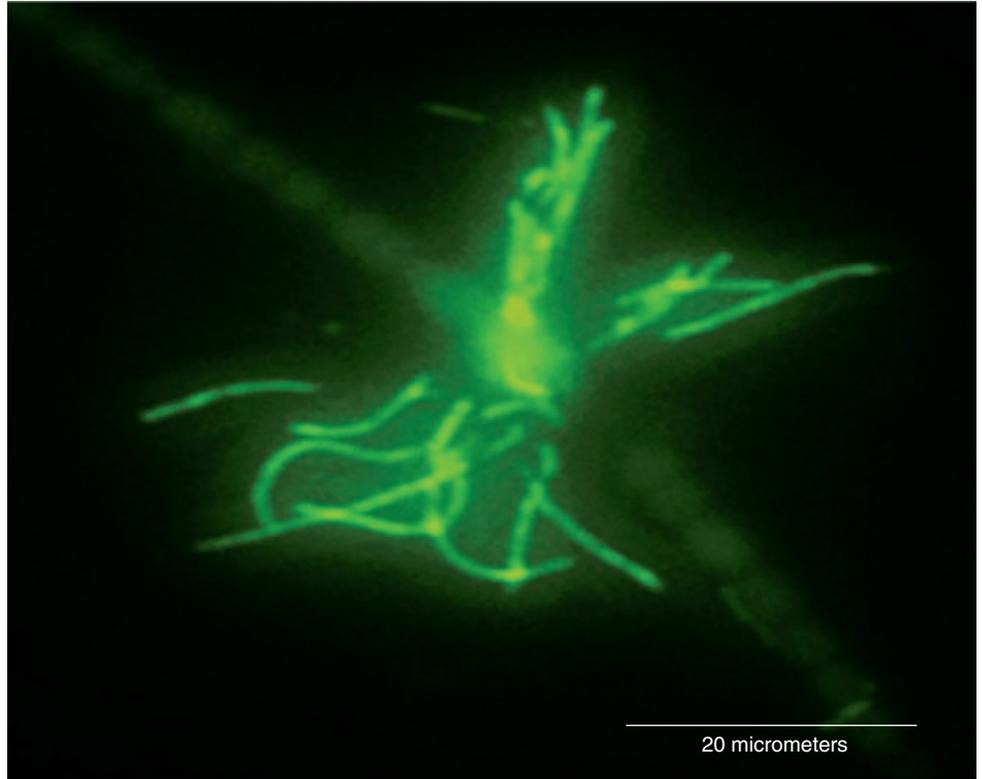


# Revealing the Identities and Functions of Microbes

**S**INCE they emerged more than 3 billion years ago, microbes—unicellular organisms such as bacteria—have populated Earth in abundance, conquering every nook and cranny of its surface and subsurface and the tissues of more complex life forms. Their ability to absorb a wide range of substances, most unrecognizable as food to humans, for basic metabolism has led the Department of Energy (DOE) to investigate using microbes to develop biofuels, clean up toxics, and sequester carbon. Researchers also want to study the interactions between disease-causing microbes and native microbe populations in humans, such as the processes that lead to tooth decay.

However, to exploit microbial traits, scientists must better understand their growth and metabolism. “We want to determine how microbes react under different environmental conditions and how they could be engineered to perform useful functions,” says environmental microbiologist Jennifer Pett-Ridge in Livermore’s Physical and Life Sciences Directorate. With funding from Livermore’s Laboratory Directed Research and Development Program and DOE’s Office of Science, Pett-Ridge and her colleagues Peter Weber, Xavier Mayali, and Steve Singer have worked with their collaborators at Stanford University to develop an imaging technique that identifies the microbes responsible for specific metabolic processes.

The team’s imaging technique, called elemental fluorescent in situ hybridization (El-FISH), combines stable isotope probing with nanometer-scale secondary-ion mass spectrometry (NanoSIMS). NanoSIMS can image trace components with a spatial resolution of 50 nanometers. Laboratory researchers use it for studies ranging from nuclear forensics to cosmochemistry. El-FISH provides even more information, showing which microbes



A new imaging technique combines fluorescent in situ hybridization (FISH) analysis, such as the image shown here, with nanometer-scale secondary-ion mass spectrometry, allowing researchers to decipher the interrelationships of different species. The two species in this FISH image are filamentous *Anabaena* cyanobacteria (barely visible) and *Rhizobium* bacteria (darker green).

use chemicals labeled with isotopes. The new technique thus allows researchers to study microbes in diverse environments, revealing the often-complex interrelationships of different species comprising microbial communities.

## Creating Chemical Images

NanoSIMS instruments scan the surface of a sample with a stream of energetic ions. These ions generate secondary ions, which are extracted by an electric field, sorted by mass, and detected. Only 18 NanoSIMS instruments exist in the world. Livermore’s machine is one of three in the nation used for biological imaging.

“A NanoSIMS instrument is similar to a light microscope,” says Pett-Ridge, “but it is tuned to specific ions to re-create a chemical image instead of a light image.” In this way, the technique reveals which cells—and exactly where in those cells—stable isotopes are incorporated.

