direct label imaging during NanoSIMS analysis (EL-FISH-NanoSIMS, HISH-SIMS) [5,11,12]. In FISH-NanoSIMS applications, the cells are commonly labelled with fluorescent dyes using conventional FISH or CARD-FISH protocols. When target cells are hindered by particles or embedded in an organic matrix or exopolymeric substances (EPS), marking with a laser microdissection microscope or using a coordinate system can ensure that the same fields of interest are imaged by fluorescence microscopy and NanoSIMS. Alternatively, an orthogonal type of microscopy (e.g. scanning electron microscopy (SEM), atomic force microscopy (AFM), or helium ion microscopy) can be used to check the morphological appearance of fluorescently labelled target cells prior to NanoSIMS analysis. For direct label imaging, a rare element such as a halogen (attached to oligonucleotide probes or tyramides) or metal (gold-labelled oligonucleotide probes or antibodies) is introduced into the target cells, these elements are then concurrently mapped during the NanoSIMS analysis (Fig. 2) [5,11-13].

Using nanoSIP to measure nutrient transfers and microbial interactions

Microbe-host interactions

The nanoSIP approach is increasingly used to provide insight on interactions between microorganisms or fungi and their animal or plant hosts. Work in this area includes some of the earliest nanoSIP studies, including a seminal study on shipworms, where N-fixing symbionts were readily identified based on their $^{15}\text{N}_2$ assimilation [1]. More recently, symbionts in reef building corals have been examined [37-39], in studies focused on demonstrating and characterizing predicted functions, such as symbiont C fixation. NanoSIP was also recently used to investigate C fixation by microbial symbionts in gutless *Olavius algarvensis* worms living in seagrass sediments. It confirmed predictions from prior metaproteomic studies, demonstrating the worm symbionts could fix CO derived from seagrass rhizome decomposition [40].

As systems become more complex, the need for additional metadata and experimental treatments increases. For example, in a study where lower termites were fed ^{13}C -cellulose, NanoSIMS images revealed ^{13}C incorporation by gut protists and their symbiotic bacteria

[41], but without additional data, the nature of this symbiosis could not be determined. In a more recent study, Tai and coworkers combined genomics and nanoSIP to show that Bacteroidales ectosymbionts fix nitrogen and supply it to their cellulose-degrading protist host within the hindgut of a wood-eating cockroach [42]. Berry and coworkers [6] took the investigation of gut microbes a step further, using isotopically labelled protein incubations (threonine), FISH, NanoSIMS imaging, community sequencing, and sterile mouse experiments to identify specific microbes that forage for host proteins in the mouse gut. This study was the first to couple single cell, isotope, and meta-'omics approaches *in vivo* in a mammalian system, and is an exemplar for future studies of interactions between uncultivated taxa in human and environmental microbiome research.

In plants, there are also many opportunities to study plant-microbe interactions using NanoSIMS. For example, root-associated mycorrhizal fungi are particularly important in root nutrient acquisition and exchange [31,33,34,43], and in Nuccio et al. [31], NanoSIMS imaging was used to illustrate mycorrhizal fungal transport of nitrogen derived from decaying organic matter, and transfer to the host plant.

Phototroph-Heterotroph Interactions

Many recent NanoSIMS studies of microbial interactions have focused on partnerships between phototrophs and heterotrophs. This emphasis reflects a growing recognition of the varied roles heterotrophs play in supporting phototrophic productivity (e.g. in the rhizosphere [31,43], phycosphere [30,44], and possibly even the endosphere [45]). But the nature of phototroph-heterotroph relationships can be complex (e.g. evolving from beneficial to opportunistic [46]) and recent work shows primary producers can also have complicated ecological roles, for example acting as organic consumers [47]. Combinations of nanoSIP and whole genome or 'omics approaches become critical as researchers attempt to unravel these relationships.

Many nanoSIP studies have explored interactions involving cyanobacteria [15,48-54]. In mats and biofilms, cyanobacteria produce an extensive organic extracellular matrix, providing the surrounding heterotrophic community with a rich source of nutrients. Using a combination of stable isotope tracing and proteomics, Stuart et al. demonstrated that mat phototrophs may assimilate their own EPS under a range of metabolic conditions [47,55]. Paired proteomics analyses point to multiple enzymes (involved in degradation of amino acids, proteins, nucleotides, and carbohydrates) that indicate the cyanobacteria use their own EPS as an energy/C source. In this study, nanoSIP results indicate that cyanobacteria can effectively compete with heterotrophic community members for organic matter in both

light and dark conditions, and that both nutrient requirements and community interactions contribute to cycling of extracellular organic matter.

