Modern microscopy in biofilm research: confocal microscopy and other approaches

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Microscopy is the only technique whereby bacterial biofilms can be studied at the single-cell level *in situ*. Our understanding of biofilm structure, physiology and control hinges on the application of confocal scanning laser microscopy and other advanced microscopic techniques. Gene expression in four dimensions (x,y,z,t), interspecies interactions, and the role of exopolymer are being defined.

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Abbreviations

CSLM	confocal scanning-laser microscopy/microscope
GFP	green fluorescent protein
HSL	homoserine lactone
SPM	scanning probe microscopy

Introduction

Biofilms (microorganisms anchored to substrata) are a ubiquitous form of bacterial growth; this recognition is relatively recent even though some of the premiere examples of microbial biofilms (e.g. dental plaque, microbial mats) have been investigated for centuries. A great obstacle to the study of biofilms — difficulty in obtaining clear photomicrographs of the often densely packed microorganisms under hydrated conditions — was overcome by a major advance in microscope instrumentation: the confocal scanning-laser microscope (CSLM) can document biofilm morphology and physiology in four dimensions under *in situ* conditions. This review is meant to orient the reader to the newer biofilm microscopy literature, primarily with respect to confocal microscopy, as well as to touch on new microscopic techniques (or new applications of older techniques).

What is confocal microscopy and why is it necessary in biofilm research?

The CSLM is a 'high-tech' epifluorescence microscope that creates a thin (~0.3 μm) plane-of-focus in which out-of-focus light has been blocked, traditionally by optical barriers (confocal apertures: 'pinholes') but also now by the physics of light absorption ('multiphoton' microscopy). Laser light is scanned across the specimen to provide intense, deeply penetrating excitation energy. Intrinsic fluorophores (biomolecules such as green fluorescent protein [GFP] or chlorophyll) or exogenous probes (e.g. fluorescently labeled antibodies) are excited, the resulting fluorescence is detected by photomultiplier tubes, and a digital image is produced. A 'stack' (series of digital XY optical sections) is automatically collected through computer-controlled alteration of the microscope's stage in the Z dimension. Sections in the XZ plane (saggital sections) can also be acquired. The stack can be computer-processed to create, for example, three-dimensional reconstructions.

Three basic designs of CSLM are commercially available. 'Real-time' instruments are optimized for video-rate data collection at the expense of axial and lateral resolution. These devices are used primarily for study of physiological phenomena on time scales of seconds or less, are generally not useful for precise optical sectioning (morphological/structural studies), and have therefore not yet found significant application to biofilm work. The other types of CSLM are point-scanning devices that offer high lateral and axial resolution but slower image collection times. The 'single-photon' CSLM has been available from several manufacturers for about 15 years and is by far the most used instrument in biological confocal microscopy. 'Multiphoton' CSLMs, which use tunable IR lasers to provide excitation energy 'packets' (multiples of photons of longer wavelengths absorbed by lower-wavelength-excitable fluors), are in their first generation of commercialization and theoretically offer several advantages over their single-photon counterparts: lower sample damage through photobleaching or heating; deeper penetration of excitation light; and absence of light-obstructing pinholes (the optical section is created solely by point-of-focus of the laser beam). Performance/reliability issues (e.g. the lasers can be difficult to tune and to stabilize) and laser price currently limit the market for such instruments. Although these issues will be resolved with time, as they were for single-photon devices, the longer excitation wavelengths of the multiphoton systems result in a twofold poorer theoretical resolution than in single-photon instruments.

In most biological disciplines, the CSLM has been used primarily to examine fixed specimens that have been stained with fluorescently labeled antibodies or organelle-specific probes. In contrast, bacterial biofilm studies began with the study of living biofilms as they grew on the surfaces of glass-walled perfusion chambers (flowcells [1,2]), thereby permitting semi-continuous non-destructive documentation of temporal changes in biofilm structure/physiology. Conventional microscopy of multilayered biofilms is possible, however, through cryo-embedding (frozen samples are embedded in a cryo-mounting media such as TissueTek) or ambient temperature embedding in a plastic resin, followed by microtome cutting; micrographs are made of the individual (optically clearer) sections. This lower-cost (a research-grade cryostat, ~$20,000, together

with a high quality epifluorescence microscope, ~$25,000, versus the >$200,000 price of a CSLM) approach to obtaining biofilm images can produce sections as thin as 2 μm: only about 10-fold thicker than what can be achieved by confocal microscopy (optical sectioning) but still of lower resolution. Fixation and dehydration required by sectioning techniques can affect three-dimensional relationships. Finally, physical sectioning, even when performed with flawless technique, can disrupt fine structure of the biofilms, thereby making spatial analysis difficult.

