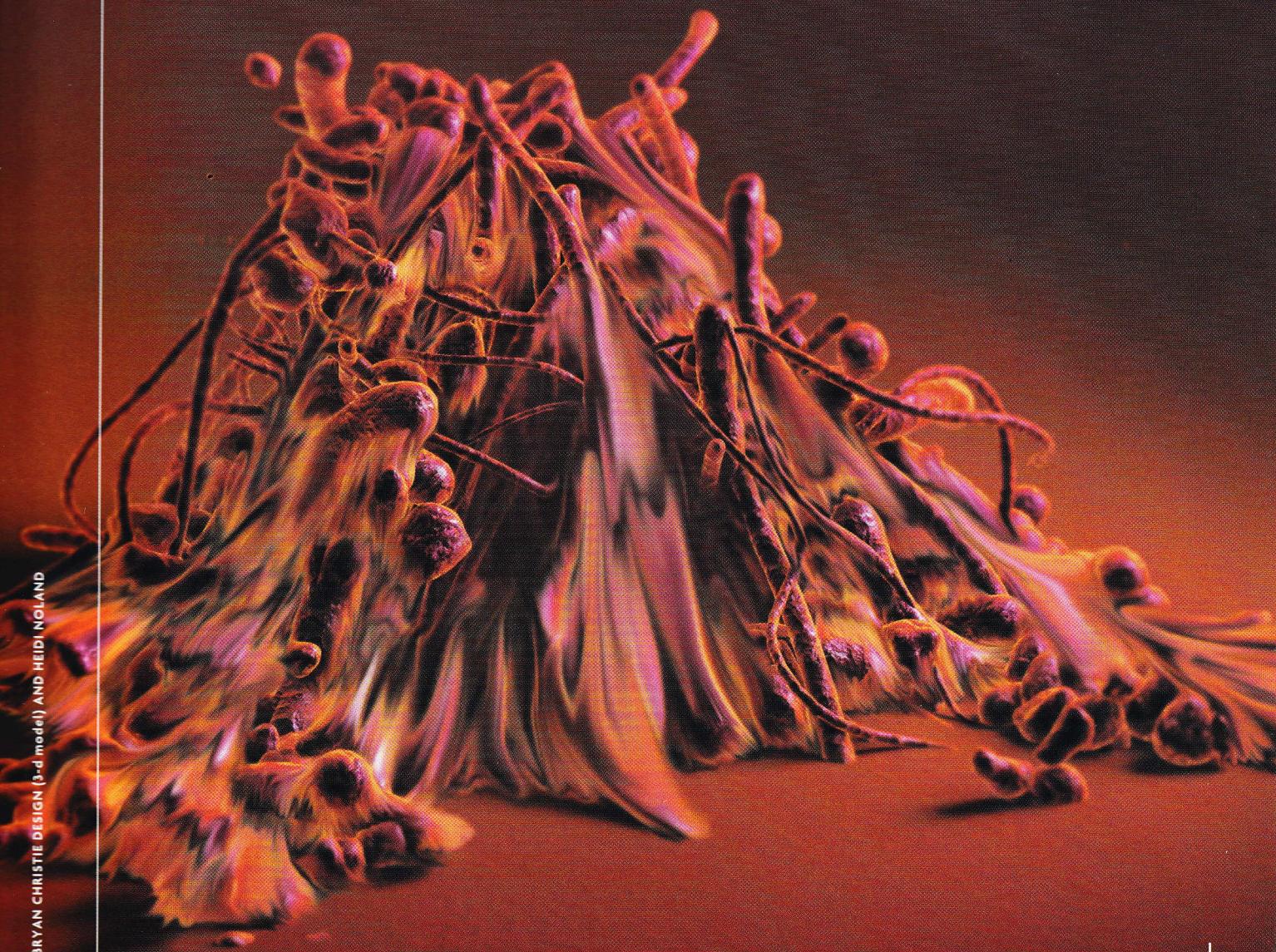


# Biofilms, Smile!

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Laser Microscopy Camera



BRYAN CHRISTIE DESIGN (3-d model) AND HEIDI NOLAND

The three-dimensional, complex interactions of a wide variety of bacteria that make up biofilms are revealed through new visualizing technologies