Other work in cyanobacteria mats has combined nanoSIP with metatranscriptomics, specific inhibitors, and CARD-FISH to illustrate carbon uptake patterns associated with different diel cycles and acetate consumers, and metabolite flows between cyanobacteria, *Chloroflexi* and sulfate reducers [48-50]. In these studies, while nanoSIP-FISH pointed to likely players and quantitatively important nutrient exchanges, the corresponding metatranscriptome and isolate genome data was critical to reconstruct the catabolic pathways involved (fermentation of glycogen by the dominant cyanobacteria and conversion of glycogen to polyhydroxyalkonates by *Chloroflexi*). Similarly, the combination of nanoSIP and genome sequencing was critical to understand the symbiotic relationship between an N-fixing unicellular cyanobacterium (whose genome has lost many common metabolic functions) and photosynthetic picoeukaryotic cells from the Haptophyta division [15]. In combination with a particularly gentle sample preparation approach (to preserve loosely partnered cells), isotope tracing with $^{15}\text{N}_2$ and H^{13}CO_3 showed these cyanobacteria provide fixed N to their eukaryotic partners in exchange for fixed C. More recent studies of this symbiosis have revealed the additional importance of Fe availability to the N-fixing partner [53].

Single cell isotope tracing becomes slightly more complicated, but even more valuable, in studies of multitrophic interactions. For example, a substantial amount of algal cell organic matter can be transferred to surrounding bacteria following viral lysis [30]. Sheik et al used a combination of nanoSIP with amplicon sequencing to track bacterial community succession following lysis of ^{13}C - and ^{15}N -enriched algal cells, which provided both ecological context and helped guide CARD-FISH targeting. Other studies have used multiple isotope tracers simultaneously (e.g. ^{13}C -inorganic carbon and $^{15}\text{N}_2$ gas), to track assimilation within a food web. The colony-forming cyanobacterium *Aphanizomenon*, common in the Baltic Sea, actively fixes N_2 , but then releases nearly half as NH_4^+ , which is taken up by surrounding prokaryotic and eukaryotic plankton, diatoms and copepods, forming the basis of the Baltic Sea food web [56].

Anaerobic Oxidation of Methane

Over the past 15 years, FISH-SIMS and more recently FISH-NanoSIMS have been used to explore the physiology of anaerobic oxidation of methane (AOM) consortia in marine sediments, via cultivation-independent incubations with ^{13}C -labelled methane and ^{15}N -ammonia, ^{15}N -nitrate and $^{15}\text{N}_2$ [4,57-61]. Syntrophic associations between methanotrophic archaea (anaerobic methanotrophs, ANME) and sulfate reducing bacteria (SRB), drive AOM in marine methane seep sediments. This system is where combined FISH and SIMS was

first applied [57], and continues to be an archetype for the approach [62]. Still, it is important to recognize that FISH-NanoSIMS alone could not resolve the nature of the syntrophic interaction between ANME and SRB, but was most powerful in combination with other microscopy techniques, physiology experiments and 'omics investigations.

The nature of the ANME-SRB syntrophy was initially elusive, with experimental evidence showing that hydrogen, methanol, formate or acetate did not served as electron donors for sulfate reduction [63]. FISH-SIMS helped reveal that ANME cells could assimilate methane-derived carbon, showed higher N assimilation by ANME vs. SRB cells when ¹⁵N-ammonia was provided, and proved that ANME cells were able to fix N₂ and transfer it to SRB [4,58,59]. A syntrophic model for AOM, through the transfer of partly-reduced sulfur compounds, was proposed based on FISH-immunolabelling-NanoSIMS results [64]. NanoSIMS analyses showed that ANME cells, identified by mapping of Au deposited by specific antibody labelling, accumulated high amounts of sulfur, correlated with the assimilation of methane-derived carbon. Identified as polysulfides by Raman microspectroscopy, these sulfur compounds are thought to act as diffusible electron shuttles between ANME and SRB cells [64].

Recently, additional NanoSIMS analyses of AOM consortia showed that biosynthetic activities of single ANME and SRB cells are unrelated to the proximity of syntrophic partners [7]. Moreover, ANME cells maintained similar biosynthetic activities when sulfate was replaced with artificial electron acceptors [62], apparently due to direct interspecies electron transfer (DIET) from ANME to SRB cells. Firm evidence in support of DIET was provided via the discovery of multi-haem cytochromes genes in the genomes of ANME, the detection of cytochromes in the extracellular space of AOM consortia, and descriptions of nanowire-like structures in thermophilic AOM cultures [7,65].

Challenges and future directions

While nanoSIP is now widely recognized as a powerful tool in microbial ecology, users must be aware of its limitations, some of which are inherent to the complexity of biological systems. Technical challenges, such as alterations of the elemental and isotopic composition of cells during hybridization protocols, are a concern for those interested in measuring truly quantitative metabolic fluxes. On the other hand, 'biological' or 'metabolic' challenges (e.g. complex microbial communities, intricate or rapid nutrient exchanges) may be overcome by multi-prong approaches that include 'omics, complementary microscopy techniques and/or physiology experiments.