Although the confocal microscope has permitted examination of intact biofilms, an embedding step is still advantageous in certain cases. Embedded biofilms are much more robust and easy to handle; this is especially important for techniques in which multiple washing steps are required (e.g. nucleic acid hybridization, see below). Agarose embedding is both simple and gives good results when used with confocal microscopy [3]. Disadvantages are that the agarose solution must be warm and that the final preparations are rather fragile due to the low (0.1%) agarose concentration. DNA-sequencing-grade acrylamide (20%) is an excellent embedding medium [4••,5,6•]; these preparations are easily handled and, for example, are suitable for 16S ribosomal probing or analysis of GFP expression. Furthermore, acrylamide is virtually non-fluorescent. Biofilms embedded by either of these methods may be fixed, dehydrated, or still living.

Microscopic investigation of biofilm architecture

The earliest applications of the CSLM to biofilm research were descriptive. Arrangement of single cells and of microcolonies within living hydrated biofilms were described with heretofore unknown precision [7], and the value of digital images became recognized (reviewed in [8]). Most of these pioneering studies were carried out with monoculture biofilms and established important models of biofilm growth or architecture, but the biofilms bore little relevance to real-world scenarios. Two exceptions of note were an *in vitro* plaque model [9] and a freshwater system (Figure 1 in [10]); these multispecies biofilms showed significant differences from then-established monoculture models. For example, they clearly demonstrated a lower biomass density at the substratum than at locations closer to the bulk-liquid interface ('inverted pyramids'), and many cells were positively stained (e.g. appeared brighter than the background) when examined using the then most commonly used staining method (fluorescein-exclusion staining [11]) in which cells usually appear darker than the background. Key studies in which nutrient composition was demonstrated to affect biofilm architecture were those of Wolfaardt *et al.* [12] and Møller *et al.* [13], and a highly multidisciplinary approach was taken by Singleton *et al.* [14] in which confocal data contributed to models of flow within biofilms. Signaling molecules such as homoserine lactones (HSLs) have been shown to play an important role in the architecture of model monoculture biofilms [15]. *Pseudomonas aeruginosa* PAO1 wild-type biofilms were thicker (but interestingly, less dense) and were more susceptible to disintegration in the presence of the surfactant sodium dodecyl sulfate than were biofilms of a mutant strain that produced no HSLs. These results suggest that signaling molecules such as HSLs can induce structural and chemical changes during biofilm development and that those changes (at least in these *Pseudomonas* strains) can make the biofilm more resistent to certain chemical treatments. Conversely, because increased antibiotic resistance of biofilms over planktonically grown cells is currently thought to involve variations on diffusion-based mechanisms (either simple diffusion resistance or exposure to antibiotic-degrading enzymes), the type of biofilm produced by the mutant strain lacking HSLs might in fact be even more resistant to antibiotic treatment than the wild-type biofilm due to the higher degree of cell-packing (less intercellular space).

As our understanding of the complexity of biofilms increases, 'simple' descriptive architectural studies are being replaced by those in which the three-dimensional organization of the biofilm is related to other information, such as species composition, relationship with the substratum, or physiology.

Relationship of architecture with species composition

The development of species-selective fluorescently labeled oligonucleotide probes has enabled microscopists to distinguish between morphologically similar cells within a biofilm (see [16] for a recent example). Although this hybridization-based methodology cannot be used non-destructively, it is a powerful tool for sorting out spatial relationships. Because the hybridization process requires dehydration, it is usually performed on polyacrylamide-stabilized biofilms; the stabilization allows some retention of three-dimensional structure, it also assures that cells are less likely to be removed during the multiple washing steps that are required, and it results in a smile that can be stored and examined at a later date. Hybridization can be combined with other techniques to elucidate physiological interactions. An elegant study [17] shows how the spatial relationship at the single-cell level of physiologically important microcolonies within wastewater-treatment biofilms can be correlated with microelectrode profiles of depth-dependent changes in concentration of soluble chemical species (nitrate/nitrite, oxygen). Even when coupled with microscopy, the use of electrodes is an invasive technique whose spatial resolution is uncertain in biofilms, however, real-time concentration data on ions of physiological importance to the biofilm outweigh these disadvantages.