**BY PAUL STOODLEY |**

As many people have discovered, failing to brush or floss for even one day will cause a buff or beige-colored stain to coat the teeth. Drag your fingernail across the stain and you get a sticky smudge of plaque biofilm. The complex communities of bacteria that form this plaque on the teeth and under the gum line are among the best-studied examples of biofilms. Still, it is only recently that advanced laser-based microscopy techniques

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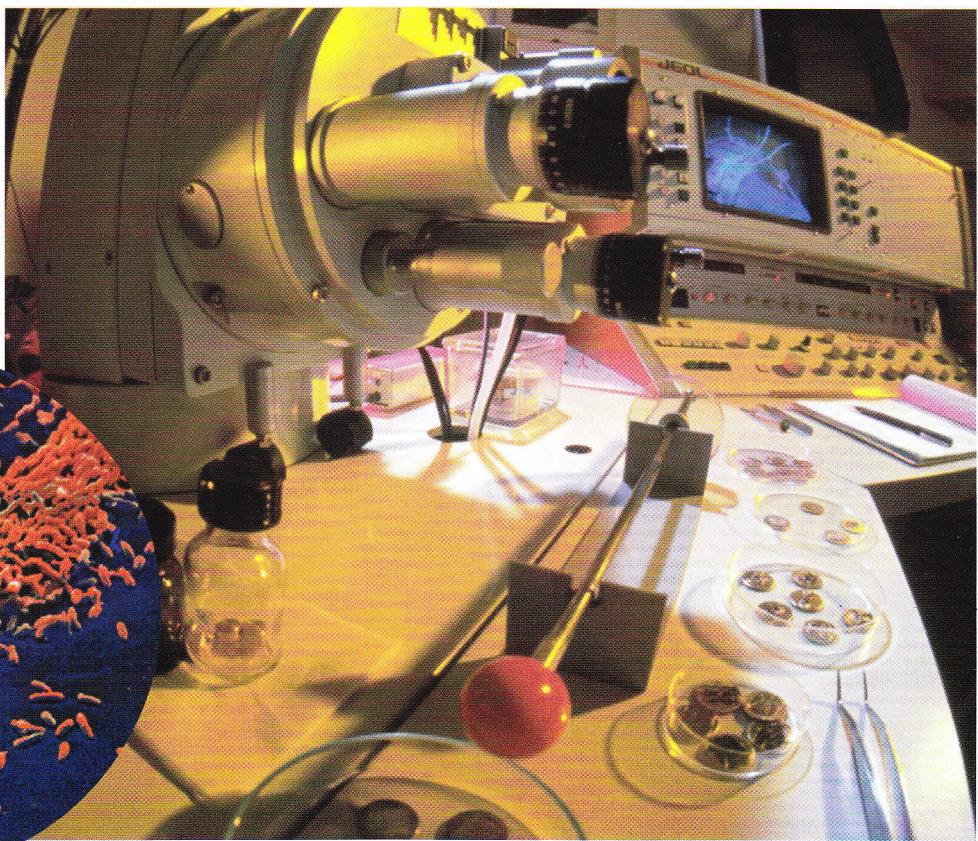
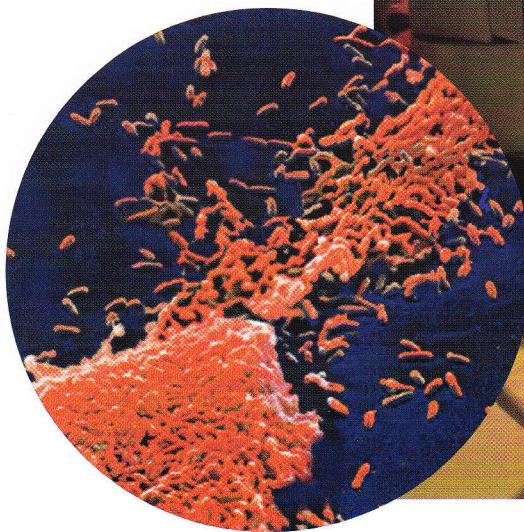
have revealed the complexity of structures and diversity of organisms living in what to the naked eye appears just a two-dimensional stain or scrape of dental debris.