214 *Cross Feeding*

215 Cross feeding occurs with the transfer of labelled metabolites from primary processors of a
216 substrate to a second group of microorganisms, and can make interpretation of nanoSIP
217 results ambiguous. The primary ways to control for this effect are 1) to use the shortest
218 possible incubations, 2) quantify the relative level of incorporation of the label, and 3) use
219 genomic data to constrain metabolic potential. Remarkably, in many environmental systems,
220 using short incubations is sufficient to avoid cross feeding. For example, nitrogen fixers tend
221 to become enriched well before other non-fixers in $^{15}\text{N}_2$ experiments [e.g. [66,67]. However,
222 we cannot take this outcome for granted for symbionts. At the extreme, terminally
223 differentiated cells within a single organism may be the site of initial isotope uptake but are
224 not the site of isotope assimilation into biomass, such as in the nitrogen-fixing heterocysts of
225 *Anabeana* [67] or field populations of *Aphanizomenon* sp. [66]. NanoSIP may also fail to
226 identify slow-growing organisms as primary substrate users. In structurally less-integrated
227 microbial systems, microorganisms using secondary metabolites released by fast-growing
228 primary users may assimilate an isotope label at the same time as slow-growing primary
229 users [e.g. [68]. Ultimately, investigators must address the potential for cross-feeding within
230 each system individually, and bring to bear accessible metadata to interpret nanoSIP results.

231 *Artefacts of Phylogenetic labelling*

232 In many nanoSIP approaches, protocols used for fixation and phylogenetic labelling have
233 numerous cell treatment steps. Recently, concerns have been raised that these treatments
234 may alter the cellular isotopic composition following SIP experiments by loss of low
235 molecular-mass compounds (permeabilization), or addition of nonlabeled elements (during
236 chemical fixation or tyramide deposition). NanoSIMS studies with ^{13}C - and ^{15}N -labelled type
237 strains showed a stepwise dilution of both carbon and nitrogen isotopic composition after
238 chemical fixation, FISH and CARD-FISH protocols [14,54]. Additional experiments suggest
239 that alteration of the isotopic composition may depend not only on the isotope in question
240 and fixation method, but also the taxa in question (J. Pett-Ridge and S. Behrens unpublished
241 results). For example, in gram-negative *Vibrio cholerae*, CARD-FISH reduced cell ^{13}C
242 enrichment by 60-80%, and $\Delta^{15}\text{N}$ values between 30-60%, relative to chemical fixation
243 alone. For gram-positive *Bacillus subtilis*, CARD-FISH treatment resulted in 55-80% lower
244 ^{13}C enrichment, and 70-75% lower $\Delta^{15}\text{N}$ values (Fig. 3). The high variance in dilution effect
245 attributable to fixation and/or FISH treatments is a particularly important consideration for
246 researchers interested in the absolute enrichment of a particular cell population.

247 Despite the caveats associated with isotopic dilution, FISH-based cell identification is still
248 widely used to target individual populations within samples of unknown microbial

composition, and preserves cell integrity for subsequent imaging analyses. This is especially true for phylogenetically cohesive populations where a confined rRNA oligonucleotide probe may be used. In samples where good community composition data exists, it may even be possible to use multiple reporter elements or fluorescent dyes (e.g. Multiplex FISH or CLASI-FISH) for simultaneous identification of multiple phylotypes [69,70] followed by NanoSIMS analysis.

Sample preparation

Sample preparation for nanoSIP experiments should attempt to conserve the ultrastructural organization of cells and, if possible, their elemental and isotopic composition and spatial distribution. NanoSIMS analyses can resolve some subcellular structures (e.g. cell wall, nucleoid, ribosomes) and these ultrastructural features may be imaged in advance with high spatial resolution by electron microscopy [32]. However, conventional sample preparation methods for TEM or SEM aiming to maintain high structural integrity of samples may lead to isotope dilution or physical rearrangement of molecules. High pressure freezing followed by freeze-substitution and sectioning is an excellent alternative to conventional protocols even for samples with a high-water content and has been used to map the distribution of ions and light elements in plant and mammalian tissue [71-74]. Similar protocols need to be developed for correlative analyses in microbiology, ideally in combination with phylogenetic labelling.

Secondary metabolites

Identification of the secondary metabolites that are transferred among microorganisms in complex systems adds additional challenges to microbe interaction studies. Since nanoSIP can only track isotopes or elements, the biochemical pathways, excreted metabolites and molecular forms by which a tracer is assimilated have to be inferred by other methods. One approach is to predict and test for secondary metabolites based on genetic potential and bulk analytical methods, and to conduct follow-up nanoSIP experiments by adding the target metabolite [49]. This combination offers direct fingerprinting of catabolic pathways, thus resolving the flow of primary intermediates within microbial communities. Another approach is correlative application of complementary single cell imaging techniques, such as Raman microspectroscopy, ToF-SIMS or scanning transmission X-ray microscopy with NanoSIMS imaging [64,75]. In experiments with known starting substrates, biochemical pathways and potential intermediates can also be inferred from orthogonal methods such as genomics, transcriptomics and proteomics.

Conclusions

The study of microbial interactions with nanoSIP is rapidly advancing and expanding, and we expect this trend to continue. The level of sophistication in these studies has increased over the last decade as questions move from “which microbe uses which substrate?” to “how does this organism interact with another and its host?”. While nanoSIP alone in an unperturbed system can identify potentially interesting interactions, data from orthogonal methods (geochemistry to ‘omics) and/or manipulation experiments are critical to providing deep understanding. The key role of nanoSIP is demonstrating that an OTU is involved in an assimilatory function or process at the single cell or subcellular scale. Because microbial interactions are critical to human health, food security and global climate, we expect to see even more studies using nanoSIP to investigate a diverse range of microbe interactions.

