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Biofilms

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Frequently it is necessary to have parts custom-made (generally by a milling process). Most universities provide shop services that can perform these operations or can refer the customer to an outside machine-shop company. For those interested in milling parts for smaller flowcells "in house," Sherline Products Inc. (2350 Oak Ridge Way, Vista, CA 92083; www.sherline.com) makes bench-top milling machines of the sophistication necessary for most work.

Note Added in Proof

The anaerobic system described herein has been used for biofilm culture of capnophilic organisms (*Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp.).[9] Recent experiments using a transformed streptococcal strain demonstrated that residual O_2 levels were sufficient to result in oxidation (fluorescence) of Green Fluorescent Protein. Modifications have resulted in a setup in which an O_2 concentration of ≤ 0.025 mg/l is maintained.[10]

[9] R. J. Palmer, Jr., unpublished.
[10] M. C. Hansen, R. J. Palmer, Jr., and D. C. White, *J. Microbiol. Meth.*, submitted.

[13] Fluorescent Probes Applied to Physiological Characterization of Bacterial Biofilms

By JOHN T. LISLE, PHILIP S. STEWART, and GORDON A. MCFETERS

Introduction

Efforts to describe the physiological activities of microbial populations have been hindered by the inability of culture-based techniques to recover a significant proportion of bacteria present in a sample[1] and by our traditional dependence on methods that yield community averages of physiological indices, such as oxygen consumption, evolution of carbon dioxide, and radiolabeled substrate incorporation into macromolecules. Progress in the development of *in situ,* intracellular fluorescent labels and probes has facilitated the rapid assessment of microbial physiology without cultivation. In the context of this article, labels are defined as fluorescent dyes that bind or interact with specific targets within the cell [e.g., 4′6-diamidino-2-phenylindole (DAPI)]. Labels most often have inherent fluorescence

[1] R. Amann, W. Ludwig, and K. Schleifer, *Microbiol. Rev.* **59,** 143 (1995).

METHODS IN ENZYMOLOGY, VOL. 310

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regardless of their being free, nonspecifically bound, or bound to their cellular target. Probes are fluorescent dyes that change their emission spectra in response to an intracellular activity (e.g., enzyme) or parameter (e.g., pH). The probes most applicable to biofilm systems are those that remain nonfluorescent until acted on by the cellular target.

The advantages of using these types of labels and probes include the direct, single cell assessment of (1) physiological status, (2) specific metabolic activities, (3) gene expression, and (4) total cell densities.[2] Additionally, when used in conjunction with fluorescent antibodies, these fluorescent markers can assess physiological activity at the species or serotype level.[3] Other nonculture-based techniques, for example, fluorescent *in situ* hybridization (FISH) using 16S rRNA probes, provide only indirect evidence pertaining to the physiological activity of the sampled community or individual cells.[4–6]

Biofilms are highly heterogeneous in their structure and composition. The traditional methods used to investigate biofilms have relied on the removal of biofilms from the substrata, followed by disaggregation. These approaches provide nominal spatial information regarding the structure of and physiological activity of individual cells within intact biofilms. Biofilm composition heterogeneity is influenced directly by constituents from the overlying bulk phase adsorbing or precipitating onto and into the biofilm matrix and intrabiofilm deposition of microbially secreted products. Individually or collectively, these constituents may influence the performance of a label or probe. Table I lists characteristics that should be considered when choosing a label or probe. Table I does not list the types of chemical modifications that have been used to stabilize and optimize a label or the permeability and fluorescence of a probe. More detailed discussions of these factors and those listed in Table I have been published elsewhere.[7–9]

Table I provides some insight into the complexities of selecting an appropriate label or probe for investigating the microbial physiology of biofilm systems. We have found that Molecular Probes, Inc. (Eugene, OR