Stable isotopes are forms of an element containing one or two extra neutrons. They are not radioactive, and cells assimilate them in exactly the same way as their more common forms. Stable isotopes that are rare in nature serve as elemental tags.

For example, the carbon-12 and nitrogen-14 in a compound can be replaced with carbon-13 and nitrogen-15, respectively, and the new mixture added to microbial systems ranging from a single species to hundreds of species. A NanoSIMS instrument tuned to these rare isotopes can locate the microbes that use the labeled compound.

EI-FISH combines NanoSIMS imaging with a variant of fluorescent in situ hybridization (FISH), a technique developed in the 1980s by Livermore bioscientists. FISH analyses involve attaching fluorescent dyes to short pieces of DNA, called probes, which bind to complementary sequences of chromosomes in a targeted species. The technique can reveal the identities and locations of different microbes existing in complex communities.

The twist of EI-FISH is that an elemental tag, such as fluorine, attached to the probe is imaged in NanoSIMS along with the stable isotopes. The cellular abundance of fluorine or bromine measured following the EI-FISH procedure typically exceeds natural background concentrations by up to 180-fold in the targeted species and is easily picked up in NanoSIMS images tuned to fluorine or bromine. “With our technique, we can look at several elements or isotopes at the same time by selectively tuning the NanoSIMS instrument,” says Pett-Ridge. “First, the carbon-13 and nitrogen-15 signals give us information about how stable isotopes are metabolized. The fluorine or bromine signal then identifies the organism performing the metabolism.”

One of the first imaging applications will be to characterize the roles of microbes living in hypersaline microbial biofilms or mats. These highly diverse, layered microbial communities develop on the surface of sediments in marine estuaries and salt ponds and

can generate hydrogen gas. In a DOE-funded collaboration with researchers at the National Aeronautics and Space Administration’s Ames Research Center and Stanford University, Pett-Ridge and her colleagues add compounds containing carbon-13 to microbial mat communities. They then follow how the bacteria take up and break down these compounds to learn about the critical links between carbon and nitrogen nutrients and the generation of hydrogen gas.

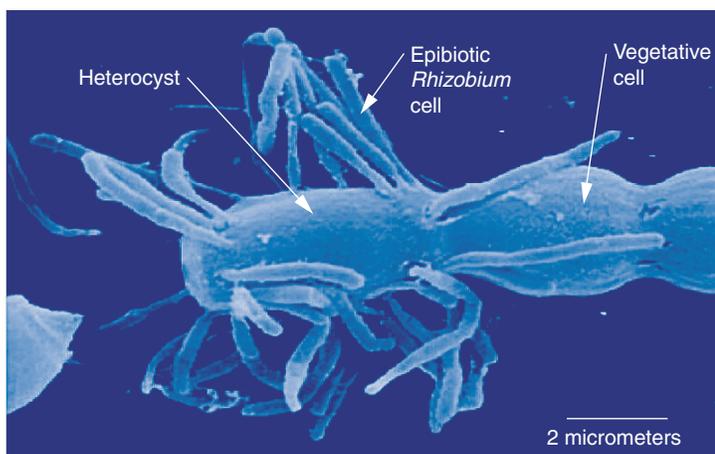
With other DOE funding, the team is partnering with Lawrence Berkeley National Laboratory and Louisiana State University to characterize the complex microbial community dwelling in the hindgut of wood-eating beetles. Like termites, these beetles have developed a symbiotic relationship with a community of gut microbes whose combined enzymes digest the complex polysaccharides and lignins of plant cell walls and produce acetate, methane, and hydrogen gas. Understanding how these microbial populations interact to break down cellulosic materials could aid large-scale industrial projects planned to convert biomass such as wood chips into hydrogen and methane biofuels.

Another promising effort is attempting to understand the metabolism of different bacteria responsible for chronic periodontitis, the leading cause of tooth decay and loss in humans. Periodontitis is linked to several bacterial species, including one called human TM7. Defining the role of TM7 during disease progression requires characterizing its ecologic niches in the mouth and revealing its metabolic functions, a task well suited to EI-FISH. The dental disease research is being conducted in conjunction with researchers at Stanford University and the Veterans Affairs Palo Alto Health Care System.