Acknowledgments. This work was supported by the Dept. of Isotope Biogeochemistry, Centre for Chemical Microscopy (ProVIS), Helmholtz Centre for Environmental Research (UFZ) and the U.S. Department of Energy Genomic Science Program under contract SCW1039 to the Physical and Life Science Directorate, Lawrence Livermore National Laboratory. Work at Lawrence Livermore National Laboratory was performed under the auspices of the U.S. Department of Energy under Contract DE-AC52-07NA27344. For Fig. 2, Dr. Hryhoriy Stryhanyuk acquired and analyzed the NanoSIMS data; the National Science Foundation (USA) provided support via NSF grant OCE-1136727 to S. Sievert. Samples for Fig 3. were provided by S. Behrens and T. Loesekann.

Figures

Figure 1. Illustration of microbial communities and associations where NanoSIMS in combination with stable isotope probing (nanoSIP) has been applied to resolve assimilation and exchange of specific metabolic substrates. These include label uptake and transfer from primary to secondary processors in planktonic communities; assimilation of carbon and nitrogen by syntrophic consortia involved in anaerobic oxidation of methane; CO₂ and N₂ fixation by environmental populations of cyanobacteria and the subsequent transfer of organic carbon and ammonia to attached microbial communities; and trophic networks in microbial communities living in the gut of higher organisms, here exemplified by cellulose-degrading protozoa and their epibionts in the gut of termites.

Figure 2. Example of direct label imaging by NanoSIMS for phylogenetic identification of target cells. In this example, key microbes responsible for autotrophic processes at deep sea vents were targeted. The NanoSIMS data is depicted as a composite RGB image showing ¹³C assimilation from ¹³C-labelled inorganic carbon (green), phylogenetic identification of the dominant autotrophic microbial group by ¹⁹F introduced in the cells via CARD-FISH (red) and ³²S as a biomass indicator (blue). Image, courtesy of Stefan Sievert.

Figure 3. The effects of fixation technique (paraformaldehyde (PFA), ethanol) and CARD-FISH on the isotopic composition (^{13}C and ^{15}N) of *Vibrio cholerae* (92A 1552 El Tor, Inaba, wild type, Rifs) and *Bacillus subtilis* (JH642) cells cultivated on 99.9 atom% $^{13}\text{C}/^{15}\text{N}$ mixed amino acids (Cambridge Isotope Laboratories) (J. Pett-Ridge and S. Behrens, unpublished data). The number of single cells analysed by NanoSIMS for each treatment is indicated on the x axis.

References

1. Lechene CP, Luyten Y, McMahon G, Distel DL: **Quantitative imaging of nitrogen fixation by individual bacteria within animal cells.** *Science* 2007, **317**:1563-1566.
2. Finzi-Hart J. & Pett-Ridge J, Weber P, Popa R, Fallon SJ, Gunderson T, Hutcheon I, Nealson K, Capone DG: **Fixation and fate of carbon and nitrogen in *Trichodesmium* IMS101 using nanometer resolution secondary ion mass spectrometry (NanoSIMS).** *Proc Nat Acad Sci USA* 2009, **106**:6345-6350.
3. Osborne CP, Freckleton RP: **Ecological selection pressures for C_4 photosynthesis in the grasses.** *Proceedings of the Royal Society B* 2009, **276**:1753-1760.
4. Dekas AE, Poretsky RS, Orphan VJ: **Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia.** *Science* 2009, **326**:422-426.
5. Musat N, Halm H, Winterholler B, Hoppe P, Peduzzi S, Hillion F, Horreard F, Amann R, Jorgensen BB, Kuypers MM: **A single-cell view on the ecophysiology of anaerobic phototrophic bacteria.** *Proc Natl Acad Sci U S A* 2008, **105**:17861-17866.
6. Berry D, Stecher B, Schintlmeister A, Reichert J, Brugiroux S, Wild B, Wanek W, Richter A, Rauch I, Decker T, et al.: **Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing.** *Proceedings of the National Academy of Sciences* 2013, **110**:4720-4725

** In this study nanoSIP was used in combination with FISH to characterize utilization of host proteins by individual bacterial cells in the mouse gut. NanoSIMS imaging was complemented by community sequencing and sterile mouse experiments.