Interactions of biofilms with the substratum

An effect of substratum hydrophobicity on biofilm morphology (attachment to the substratum) as displayed by a marine bacterium was described by Dalton *et al.* [18], and

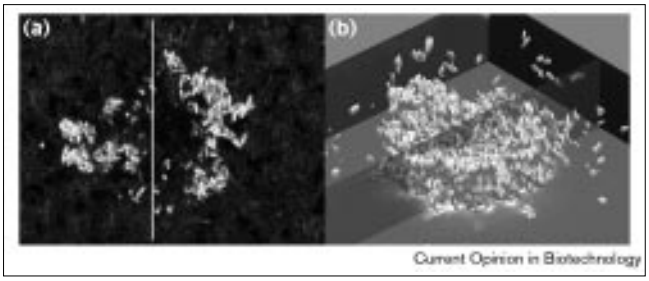
biofilms grown within crevices demonstrated continued growth (in contrast with their non-crevice-protected counterparts) after exposure to a disinfectant [19]. Effects of biofilms on materials can also be assessed. For example, the corrosion of mild steel seems to be reduced when actively metabolizing pure-culture aerobic biofilms are present [20]. This study also points out a problem that can be associated with the use of multicomponent stains: indeterminate staining, in which a stain mixture that should yield cells of two distinct colors can result in some cells whose color is in fact a mixture of the two stains. Examination of implantable medical devices is a clinically relevant area in which traditional electron microscopy has produced outstanding images of difficult samples [21]. CSLM images of implants can, however, be difficult to interpret [22,23], perhaps because they are often acquired at low resolution [24] to maximize the viewable area on the implant. Imaging of bacteria on living substrata is possible as shown by an examination of bacterial attachment to lettuce leaves [25].

Microscopic investigation of biofilm physiology and chemistry

That a gene becomes active is of interest, but it is also important when it becomes inactive, and both processes will be influenced by location of cells with respect to the bulk liquid and to other cells in the biofilm; a microscopic approach is the only manner in which time-resolved three-dimensional data can be obtained. Prokaryotic gene activity is often detected through a reporter enzyme system: an enzyme gene (e.g. the *lacZ* gene whose product is β-galactosidase or the *lux* gene whose product is bacterial luciferase) whose transcription is coupled with that of the gene of interest. To probe the activity of a *lacZ*-coupled gene of interest, substrates are employed that are colorless until enzymatically hydrolyzed. The *lacZ* system has been used with epifluorescence microscopy [26,27], but confocal applications have been limited because of difficulty in finding acceptable substrates: they must be cell-permeant, preferably visible-light-excitable, and the resulting fluor should be non-diffusable [4••]. Luciferase produces light rather than fluorescence, and can be detected using sensitive cameras [28,29]. Quantitative light detection has been used to show single-cell differences in natural bioluminescence by attached cells of *Vibrio* spp. [30], and to show heterogeneity of toluene degradation (*tod/lux* reporter) within a genetically engineered *Pseudomonas putida* biofilm [31•]. Luciferase activity can be axially resolved (RJ Palmer, unpublished data), but to a much poorer degree than that of confocal fluorescence detection.

If the product of the gene of interest is an enzyme, the opportunity for direct detection exists. For example, alkaline phosphatase activity has been monitored within a biofilm using a substrate that changes its fluorescence emission after hydrolysis [32•]. Cryosectioning was used to prepare 5 μm thick sections for epifluorescence microscopy, and the region of phosphatase expression within the biofilm was shown to relate to accessibility of the cells to nutrients.