Scientists began to study dental biofilms in the 1940s by scraping them from the surfaces on which they were growing, mixing them up and culturing them on agar or broth to find out which types of organisms were in

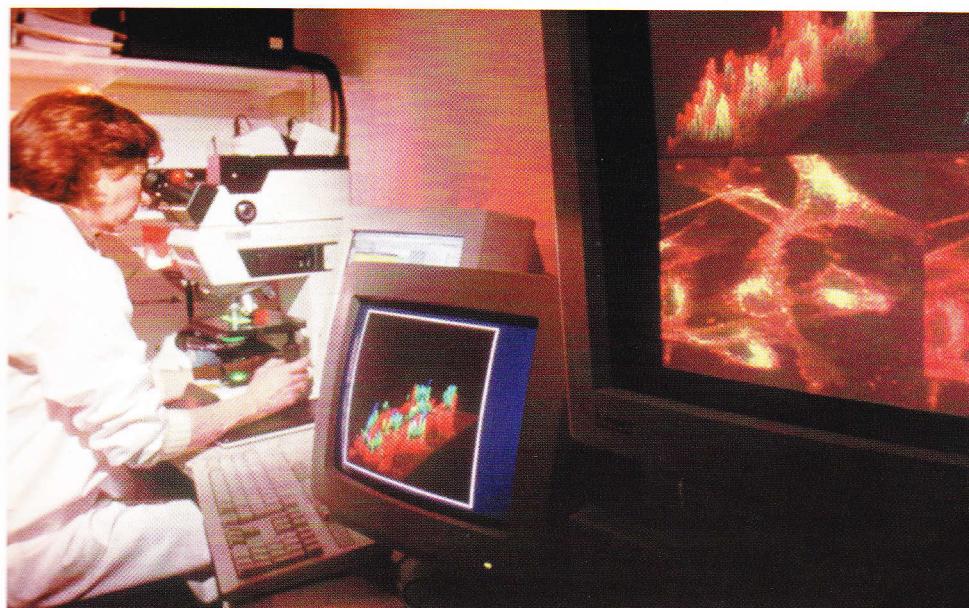
the biofilm and how they colonized the surface over time. It was found that in dental plaques there was a succession of organisms, much like those found in macro ecosystems where primary colonizers modify the local environment for other organisms to later colonize. For example, streptococci are early colonizers of the teeth. Among this group is the sticky *Streptococcus mutans*. This organism grows on sugar in the food we eat, which it ferments and produces acid as a waste product. This acid can dissolve tooth enamel, causing caries or cavities. As the local environment becomes more acidic, other groups of acid-tolerant organisms such as lactobacilli can thrive, exacerbating the problem since they also produce acid. As the first colonizers proliferate, they create an environment receptive to the next wave of colonizers from the saliva and the tongue.

Using molecular techniques scientists can identify the different bacteria by genetic fingerprinting. It is now known that more than 500 types of microorganisms, including bacteria, fungi and even amoebas, can live in dental plaque. Many of the bacteria in the plaque use oxygen to respire. Because there is

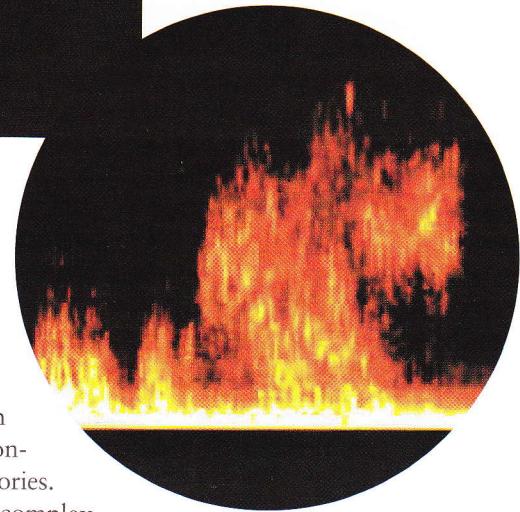
**THE INTRODUCTION OF THE**  
scanning electron microscope (SEM) in the 1950s provided views of common biofilms, such as the *Pseudomonas aeruginosa* pictured here. The single cells that make up the biofilm are approximately two microns (one millionth of a meter) in length. *P. aeruginosa* is commonly found in soil and industrial piping, and is implicated in infections of the lungs, eyes and skin burns.



SEM OF PSEUDOMONAS AERUGINOSA. © PAUL STOODLEY, AUTHOR, LICENSED FOR USE, ASM MICROBLENDARY (left); GRULIK HARAMATY/PHOTOTAKE (right)  
(LINKED TO [HTTP://WWW.MICROBLENDARY.ORG](http://www.microblendary.org))



**THE CONFOCAL SCANNING**  
laser microscope (CSLM) was adapted to biofilm study in the early 1990s and allowed scientists to observe samples in their fully hydrated state and to view the space between the bacterial colonies. Additionally, samples such as the mixed species biofilm pictured below, common to the environment and industrial piping and linked to hospital infections, could be tagged with fluorescent stains to distinguish bacterial species within the biofilm.