7. McGlynn SE, Chadwick GL, Kempes CP, Orphan VJ: **Single cell activity reveals direct electron transfer in methanotrophic consortia.** *Nature* 2015, **526**:531-535

** In this study biosynthetic activities of single cells within AOM consortia were determined by NanoSIMS analysis. The nanoSIP data, together with genome sequencing, redox

- staining of extracellular cytochromes and modelling, was essential to propose a direct electron transfer between ANME and SRB cells as the basis of the AOM syntrophy.
8. Milucka J, Kirf M, Lu L, Krupke A, Lam P, Littmann S, Kuypers MMM, Schubert CJ: **Methane oxidation coupled to oxygenic photosynthesis in anoxic waters.** *ISME J* 2015, **9**:1991-2002.
 9. Pett-Ridge J, Weber PK: **NanoSIP: NanoSIMS applications for microbial biology.** *Methods Mol Biol* 2012, **881**:375-408.
 10. Lechene C, Hillion F, McMahon G, Benson D, Kleinfeld A, Kampf JP, Distel D, Luyten Y, Bonventre J, Hentschel D, et al.: **High-resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry.** *Journal of Biology* 2006, **5**:20.
 11. Li T, Wu TD, Mazeas L, Toffin L, Guerquin-Kern JL, Leblon G, Bouchez T: **Simultaneous analysis of microbial identity and function using NanoSIMS.** *Environmental Microbiology* 2008, **10**:580-588.
 12. Behrens S, Losekann T, Pett-Ridge J, Weber PK, Ng W, Stevenson BS, Hutcheon ID, Relman DA, Spormann AM: **Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced Element Labeling-catalyzed reporter deposition Fluorescence In Situ Hybridization (EL-FISH) and NanoSIMS.** *Applied and Environmental Microbiology* 2008, **74**:3143.
 13. Kubota K, Morono Y, Ito M, Terada T, Itezono S, Harada H, Inagaki F: **Gold-ISH: A nano-size gold particle-based phylogenetic identification compatible with NanoSIMS.** *Systematic and Applied Microbiology* 2014, **37**:261-266.
 14. Musat N, Stryhanyuk H, Bombach P, Adrian L, Audinot J-N, Richnow HH: **The effect of FISH and CARD-FISH on the isotopic composition of ¹³C- and ¹⁵N-labeled *Pseudomonas putida* cells measured by nanoSIMS.** *Systematic and Applied Microbiology* 2014, **37**:267-276
- * This comprehensive study documents the effects of cell fixation and hybridization techniques on the isotopic composition of labelled cells. The dilution effect was more pronounced for CARD-FISH vs. FISH protocols, and appeared to be influenced by the physiological state of the cells.
15. Thompson AW, Foster RA, Krupke A, Carter BJ, Musat N, Vaulot D, Kuypers MMM, Zehr JP: **Unicellular Cyanobacterium Symbiotic with a Single-Celled Eukaryotic Alga.** *Science* 2012, **337**:1546-1550.
 16. Mayali X, Weber PK, Brodie EL, Mabery S, Hoeprich P, Pett-Ridge J: **High-throughput isotopic analysis of RNA microarrays to quantify microbial resource use** *ISME Journal* 2012, **6**:1210-1221.
 17. Lueders T, Dumont MG, Bradford L, Manefield M: **RNA-stable isotope probing: from carbon flow within key microbiota to targeted transcriptomes.** *Curr Opin Biotechnol* 2016, **this volume**.
 18. Jehmlich N, Vogt C, Lünsmann V, Richnow HH, von Bergen M: **Protein-SIP in environmental studies.** *Curr Opin Biotechnol* 2016, **41**:26-33.
 19. Wegener G, Kellermann MY, Elvert M: **Tracking activity and function of microorganisms by stable isotope probing of membrane lipids.** *Curr Opin Biotechnol* 2016, **41**:43-52.
 20. Jiang H, Favaro E, Goulbourne CN, Rakowska PD, Hughes GM, Ryadnov MG, Fong LG, Young SG, Ferguson DJP, Harris AL, et al.: **Stable isotope imaging of biological samples with high resolution secondary ion mass spectrometry and complementary techniques.** *Methods* 2014, **68**:317-324.
 21. Saka SK, Vogts A, Kröhnert K, Hillion F, Rizzoli SO, Wessels JT: **Correlated optical and isotopic nanoscopy.** *Nat Commun* 2014, **5**:

** This was the first correlative application of STED microscopy and nanoSIP to study the protein turnover at sub-cellular level. Dealing with cultured hippocampal neurons, this