Figure 1



GFP as a gene-expression reporter. *Pseudomonas putida* R1 (containing a GFP-encoding expression cassette under control of the Pm promoter from the TOL meta pathway of toluene degradation) cells growing together with seven other species in a flowcell on 3-methylbenzoate (Pm is induced). **(a)** Single confocal slice at the substratum. GFP-expressing cells are white; the other bacteria are faintly visible (gray) due to slight autofluorescence. **(b)** The cone-shaped colony is viewed from the substratum in three-dimensional rendering. Autofluorescent cells have been filtered out by image processing. The 3D image is composed of 26 individual slices and rendered using Spyglass software (Fortner Software LLC, Sterling, VA, USA).

Application of confocal microscopy to three-dimensional localization of non-enzyme reporter systems, notably GFP has brought significant advances to the understanding of gene activity in biofilms. Ribosome content is an indicator of growth potential (and is closely correlated to growth rate in exponentially growing cultures), measurements of ribosome-hybridization-signal intensity have therefore been used for *in situ* determination of cellular activity [33]. The physiological state of cells in biofilms can be assessed using specially designed promoter–reporter systems which fluoresce only in actively dividing cells. A recent study demonstrated the growth activity and potential of *Pseudomonas* cells within a biofilm. Briefly, an expression cassette that contained GFP under control of a ribosomal rRNA promoter (growth-phase regulated; active only when cells are growing) was constructed, and the growth phase of individual cells in the developing biofilm was monitored. Ribosome synthesis rate and ribosome content were determined *in situ* giving a snapshot of the cells' physiological state (C Sternberg *et al.*, unpublished data; C Ramos *et al.*, unpublished data). Exchange of genetic information (plasmid transfer [34]) and 'metabolic webs' [6•] (Figure 1) can be similarly mapped in three-dimensions. Recently, microautoradiography was used in combination with ribosomal probing to visualize activity-identity relations in microbial communities [35].

Confocal microscopy has been adeptly applied to the examination of free-radical-based disinfection [36]. A non-fluorescent compound was employed which became fluorescent after reaction with hydroxyl radicals or superoxide and excitation with 365 nm light. A time series of saggital sections showed that free radicals were generated

within the biofilm during exposure to potassium monopersulfate, presumably from the action of catalysts incorporated into the substratum. Radical generation was suggested to be the cause of a depth-dependent killing of biofilm bacteria exposed to the persulfate.

It is important to recognize that confocal microscopy (or any epifluorescence technique) cannot generally be used to visualize unstained (nonfluorescent) material, such as the exopolymer matrix in which biofilm cells are embedded. Hence, conclusions regarding presence/absence/changes in the extracellular matrix when that matrix was not specifically stained are based primarily on assumptions. Exopolymer and its relationship to biofilm structure/function can, however, be explored using appropriate techniques (e.g. fluorescent lectin staining [13,37•]).

Emerging techniques

The environmental scanning electron microscope (ESEM) is a low-vacuum (near atmospheric pressure) scanning electron microscope which can be used to image hydrated samples. While the images are essentially topographical, the technique has been used to demonstrate the degree of exopolymer hydration in manganite-reducing biofilms through comparison with dehydrated samples [38].

Scanning probe microscopy (SPM) comprises a suite of instruments that move a cantilevered probe across a surface in contact or near-contact modes. Force microscopy measures the attraction/repulsion of the probe tip at resolutions up to the atomic level (atomic force microscopy). Variations in tip shape/composition, or in the way tip–surface interaction is modulated, give different resolutions and modes of interaction (force modulation microscopy, electrostatic force microscopy, magnetic force microscopy, phase detection microscopy). Although the images from these techniques are topographical renderings of the tip-surface interaction (meaning that the topographical information actually represents numerical physicochemical data), common applications are simply topographical (obtaining the shape of the surface is the final goal of the imaging process). SPM has produced images of dried bacteria that resemble those acquired with scanning electron microscopy [39], but with greater detail at similar magnification (especially in three dimensions) and without extensive sample preparation, such as critical point drying and coating. Gross antibiotic-induced changes in the cell wall of a bacterium have been demonstrated [40]. Water is integral to biological structures, and SPM is now being applied to hydrated, living biological specimens [41], although tip contamination must be considered [42]. In an elegant application of force microscopy to bacterial structure, the influence of lipopolysaccharide composition on relative strength-of-interaction between cells and substrata has been quantified in aqueous solution [43••].

