**The future of single-cell environmental microbiology**

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One of the key objectives in environmental microbiology is to couple identity and function of microorganisms in soil, water, sediment or other ecosystems. The field has come a long way over the past decade, both with respect to 'who is there?' and to 'what are they doing?' The coupling between identification and activity, however, remains the weakest point. Existing approaches need to be further developed and combined. It is a dream to one day do experiments with prokaryotes the way that they are done with higher animals or plants, at the level of the individual organism.

There are indeed already methods in use that enable this to a certain extent. These may be based on pulse-chase experiments during which the microorganisms have been fed a radioactive or an isotopically heavy meal. Those cells that have taken up the substrate may subsequently be identified, e.g. by a combination of microautoradiography and fluorescence *in situ* hybridization (MAR-FISH). MAR-FISH has the advantage that the active substrate uptake can be related to cells. This is not the case with most methods that combine stable isotope tracers and the analysis of DNA, RNA or biomarkers. The fact that MAR uses radioactivity, however, limits its use to those elements that have a radioisotope with a suitable half-life and excludes the study of other elements such as nitrogen.

It was therefore a methodological breakthrough when Orphan and coworkers (Orphan *et al*., 2001) combined FISH with secondary ion mass spectrometry (SIMS). The aim was to show whether those aggregates of archaea and sulfate reducing bacteria, which Boetius and coworkers had discovered 1 year before in sediments where anaerobic oxidation of methane predominated, were indeed living on methane. By analysing FISH-stained aggregates with SIMS it was shown that their highly $^{13}C$-depleted cell carbon carried the distinct isotopic signal of methane. It was a limitation of this FISH-SIMS method, however, that the size (~10–15 μm) of the ion beam used to sputter biomass and generate secondary ions to be analysed by mass spectrometry exceeded the average diameter of a microbial cell (~1 μm). This problem has recently been solved, however, by the development of the so-called nanoSIMS, which for the first time makes it possible to determine the chemical, radioisotopic or stable isotopic composition of biomass at the submicron level (Lechene *et al*., 2006; McMahon *et al*., 2006). This technique in combination with pulse-chase experiments with radioactive or stable isotope labelled substrates opens up a vast new area of research possibilities in single-cell environmental microbiology.

The nanoSIMS has a beam size of ~50 nm which is sufficiently small to analyse individual cells or even parts of a cell. It is only mildly destructive as it removes only the upper one to three atomic layers (~1 nm) to obtain sufficient sputtered (vaporized) biomass for elemental and isotopic analysis. On the other hand, it is also possible to sputter the entire cell when whole cell isotopic analysis is needed. In a pulse-chase experiment using a stable isotope labelled substrate the individual microbial cells that have assimilated the substrate in an environmental sample can be identified from their isotopic enrichment after 24 h of incubation, assuming a growth rate of just one doubling per week and a typical labelling percentage of the substrate of *c*. 50%. Some of the first such experiments in a $^{15}N_2$-fixing bacterial culture have shown large intercellular and intracellular differences in the degree of $^{15}N$ labelling. Thus, even the cells in a laboratory culture are not all alike but consist of billions of individuals, each with their different activities and life stages.

The high sensitivity and spatial resolution of the nanoSIMS opens a novel possibility for coupling phylogenetic identity and metabolic function in studies of mixed microbial communities from the environment. We propose that by replacing the fluorescent oligonucleotide probes used for FISH with isotopically labelled probes (stable or radioactive) or halogenated probes, individual hybridized cells can be directly identified by nanoSIMS. The hybridization procedure is essentially the same as that used for FISH and the same oligonucleotide probes can be used. The main difference is that stable isotope or radioactive elements are coupled to the probe or halogenated probes are used instead of a fluorescent dye. By combining this new type of probing with a pulse-chase experiment using an isotope labelled substrate (e.g. with $^{13}C$ or $^{15}N$) the metabolically active cells can at the same time be phylogenetically identified during a single scan in the nanoSIMS. To describe such techniques, we may need to think of new -ISH names in parallel to FISH, like 'RISH' (radioisotope *in situ* hybridization), 'SISH' (stable isotope *in situ* hybridization) or 'HISH' (halogen *in situ* hybridization).