- study highlighted the relevance of NanoSIMS to analyze the isotopic composition of sub-cellular structures in cell biology.
22. Kleinfeld AM, Kampf JP, Lechene C: **Transport of ^{13}C -oleate in adipocytes measured using multi imaging mass spectrometry.** *Journal of the American Society of Mass Spectrometry* 2004, **15**:1572–1580.
 23. Champion C, Elbast M, Wu TD, Colas-Linhart N: **Thyroid cell irradiation by radioiodines: A new Monte Carlo electron track-structure code.** *Brazilian Archives of Biology and Technology* 2007, **50**:135-144.
 24. Romer W, Wu TD, Duchambon P, Amessou M, Carrez D, Johannes L, Guerquin-Kern JL: **Sub-cellular localisation of a N-15-labelled peptide vector using NanoSIMS imaging.** *Applied Surface Science* 2006, **252**:6925-6930.
 25. Kraft ML, Fishel SF, Marxer CG, Weber PK, Hutcheon ID, Boxer SG: **Quantitative analysis of supported membrane composition using the NanoSIMS.** *Applied Surface Science* 2006, **252**:6950-6956.
 26. Galli Marxner C, Kraft ML, Weber PK, Hutcheon I, Boxer SG: **Supported membrane composition analysis by secondary ion mass spectrometry with high lateral resolution.** *Biophys J* 2005, **88**:2965-2975.
 27. Kraft ML, Weber PK, Longo ML, Hutcheon ID, Boxer SG: **Phase Separation of Lipid Membranes Analyzed with High-Resolution Secondary Ion Mass Spectrometry.** *Science* 2006, **313**:1948-1951.
 28. Jaekel U, Musat N, Adam B, Kuypers M, Grundmann O, Musat F: **Anaerobic degradation of propane and butane by sulfate-reducing bacteria enriched from marine hydrocarbon cold seeps.** *ISME J* 2013, **7**:885-895.
 29. Probst AJ, Weinmaier T, Raymann K, Perras A, Emerson JB, Rattei T, Wanner G, Klingl A, Berg IA, Yoshinaga M, et al.: **Biology of a widespread uncultivated archaeon that contributes to carbon fixation in the subsurface.** *Nat Commun* 2014, **5**.
 30. Sheik AR, Brussaard CPD, Lavik G, Lam P, Musat N, Krupke A, Littmann S, Strous M, Kuypers MMM: **Responses of the coastal bacterial community to viral infection of the algae *Phaeocystis globosa*.** *ISME J* 2014, **8**:212-225.
 31. Nuccio EE, Hodge A, Pett-Ridge J, Herman DJ, Weber PK, Firestone MK: **An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition.** *Environmental Microbiology* 2013, **15**:1870-1881, **Cover Image**.
 32. Hong-Hermesdorf A, Miethke M, Gallaher SD, Kropat J, Dodani SC, Barupala D, Chan J, Domaille DW, Shirasaki DI, Loo JA, et al.: **Selective sub-cellular visualization of trace metals identifies dynamic sites of Cu accumulation in *Chlamydomonas*.** *Nature Chemical Biology* 2014, **10**:1034-1042.
 33. Bougoure J, Ludwig M, Brundrett M, Cliff J, Clode P, Kilburn M, Grierson P: **High-resolution secondary ion mass spectrometry analysis of carbon dynamics in mycorrhizas formed by an obligately myco-heterotrophic orchid.** *Plant, Cell & Environment* 2014, **37**:1223-1230.
 34. Kuga Y, Sakamoto N, Yurimoto H: **Stable isotope cellular imaging reveals that both live and degenerating fungal pelotons transfer carbon and nitrogen to orchid protocorms.** *New Phytologist* 2014, **202**:594-605.
 35. Musat N, Foster R, Vagner T, Adam B, Kuypers MMM: **Detecting metabolic activities in single cells, with emphasis on nanoSIMS.** *FEMS Microbiology Reviews* 2012, **36**:486-511.
 36. Fike DA, Gammon CL, Ziebis W, Orphan VJ: **Micron-scale mapping of sulfur cycling across the oxycline of a cyanobacterial mat: a paired nanoSIMS and CARD-FISH approach.** *ISME Journal* 2008, **2**:749-759.
 37. Ceh J, Kilburn MR, Cliff JB, Raina J-B, van Keulen M, Bourne DG: **Nutrient cycling in early coral life stages: *Pocillopora damicornis* larvae provide their algal symbiont (*Symbiodinium*) with nitrogen acquired from bacterial associates.** *Ecology and Evolution* 2013, **3**:2393-2400.