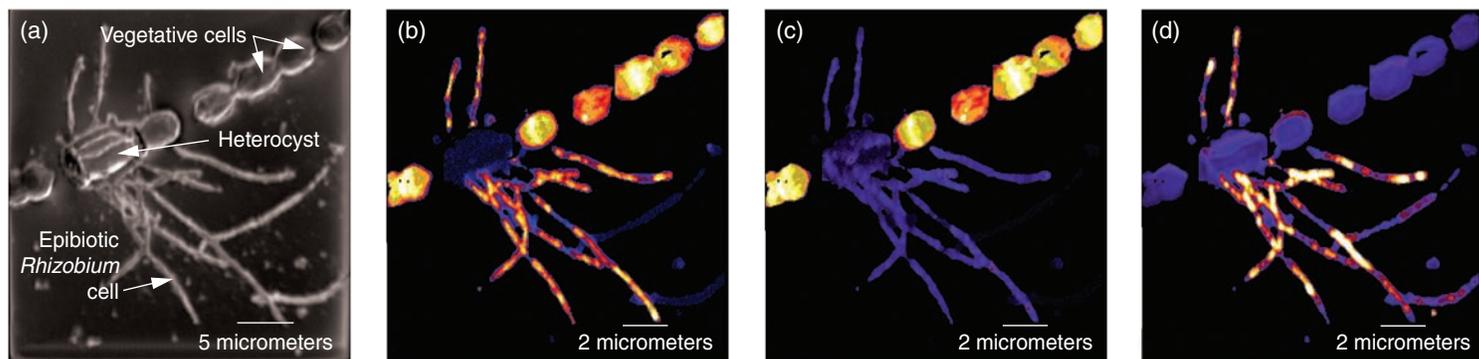
### Studying a Microbial Community

In a proof-of-concept demonstration of the EI-FISH technique, the research team studied a two-species community, or coculture, of filamentous *Anabaena* cyanobacteria (blue-green algae) and epibiotic (living on the surface of another species) bacteria from the family *Rhizobium*. Although many cyanobacteria species are free-living, some are closely associated with other organisms, including plants, algae, fungi, and other bacteria. Understanding these associations provides important insights into the ways microbes metabolize and share nutrients.

Filaments of *Anabaena* cyanobacteria consist of two kinds of cells: Vegetative cells conduct photosynthesis, converting atmospheric carbon dioxide to oxygen. Heterocysts fix atmospheric nitrogen, converting nitrogen gas to ammonium. A typical cyanobacteria filament has 15 to 20 vegetative cells between each heterocyst. Scientists have detected neither photosynthesis nor nitrogen fixation in *Rhizobium* cells when grown on their own. However, electron microscope images indicate that when these cells are grown with *Anabaena*, *Rhizobium* rods radiate from the junction between *Anabaena*’s vegetative and heterocyst cells. (See the figure at left.)



In this micrograph, *Rhizobium* cells appear as rods radiating from the junction between *Anabaena*’s heterocysts, which convert atmospheric nitrogen to ammonium, and vegetative cells, which conduct photosynthesis. (Courtesy of Bradley S. Stevenson and John B. Waterbury, from “Isolation and Identification of an Epibiotic Bacterium Associated with Heterocystous *Anabaena* Cells,” *Biological Bulletin*, 2006, pp. 73–77.)



Elemental fluorescent in situ hybridization (EI-FISH) was used to examine (a) a chain of eight *Anabaena* cyanobacterial cells with elongated *Rhizobium* bacterial cells attached. (b) Fluorine identifies the organisms. Isotope images show the relative uptake of (c) newly fixed carbon (carbon converted from carbon dioxide to organic materials) and (d) newly fixed nitrogen (converted from its atmospheric gas to nitrogen compounds). Isotope enrichment ranges from yellow (maximum) to orange, red, purple, and blue.

To better examine this two-species system, the team imaged the assimilation and flow of nutrients, in particular, the exchange of carbon and nitrogen molecules, between the two bacteria. A succession of NanoSIMS images (shown above) reveals that carbon-13 is incorporated into cells through photosynthesis and nitrogen-15 through nitrogen fixation. The team found that heterocysts export fixed nitrogen to vegetative cells, but only vegetative cells incorporate fixed carbon (from carbon dioxide) into carbon compounds. In addition, the team's results suggest that *Anabaena* cells transfer significant quantities of both carbon and nitrogen compounds to *Rhizobium* cells. Most of this nutrient exchange appears to occur at the junction between heterocysts and vegetative cells, the place where *Rhizobium* cells attach.

### Tracking Isotopes within Cells

The researchers also studied the accumulation and distribution of isotopes when either nitrogen fixation or photosynthesis was inhibited with materials that inactivate essential cell enzymes. These experiments strengthened their conclusion that *Rhizobium* cells cannot perform nitrogen fixation or photosynthesis. Therefore, the stable isotopic labels (carbon-13 and nitrogen-15) found in *Rhizobium* must be derived from the host.

According to Pett-Ridge, this high degree of attachment specificity at the heterocyst-vegetative cell junction indicates

*Rhizobium* cells possess sensors that can discriminate between cell types. It remains unclear if *Rhizobium* has deleterious effects on the host (parasitism) or provides benefits (symbiosis).

EI-FISH is attracting interest from biological researchers worldwide. Over the next few years, scientists may use the technique to engineer bacteria for producing biofuels or to study microbial communities and host-pathogen interactions in nature and the human body. The methodology could also advance understanding of how microbes break down and sequester carbon dioxide in the soil, immobilize toxic metals, and biodegrade hazardous organic pollutants. Clearly, organisms that have evolved over 3 billion years may have some secrets to help the most recent arrival on Earth—humankind.

—Arnie Heller

**Key Words:** biofuels, elemental fluorescent in situ hybridization (EI-FISH), microbe, nanometer-scale secondary-ion mass spectrometry (NanoSIMS), stable isotope.

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