There are, of course, many more possibilities for using this approach in combination with the powerful toolbox of DNA, RNA, biomarker, and protein based techniques. We have used the pulse-chase experiment here only as an example. The nanoSIMS already has amazing specifications compared with more conventional SIMS instruments. Sample preparation is comparable to simple electron microscopy, or cells can even be filtered on a gold-sputtered polycarbonate filter and dried, which makes the sample preparation similar to that of normal FISH hybridization. The sensitivity for detecting $^{14}C$ following a radiotracer experiment is at least 1000 times that of microautoradiography (Lechene *et al*., 2006). The stan-

 

dard deviation for stable isotope analyses can be better than ±1‰. So, what's the catch? Well, running this instrument requires extra mass spectrometric and general instrumental expertise, and the price (≥ 2 million euros) is certainly prohibiting for most laboratories. So far little more than a dozen instruments are up and running and they are mostly used for research in material sciences, cosmochemistry, geology and biology. We predict that in the future the nanoSIMS will show up also on the wish-list of many environmental microbiologists.

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## Single-cell genomics

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A burgeoning technology that will revolutionize the analysis of microbial communities will be the ability to obtain a complete genome sequence from an individual bacterial cell. At present, the majority of published genome sequences represent bacteria that can be grown in culture. But as most bacteria live in close proximity to or in contact with other organisms and most cannot be propagated in pure culture, there are still severe limitations to the genomic analysis of the most abundant and most diverse group of organisms on the planet. Certain cultivation issues have been minimized by applying procedures (such as filtration, centrifugation, enzymatic treatment or library screening) that return relatively pure samples of DNA in quantities sufficient for genome sequencing. Although contaminating DNA might sometimes offer insights into community or cellular interactions and contents, it certainly does not make the job of genome assembly any easier. The genomic analysis of microbial communities is further complicated by the fact that each of the species can harbour substantial amounts of genetic variation. So even in the event that species-specific DNA can be recovered, it is difficult to ascertain how polymorphisms might assort among the various lineages within the sample.

Such problems will be circumvented by single-cell genomics. These procedures will eliminate the need to cultivate or mechanically purify large samples of cells, to recognize contaminating sequences, or to presume *anything* about the contents of an individual genome. Imagine isolating a single bacterial cell in the morning and obtaining its complete, gap-free and annotated sequence genome just after lunch. Okay, the technology is not quite there, but it is close. Single cells have been isolated by various methods, such as optical tweezers, flow-sorting, and serial dilution. The DNA from individual cells seems sufficient for some form of whole-genome amplification (e.g. Raghunathan *et al*., 2005). Finally, clone-free sequencing methods are producing volumes of DNA sequence information in a matter of hours (e.g. Margulies *et al*., 2005). And in fact, there is already one report of its execution (Zhang *et al*., 2006). Mark my words, by next year you will be contemplating grant proposals, and in 2 years reviewing manuscripts, that invoke this technology.

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## Moving to a higher level of abstraction

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I vividly remember a point in 1978 when, as a computer science professor, I told a student that the significance of microprocessors was grossly oversold. Since then, I have tried to be somewhat more cautious in my predictions. However, in 1994 I did predict that over 100 genomes would be sequenced by the turn of the century, and thanks largely to the efforts of Craig Venter, that prediction turned out to be reasonably accurate. To increase my odds of success, in this article I will comment only on the immediate future of bacterial genome annotation and include one rather specific prediction for a change of paradigm.

The field of biology is advancing rapidly due to acquisition of data in many forms. The underlying driving force is certainly the continuing drop in costs of sequencing. The annotation and exploration of hundreds, and within

 