38. Kopp C, Meibom A, Beyssac O, Stolarski J, Djediat S, Szlachetko J, Domart-Coulon I: **Calcareous sponge biomineralization: Ultrastructural and compositional heterogeneity of spicules in *Leuconia johnstoni* Carter, 1871.** *Journal of Structural Biology* 2011, **173**:99-109.
39. Lema KA, Clode PL, Kilburn MR, Thornton R, Willis BL, Bourne DG: **Imaging the uptake of nitrogen-fixing bacteria into larvae of the coral *Acropora millepora*.** *ISME J* 2015.
40. Kleiner M, Wentrup C, Holler T, Lavik G, Harder J, Lott C, Littmann S, Kuypers MMM, Dubilier N: **Use of carbon monoxide and hydrogen by a bacteria–animal symbiosis from seagrass sediments.** *Environmental Microbiology* 2015, **17**:5023-5035
- * Kleiner et al used nanoSIP in correlation with metaproteomic analyses to elucidate the energy sources of symbionts of gutless oligochaetes living in seagrass sediments.
41. Carpenter KJ, Weber PK, Davisson ML, Pett-Ridge J, Haverty MI, Keeling PJ: **Correlated SEM, FIB-SEM, TEM, and NanoSIMS Imaging of Microbes from the Hindgut of a Lower Termite: Methods for In Situ Functional and Ecological Studies of Uncultivable Microbes.** *Microscopy and Microanalysis* 2013, **19**:1490-1501.
42. Tai V, Carpenter KJ, Weber PK, Nalepa CA, Perlman SJ, Keeling PJ: **Genomic and NanoSIMS analyses of nitrogen-fixing bacterial ectosymbionts of a protist inhabiting a wood-eating cockroach.** *Applied and Environmental Microbiology* 2016, **in press**.
43. Kaiser C, Kilburn MR, Clode PL, Fuchslueger L, Koranda M, Cliff JB, Solaiman ZM, Murphy DV: **Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation.** *New Phytologist* 2015, **205**:1537-1551
- * Here nanoSIP was applied to resolve the in situ flows of recently photoassimilated carbon and its transfer from plant roots to soil microbes. .
44. de-Bashan L, Mayali X, Bebout B, Weber P, Detweiler A, Hernandez J, Prufert-Bebout L, Bashan Y: **Establishment of stable synthetic mutualism without co-evolution between microalgae and bacteria demonstrated by mutual transfer of metabolites (NanoSIMS isotopic imaging) and persistent physical association (Fluorescent in situ hybridization).** *Algal Research* 2016, **15**:179-186.
45. Nomaki H, Bernhard JM, Ishida A, Tsuchiya M, Uematsu K, Tame A, Kitahashi T, Takahata N, Sano Y, Toyofuku T: **Intracellular isotope localization in *Ammonia* sp. (Foraminifera) of oxygen-depleted environments: results of nitrate and sulfate labeling experiments.** *Frontiers in Microbiology* 2016, **7**.
46. Seyedsayamdost MR, Case RJ, Kolter R, Clardy J: **The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*.** *Nat Chem* 2011, **3**:331-335.
47. Stuart RK, Mayali X, Lee JZ, Craig Everroad R, Hwang M, Bebout BM, Weber PK, Pett-Ridge J, Thelen MP: **Cyanobacterial reuse of extracellular organic carbon in microbial mats.** *ISME J* 2015:
- ** This study used an original combination of proteomics and nanoSIP. Proteomic were applied to identify cyanobacterial extracellular enzymes, while nanoSIP was used to track isotopically labelled EPS into single cells.
48. Burow LC, Woebken D, Marshall IPG, Singer SW, Pett-Ridge J, Prufert-Bebout L, Spormann AM, Bebout BM, Weber PK, Hoehler TM: **Identification of Desulfobacterales as primary hydrogenotrophs in a complex microbial mat community.** *Geobiology* 2014, **12**:221-230.
49. Burow LC, Woebken D, Marshall IPG, Lindquist EA, Bebout BM, Prufert-Bebout L, Hoehler TM, Tringe SG, Pett-Ridge J, Weber PK, et al.: **Anoxic carbon flux in**

- photosynthetic microbial mats as revealed by metatranscriptomics. *ISME J* 2013, **7**:817-829.
50. Lee JZ, Burow LC, Woebken D, Everroad RC, Kubo MD, Spormann AM, Weber PK, Pett-Ridge J, Bebout BM, Hoehler TM: **Fermentation couples Chloroflexi and sulfate-reducing bacteria to Cyanobacteria in hypersaline microbial mats.** *Frontiers in Microbiology* 2014, **5**.
51. Woebken D, Burow LC, Prufert-Bebout L, Bebout BM, Hoehler TM, Pett-Ridge J, Spormann AM, Weber PK, Singer SW: **Identification of a novel cyanobacterial group as active diazotrophs in a coastal microbial mat using NanoSIMS analysis.** *The ISME Journal* 2012, **6**:1427-1439.
52. Krupke A, Musat N, LaRoche J, Mohr W, Fuchs BM, Amann RI, Kuypers MMM, Foster RA: **In situ identification and N₂ and C fixation rates of uncultivated cyanobacteria populations.** *Systematic and Applied Microbiology* 2013, **36**:259-271.
53. Krupke A, Mohr W, LaRoche J, Fuchs BM, Amann RI, Kuypers MMM: **The effect of nutrients on carbon and nitrogen fixation by the UCYN-A-haptophyte symbiosis.** *ISME J* 2015, **9**:1635-1647.
54. Woebken D, Burow LC, Behnam F, Mayali X, Schintlmeister A, Fleming ED, Prufert-Bebout L, Singer SW, Cortes AL, Hoehler TM, et al.: **Revisiting N₂ fixation in Guerrero Negro intertidal microbial mats with a functional single-cell approach.** *ISME J* 2014, **9**:485-496
- * This study investigated the dilution effect that CARD-FISH may have on the isotopic composition of bacterial cells. A dilution of both 4¹³C and 4¹⁵N was observed. Also, the dilution effect appeared to vary with the phylotype.
55. Stuart RK, Mayali X, Boaro A, Zemla A, Everroad RC, Nilson D, Weber PK, Lipton M, Pett-Ridge J, Thelen MP: **Light regimes shape utilization of extracellular organic C and N in a cyanobacterial biofilm.** *mBio* 2016, in press.
56. Adam B, Klawonn I, Sveden JB, Bergkvist J, Nahar N, Walve J, Littmann S, Whitehouse MJ, Lavik G, Kuypers MMM, et al.: **N₂-fixation, ammonium release and N-transfer to the microbial and classical food web within a plankton community.** *ISME J* 2016, **10**:450-459
- * NanoSIMS was used to show role of colony-forming cyanobacteria on the ecosystem productivity and biogeochemical processes at short time scales. A surprising finding was that the cyanobacterium *Aphanizomenon* was excreting about 450% of its fixed nitrogen as ammonium, further assimilated by the prokaryotic and eukaryotic community.
57. Orphan VJ, House CH, Hinrichs KU, McKeegan KD, DeLong EF: **Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis.** *Science* 2001, **293**:484-487.
58. Dekas AE, Chadwick GL, Bowles MW, Joye SB, Orphan VJ: **Spatial distribution of nitrogen fixation in methane seep sediment and the role of the ANME archaea.** *Environmental Microbiology* 2014, **16**:3012-3029.
59. Orphan VJ: **Methods for unveiling cryptic microbial partnerships in nature.** *Current Opinion in Microbiology* 2009, **12**:231-237.
60. Dekas AE, Connon SA, Chadwick GL, Trembath-Reichert E, Orphan VJ: **Activity and interactions of methane seep microorganisms assessed by parallel transcription and FISH-NanoSIMS analyses.** *ISME J* 2016, **10**:678-692.
61. Green-Saxena A, Dekas AE, Dalleska NF, Orphan VJ: **Nitrate-based niche differentiation by distinct sulfate-reducing bacteria involved in the anaerobic oxidation of methane.** *ISME J* 2014, **8**:150-163.
62. Scheller S, Yu H, Chadwick GL, McGlynn SE, Orphan VJ: **Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction.** *Science* 2016, **351**:703-707.