Chemical microscopy (Raman/infrared microscopy) provides spatially resolved (line scanning, global imaging) chemical information on the specimen: spectroscopy through the microscope. As in any microscope technique, spatial resolution is finally limited by the wavelengths of light used. IR microscopy (a broad-band absorption technique) has historically used wavelengths (~2800–10,000 nm) too long for resolution of a single bacterial cell; spatial resolution is at best tens-of-micrometers. Raman microscopy (a light-scattering technique which measures small shifts in photon energy around a single wavelength) using visible or near-IR lasers, however, can resolve single bacterial cells [44•]. Furthermore, several commercial confocal Raman microscopes exist that theoretically allow the chemical nature of biofilms to be investigated in the axial as well as the lateral dimensions.

Correlative microscopy is the use of two or more techniques to investigate the same (or parallel) specimens. Relatively easily performed with fixed specimens, CSLM images have been combined with electron micrographs to examine biofilm architectural features [3]. The approach can also be employed with living biofilms; for example, Palmer and co-workers [30,31•] have combined photon-counting microscopy (to measure luciferase activity, i.e. gene expression) with laser confocal microscopy to analyze, firstly, viability in *Vibrio* species [30] and, secondly, membrane potential in genetically engineered *P. putida* cells [31•]. The potential for combination of the scanning probe methods or Raman methods with epifluorescence techniques (including confocal) promises major advances in our understanding of biofilm structure/function.

Conclusions

Microscopy has been rediscovered as a highly valuable approach to all biological questions, and it is indispensable for investigation of biofilms. Use of real-time gene reporters in unfixed samples will provide the most significant information on biofilm physiology. *In vitro* model systems will continue to be important, but time-resolved data collection and examination of real-world samples will drive conceptual development.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Palmer R Jr: **Microscopy flowcells: perfusion chambers for real-time study of biofilms.** In *Biofilms*. Edited by Doyle R. Orlando: Academic Press; 1999:in press. [Abelson J, Simon M (Series Editors): *Methods in Enzymology Series*.]
2. Kolenbrander P, Andersen R, Kazmerzak K, Wu R, Palmer R Jr: **Spatial organization of oral bacteria in biofilms.** In *Biofilms*. Edited by Doyle R. Orlando: Academic Press; 1999:in press. [Abelson J, Simon M (Series Editors): *Methods in Enzymology Series*.]
3. Massol-Deyá A, Whallon J, Hickey R, Tiedje J: **Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater.** *Appl Environ Microbiol* 1995, **61**:769-777.
4. •• Christensen B, Sternberg C, Andersen J, Palmer R Jr, Nielsen A, Givskov M, Molin S: **Molecular tools for the study of biofilm physiology.** In *Biofilms*. Edited by Doyle R. Orlando Academic Press;

1999:in press. (Abelson J, Simon M [Series Editors]: *Methods in Enzymology Series.*)
Detailed 'instruction manual' on fixation, embedding and use of reporters in biofilms.

5. Sternberg C, Christensen B, Møller S, Andersen J, Molin S: **Localisation of cells active in gene expression and gene transfer in intact bacterial biofilms.** In *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons.* Edited by Hastings J, Kricka L, Stanley P. Chichester: John Wiley and Sons; 1996:371-374.

6. • Møller S, Sternberg C, Andersen J, Christensen B, Molin S: ***In situ* gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members.** *Appl Environ Microbiol* 1998, **64**:721-732.
Promoter activity and identity in a seven-species biofilm: spatial and metabolic interaction of two species during toluene degradation. The spatial organization and chemical nature of biofilm exopolymer was revealed by staining with fluorescently tagged lectins.

7. Lawrence J, Korber D, Hoyle B, Costerton J, Caldwell D: **Optical sectioning of microbial biofilms.** *J Bacteriol* 1991, **173**:6558-6567.

8. Caldwell D, Korber D, Lawrence J: **Confocal laser microscopy and digital image analysis in microbial ecology.** *Adv Microb Ecol* 1992, **12**:1-67.

9. Palmer R Jr, Caldwell D: **A flowcell for the study of plaque removal and regrowth.** *J Microbiol Methods* 1995, **24**:171-182.