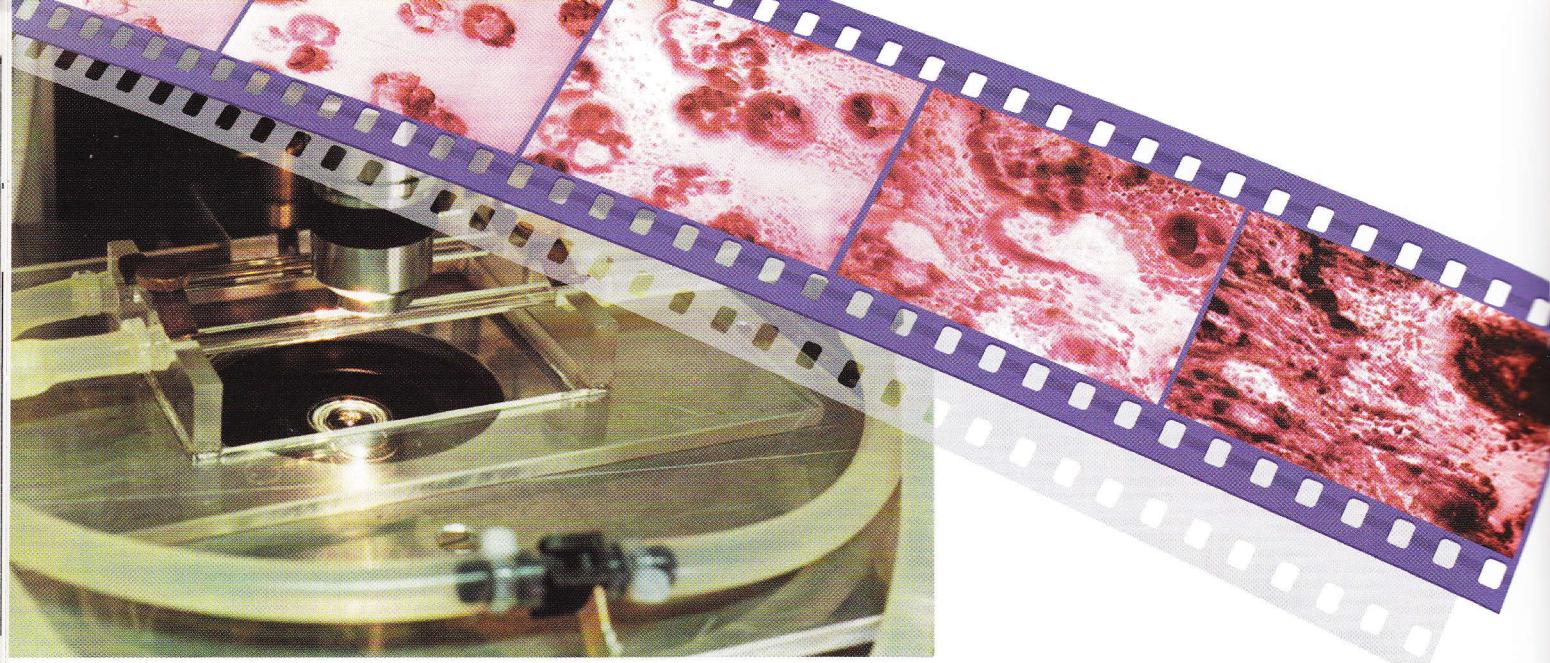
usually an abundance of nutrients in the mouth but only a limited supply of oxygen if the plaque is allowed to build up, the oxygen gets used up, creating the anaerobic conditions required for a group of Gram-negative anaerobes which cause gingivitis and eventually periodontitis. Common among these villains are *Porphyromonas gingivalis*, *Treponema denticola* and *Bacteroides forsythus*. To make matters worse, there is mounting evidence that pathogens living in dental plaque biofilms can enter the bloodstream, a task made easier via entry through the bleeding gums caused by the disease of their own making, increasing risk of heart disease, stroke and bacteremia. To gain information to combat disease-causing bacteria living in a biofilm, while also avoiding destroying nonpathogenic bacteria that may serve to keep dangerous bacteria in check, scientists are developing more refined methods to visualize biofilms.