63. Nauhaus K, Boetius A, Krüger M, Widdel F: **In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area.** *Environmental Microbiology* 2002, **4**:296-305.
64. Milucka J, Ferdelman TG, Polerecky L, Franzke D, Wegener G, Schmid M, Lieberwirth I, Wagner M, Widdel F, Kuypers MMM: **Zero-valent sulphur is a key intermediate in marine methane oxidation.** *Nature* 2012, **491**:541-546.
65. Wegener G, Krukenberg V, Riedel D, Tegetmeyer HE, Boetius A: **Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria.** *Nature* 2015, **526**:587-590.
66. Ploug H, Musat N, Adam B, Moraru CL, Lavik G, Vagner T, Bergman B, Kuypers MMM: **Carbon and nitrogen fluxes associated with the cyanobacterium *Aphanizomenon* sp. in the Baltic Sea.** *ISME J* 2010, **4**:1215-1223.
67. Popa R, Weber PK, Pett-Ridge J, Finzi JA, Fallon SJ, Hutcheon ID, Nealson KH, Capone DG: **Carbon and nitrogen fixation and metabolite exchange in and between individual cells of *Anabaena oscillarioides*.** *ISME Journal* 2007, **1**:354-360.
68. Kleindienst S, Herbst F-A, Stagars M, von Netzer F, von Bergen M, Seifert J, Peplies J, Amann R, Musat F, Lueders T, et al.: **Diverse sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade are the key alkane degraders at marine seeps.** *ISME J* 2014, **8**:2029-2044.
69. Thurnheer T, Gmür R, Guggenheim B: **Multiplex FISH analysis of a six-species bacterial biofilm.** *Journal of Microbiological Methods* 2004, **56**:37-47.
70. Valm AM, Welch JLM, Rieken CW, Hasegawa Y, Sogin ML, Oldenbourg R, Dewhirst FE, Borisy GG: **Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging.** *Proceedings of the National Academy of Sciences* 2011, **108**:4152-4157.
71. Moore KL, Lombi E, Zhao F-J, Grovenor CRM: **Elemental imaging at the nanoscale: NanoSIMS and complementary techniques for element localisation in plants.** *Analytical and Bioanalytical Chemistry* 2011, **402**:3263-3273.
72. Moore KL, Schroeder M, Wu Z, Martin BGH, Hawes CR, McGrath SP, Hawkesford MJ, Ma JF, Zhao F-J, Grovenor CRM: **High-Resolution Secondary Ion Mass Spectrometry Reveals the Contrasting Subcellular Distribution of Arsenic and Silicon in Rice Roots.** *Plant Physiology* 2011, **156**:913-924.
73. Zhao F-J, Moore KL, Lombi E, Zhu Y-G: **Imaging element distribution and speciation in plant cells.** *Trends in Plant Science* 2014, **19**:183-192.
74. Smart KE, Smith JAC, Kilburn MR, Martin BGH, Hawes C, Grovenor CRM: **High-resolution elemental localization in vacuolate plant cells by nanoscale secondary ion mass spectrometry.** *The Plant Journal* 2010, **63**:870-879.
75. Keiluweit M, Bougoure JJ, Zeglin LH, Myrold DD, Weber PK, Pett-Ridge J, Kleber M, Nico PS: **Nano-scale investigation of the association of microbial nitrogen residues with iron (hydr)oxides in a forest soil O-horizon.** *Geochimica et Cosmochimica Acta* 2012, **95**:213-226.