10. Costerton J, Lewandowsi Z, DeBeer D, Caldwell D, Korber D, James G: **Biofilms, the customized microniche.** *J Bacteriol* 1994, **176**:2137-2142.

11. Caldwell D, Korber D, Lawrence J: **Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy.** *J Microbiol Methods* 1992, **15**:249-261.

12. Wolfaardt G, Lawrence J, Robarts R, Caldwell S, Caldwell D: **Multicellular organization in a degradative biofilm community.** *Appl Environ Microbiol* 1994, **60**:434-466.

13. Møller S, Korber D, Wolfaardt G, Molin S, Caldwell D: **Impact of nutrient composition on a degradative biofilm community.** *Appl Environ Microbiol* 1997, **63**:2433-2438.

14. Singleton S, Treolar R, Warren R, Watson G, Hodgson R, Allison C: **Methods for microscopic characterization of oral biofilms: analysis of colonization, microstructure, and molecular transport phenomena.** *Adv Dent Res* 1997, **11**:133-149.

15. Davies D, Parsek M, Pearson J, Iglewski B, Costerton J, Greenberg E: **The involvement of cell-to-cell signals in the development of a bacterial biofilm.** *Science* 1998, **280**:295-298.

16. Manz W, Eisenbrecher M, Neu T, Szewzyk U: **Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides.** *FEMS Microb Ecol* 1998, **25**:43-61

17. Schramm A, Larsen L, Revsbech N, Ramsing N, Amann R, Schleifer KH: **Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes.** *Appl Environ Microbiol* 1996, **62**:4641-4647.

18. Dalton H, Poulsen L, Halasz P, Angles M, Goodman A, Marshall K: **Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure.** *J Bacteriol* 1994, **176**:6900-6906.

19. Korber D, Choi A, Wolfaardt G, Ingham S, Caldwell D: **Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to trisodium phosphate.** *Appl Environ Microbiol* 1997, **63**:3352-3358.

20. Jayaraman A, Sun A, Wood T: **Characterization of axenic *Pseudomonas fragi* and *Escherichia coli* biofilms that inhibit corrosion of SAE 1018 steel.** *J Appl Microbiol* 1998, **84**:485-492.

21. Gregory M, Hadley G: **The evolution of biofilms in venous access devices implanted in children with Wilm's tumour.** *Pediatr Surg Int* 1998, **13**:400-405.

22. Gorman S, Mawhinney W, Adair C, Issouckis M: **Confocal laser scanning microscopy of peritoneal catheter surfaces.** *J Med Microbiol* 1993, **38**:411-417.

23. Inglis T, Tit-Meng L, Mah-Lee N, Ee-Koon T, Kok-Pheng H: **Structural features of tracheal tube biofilm formed during prolonged mechanical ventilation.** *Chest* 1995, **108**:1049-1052.

24. Gorman S, Adair C, Mawhinney W: **Incidence and nature of peritoneal catheter biofilm determined by electron and confocal laser scanning microscopy.** *Epidemiol Infect* 1994, **112**:551-559.

25. Seo K, Frank J: **Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by confocal scanning laser microscopy.** *J Food Prot* 1999, **62**:3-9.

26. Robinson P, Walker J, Keevil C, Cole J: **Reporter genes and fluorescent probes for studying the colonisation of biofilms in a drinking water supply line by enteric bacteria.** *FEMS Micro Lett* 1995, **129**:183-188.

27. Davies D, Geesey G: **Regulation of the alginate biosynthetic gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture.** *Appl Environ Microbiol* 1995, **61**:860-867.

28. Palmer R Jr, Phiefer C, Burlage R, Sayler G, White D: **Single-cell bioluminescence and GFP in biofilm research.** In *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons.* Edited by Hastings JW, Kricka LJ, Stanley PE. Chichester: John Wiley and Sons; 1996:445-450.

29. Palmer R Jr, White D: **Spatially resolved, quantitative determination of luciferase activity by photon-counting microscopy.** In *Biofilms.* Edited by Doyle R. Orlando: Academic Press; 1999:in press. [Abelson J, Simon M (Series Editors): *Methods in Enzymology Series.*]

30. Phiefer C, Palmer R Jr, White D: **Comparison of relative photon flux from single cells of the bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* using photon-counting microscopy.** *Luminescence* 1999: in press.