## Improving Visualization Techniques

In addition to counting and identifying the types of bacteria in dental plaques, and indeed biofilms generally, researchers were also interested in finding out what these biofilms looked like. However, direct observation of bacteria in biofilms was limited to simple light microscopes, usually after the bacteria were scraped from the tooth surface and smeared on a microscope slide, thus destroying the

biofilm structure and mixing up all the species. The development of the scanning electron microscope (SEM) in the 1950s led the way to observing biofilms on natural surfaces that were small enough to be removed from the environment or those grown in laboratories.

These images revealed a complex arrangement of bacterial cells held together by strands of slime that were produced by the bacteria themselves. Although producing beautifully clear, high-resolution images, the SEM was a high-vacuum technology that required biofilm dehydration. Because biofilms often contain more than 90 percent water, dehydration causes the elaborate structures to collapse, the slime matrix to shrink into fibrous strands and cracks to form throughout the structure. However, the shapes of the individual bacteria within the biofilm were retained. These included cocci (spheres), rods (tubes with rounded ends), spirochetes (corkscrew twists), filaments (tangled strands), and vibrios (half-twists), hinting at the complexity of the community living in the dental plaque biofilm. However, these results provided limited information because the hundreds of species in dental plaque biofilms come in a limited variety of shapes while exhibiting very different pathogenicity. Crude morphological information permitted researchers to begin to distinguish the



#### DIGITAL TIME-LAPSE

microscopy (DTLM) allows biofilm movement to be observed over time. The above mixed-species sample, captured via bright-field microscopy and grown in a glass flow cell similar to the one shown above, was subjected to turbulent water flow. The biofilm starts with distinct clumps of bacteria that multiply and produce a slime matrix of carbohydrates, proteins and nucleic acids. The biofilm matrix is interspersed with water channels.

#### MORE TO EXPLORE

**Dental Plaque Revisited: Oral Biofilms in Health and Disease.** Edited by H. H. Newman and M. Wilson. Eastman Dental Institute, University College London. Bioline, 1999.

**Microbial Biofilms.** Edited by J. W. Costerton and H. M. Lappin-Scott. Cambridge University Press, 1995.

Biofilm images and time-lapse movies showing biofilm behaviors can be found at the Center for Biofilm Engineering at Montana State University at <http://www.erc.montana.edu/Res-Lib99-SW/Movies/default.htm> and the American Society for Microbiology Microbe Library at [www.microbelibrary.org](http://www.microbelibrary.org) [search for "biofilm"].

arrangement of different types of bacteria within a biofilm while genetic fingerprinting tantalizingly pointed to the existence of hundreds of different species in biofilms, including dental plaque. Other visualization techniques were needed.

In the early 1990s, researchers began to use the confocal scanning laser microscope (CSLM) to study biofilms. Like the SEM, the CSLM can take high-resolution pictures, but it does not require dehydration and so microscopic biofilms could be seen for the first time in their fully hydrated state. Additionally, the laser technology allowed various fluorescent stains to be designed so that different bacteria or components of the biofilm, such as carbohydrates or proteins in the slime matrix, could be distinguished. The new technique allowed researchers to see where a particular bacterial species lived in a biofilm. In addition to the complexity of many types of bacteria living in close proximity, much like a macroscopic ecosystem such as a rain forest, desert or plain, the CSLM could focus on the shape of the entire biofilm, and so revealed different structures of biofilms, including "mushrooms," "dunes," "filaments," "fronds," "hollow mounds," "ridges," "ripples," "streamers" and "stacks." Dental plaques in particular harbor a structure called "corn cobs" formed by some bacteria.

CLSM allowed visualization of not only the biofilms but also the spaces in between bacterial clumps. In many cases it was found that colonies are separated by channels through which water can flow, bringing nutrients to the microbes in the biofilm. This

suggests that such structural patterns may not be incidental. The use of CSLM to guide microelectrodes for measuring oxygen in specific locations in the biofilm was pioneered by Dirk deBeer and colleagues in 1994 at the Center for Biofilm Engineering at Montana State University. Their research showed that the channels could even supply oxygen from beneath the "caps" of mushroom-shaped structures so that localized anaerobic regions developed not necessarily at the bottom of the biofilm but sometimes away from the surface at the centers of the protruding structures in the otherwise aerobic region. This changes our concept of how aerobic and anaerobic organisms may be distributed in the biofilm and helps explain why anaerobic bacteria in dental plaques may sometimes be found above the gum line. It is possible that these anaerobic bacteria act as the initial source infecting the periodontal pocket, where the anaerobes can then thrive in a niche less likely to be disturbed and eventually cause inflammation.