31. • Palmer R Jr, Applegate B, Burlage R, Sayler G, White D: **Heterogeneity of gene expression and activity in bacterial biofilms.** In *Bioluminescence and Chemical Luminescence: Perspectives for the 21st Century. Proceedings of the 10th International Symposium on Bioluminescence and Chemiluminescence: 1998 September 4–8; Bologna.* Edited by Roda A, Pazzagli M, Kricka LJ, Stanley PE. Chichester: John Wiley and Sons; 1999:609-612.
The authors demonstrate correlative microscopy using living biofilms. This shows the value of having multiple lines of evidence for assessing bacterial activity and that gene expression within a pure-culture microcolony can be heterogeneous.

32. • Huang C-T, Xu K, McFeters G, Stewart P: **Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation.** *Appl Environ Microbiol* 1998, **64**:1526-1531.
Excellent use of cryo-embedding methods and of enzymatic reporters to dissect biofilm physiology in three dimensions.

33. Poulsen L, Ballard G, Stahl D: **Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms.** *Appl Environ Microbiol* 1993, **59**:1359-1360.

34. Christensen B, Sternberg C, Andersen J, Eberl L, Møller S, Givskov M, Molin S: **Establishment of new genetic traits in a microbial biofilm community.** *Appl Environ Microbiol* 1998, **64**:2247-2255.

35. Lee N, Nielsen P, Andreasen K, Juretschko S, Nielsen J, Schleifer K-H, Wagner M: **Combination of fluorescent *in situ* hybridization and microautoradiography, a new tool for structure-function analyses in microbial ecology.** *Appl Environ Microbiol* 1999, **65**:1289-1297.

36. Wood P, Caldwell D, Evan E, Jones M, Korber D, Wolfhaardt G, Wilson M, Gilbert P: **Surface-catalysed disinfection of thick *Pseudomonas aeruginosa* biofilms.** *J Appl Microbiol* 1998, **84**:1092-1098.

37. • Wolfaardt G, Lawrence J, Robarts R, Caldwell D: ***In situ* characterization of biofilm exopolymers involved in the accumulation of chlorinated organics.** *Microb Ecol* 1998, **35**:213-223.
Visualization of exopolymer and elucidation of its role in biofilm physiology.

38. Larson I, Little B, Nealson K, Ray R, Stone A, Tian J: **Magnetite reduction by *Shewanella putrifaciens* MR-4.** *Am Mineralogist* 1998, **83**:1564-1572.

39. Umeda A, Saito M, Amako K: **Surface characteristics of Gram-negative and Gram-positive bacteria in an atomic force microscope image.** *Microbiol Immunol* 1998, **42**:159-164.

40. Braga P, Ricci D: **Atomic force microscopy: application to investigation of *Escherichia coli* morphology before and after exposure to cefodizime.** *Antimicrob Agents Chemother* 1998, **42**:18-22.

41. Kuznetsov Y, Malkin A, McPherson A: **Atomic force microscopy studies of living cells: visualization of motility, division, aggregation, transformation, and apoptosis.** *J Struct Biol* 1997, **120**:180-191.

42. Schaus S, Henderson E: **Cell viability and probe-cell membrane interactions of XR1 glial cells imaged by atomic force microscopy.** *Biophys J* 1997, **73**:1205-1214.

43. Razatos A, Ong Y-L, Sharma M, Georgiou G: **Molecular
•• determinants of bacterial adhesion monitored by atomic force microscopy.** *Proc Natl Acad Sci USA* 1998, **95**:11059-11064.
Correlation of lipopolysaccharide chemistry to adhesion strength in aqueous solution using force microscopy and bacteria-coated tips. Clever methodology used to generate real-world biological data with SPM.

44. Colarusso P, Whitley A, Levin I, Lewis E: **Raman microscopy and
• imaging of inorganic and biological materials with liquid crystal tunable filters.** In *Biomedical Applications of Raman Spectroscopy. Proceedings of SPIE, vol 3608.* Edited by Morris MD. Bellingham: SPIE Press; 1999:in press.
Bacterial cells imaged by Raman microscopy; demonstrates potential for non-destructive investigation of biofilm chemistry at single-cell resolution.