With the improved technique to investigate biofilm structures and function, researchers turned to developing ways of growing biofilms in the laboratory. These devices range from the simple, such as a glass slide immersed in an inoculated beaker, to complex flow systems in which the flow rate and nutrients can be tightly controlled in an attempt to mimic actual environments. These set-ups enable researchers to elucidate the factors that determine the growth and development of biofilms. To document and investigate the development of biofilms as they

grew, scientists designed flow cells made of glass or containing a glass observation window that could be positioned on the stage of a CSLM, allowing the biofilm to be observed directly under flowing conditions.

Observations of biofilms in various types of flow cells have shown that many environmental and genetic factors come into play in shaping biofilms; these include the types of microorganisms in the biofilm, the types and amount of nutrients, the flow conditions and the production of cell signal molecules through which the bacteria can coordinate their behavior.

## Moving Pictures

Another advantage of being able to observe biofilms directly under the microscope was that behaviors over time became apparent, ranging from the twitching motility of single cells sliding around on the surface to the rapid oscillation of streamers in the flowing water. Digital time-lapse microscopy (DTLM) has revealed other behaviors that occur too slowly to be seen with the naked eye. Mature biofilms that appear unchanged from day to day are actually very dynamic. Cell clusters containing thousands of cells can continually grow and detach from biofilms. These detachment events can be quantified by digitally subtracting each image from the preceding image to reveal the changes that occurred on the surface during the intervening time.

Under higher shear flows biofilms can also move downstream over a surface while remaining attached to the surface. Because biofilm cells are often more resistant to antimicrobial agents than are suspended cells, this condition may allow the biofilm to spread while remaining associated with the surface and yet still retain the protective properties of biofilm formation. The resistance of biofilm microorganisms is thought to be due to a number of variables, including protection by the slime matrix, lower rates of metabolism in the biofilm, stationary-phase stress response and the surface-associated up-regulation of genes encoding enzymes that can destroy certain classes of antibiotics such as beta-lactams, including penicillin. Although the movement may be slow—less than a millimeter per hour—because of the large concentration of bacteria living in biofilms, which can be more than 10 million cells per

square centimeter, this movement may represent a large downstream flux of organisms.

The movement of biofilms along a surface can occur through the rolling and sliding of cell clusters that detach at the upstream edge, presumably when the fluid shear overcomes the adhesive force, roll over and reattach on the downstream side, presumably where the adhesive force is now greater than the fluid shear in the wake region created as the fluid flows around the cell cluster. Biofilms can also form highly organized "ripple"-like structures, which in time-lapse imaging appear to flow downstream like the ripples that form on a thin body of flowing water. The notion of biofilms as dynamic, moving structural entities rather than static "coats of slime" has significant ramifications for not only how we view and model biofilms, but also how we may successfully control them. As developments in CSLM and DTLM continue to be used for the study of biofilms, in both flow cells and natural environments, it is certain that other fascinating dynamic behavior will be revealed over a spectrum of spatial and temporal scales.

Direct microscopy of environmental surfaces *in situ* is currently difficult. The sensitivity of microscopes to moisture, temperature and vibration and the power requirements of laser microscopes all restrict field use. Also, microscopes are generally sensitive to irregular surfaces, which cause image distortion. However, it may be possible that flexible waterproof endoscopic-type confocal microscopes may be developed that can peer directly into the mouth to look at the colonizing biofilms directly and in great detail. David Dickensheets, in the Electrical Engineering Department at Montana State University, has designed a fiber-optics-based fluorescent confocal endoscope with a two-millimeter-diameter tip. His goal, which he estimates is several years away, is to get the instrument's resolution down to 0.5 microns in order to see single cells. As visualization methods continue to evolve, it is almost a certainty that our knowledge of the variety and intricacies of biofilms will also expand.

**PAUL STOODLEY, Ph.D.**, is assistant research professor at the Center for Biofilm Engineering at Montana State University, Bozeman. He is the principle scientific consultant for BioSurface Technologies Corp. in Bozeman, Mont.