[2] G. McFeters, F. Yu, B. Pyle, and P. Stewart, *J. Microbiol. Methods* **21,** 1 (1995).
[3] B. Pyle, S. Broadaway, and G. McFeters, *Appl. Environ. Microbiol.* **61,** 2614 (1995).
[4] L. Poulsen, G. Ballard, and D. Stahl, *Appl. Environ. Microbiol.* **59,** 1354 (1993).
[5] S. Williams, Y. Hong, D. Danavall, M. Howard-Jones, D. Gibson, M. Frischer, and P. Verity, *J. Microbiol. Methods* **32,** 225 (1998).
[6] C. Buswell, Y. Herlihy, L. Lawrence, J. McGuiggan, P. Marsh, C. Keevil, and S. Leach *Appl. Environ. Microbiol.* **64,** 733 (1998).
[7] R. Haugland, "Handbook of Fluorescent Probes and Research Chemicals." Molecular Probes, Eugene, OR, 1996.
[8] J. Slavik, "Fluorescent Probes in Cellular and Molecular Biology." CRC Press, Boca Raton FL, 1994.
[9] P. Stewart, *Biotech. Bioeng.* **59,** 261 (1998).

TABLE I
CHARACTERISTICS TO CONSIDER WHEN SELECTING LABELS OR PROBES

Label or probe characteristic	Contributing factors	Effect on label or probe[a]
Preparation solution	pH, water, ethanol, methanol, Tris–EDTA, dimethyl sulfoxide, growth medium	±
Excitation and emission	Large Stokes shift	+
	Quenching	−
	pH	±
Permeability and retention in biofilms	Thick biofilms (> monolayers)	−
	Thin biofilms (monolayers)	+
	Cationic, anionic or neutral charge	±
	Hydrophilic or hydrophobic	±
	pH	±
	Molecular weight (effective diffusion coefficient)	±
Permeability and retention within cells	Active or facilitated diffusion	+
	Uni- or bidirectional active transport	±
	Acetylated modifications	+
	Cationic, anionic, or neutral charge	±
	Hydrophilic or hydrophobic	±
	pH	±
	Molecular weight	±

[a] +, increases label or probe efficiency; −, decreases label or probe efficiency; and ±, may increase or decrease a label or the efficiency of a probe dependent on local conditions.

http://www.probes.com) offers an extensive selection of labels, probes, and technical information for these types of applications.

Biofilm Recovery and Microscopic Visualization

Cryosectioning and Fluorescent Microscopic Visualization

The visualization of monolayer biofilms is accomplished easily by either light or fluorescence microscopy. However, due to the resolution limits of optical microscopy, studies on thicker biofilms require their removal from the substratum prior to visualization. Cryosectioning effectively removes

biofilms from substrata, while retaining their complex structures.[10,11] This technique allows the visualization of biofilm cross sections using fluorescent microscopy. A similar technique has been developed for sectioning sludge granules.[12]

1. Biofilms should be grown on a substratum that will be able to withstand exposure to dry ice for 20 min. A commonly used substratum is 316L stainless steel (17 × 75 mm). A larger size can be used, but one must be able to manipulate the substratum easily without physically disrupting the biofilm or inhibiting the freezing of the specimen.
2. Transfer the biofilm sample slide to a dry ice slab, biofilm side up.
3. Immediately dispense a thick layer of Tissue-Tek OCT compound (Miles, Inc., Diagnostics Division, Elkhart, IN) on top of the biofilm. Allow the sample to freeze until the entire sample turns opaque white.
4. Gently bend the substratum to pop off the frozen sample and immediately replace the embedded biofilm sample on the dry ice with the surface that was attached to the substratum pointed up.
5. Dispense a thick layer of OCT compound on this surface of the biofilm sample and allow to freeze until the sample turns opaque white. Label the substratum side of the sample with a permanent marker.
6. Wrap the embedded biofilm sample in aluminum foil and store at −70°.
7. Mount the frozen biofilm sample on a precooled specimen chuck and then slice sections (5 μm thick) using a cryostat operated at −20°. Some trimming may be required to obtain a smooth surface, prior to collecting sections for visualization. Collect the biofilm sections onto polylysine-coated slides, such as Superfrost Plus slide (Fisher Scientific, Pittsburgh, PA).
8. The biofilm sections can then be visualized directly using fluorescence microscopy. If the addition of a coverslip is required, the mounting medium should be evaluated as to its ability to quench, dissolve, or release the label or probe.

[10] F. Yu, G. Callis, P. Stewart, T. Griebe, and G. McFeters, *Biofouling* **8**, 85 (1994).
[11] C. Huang, P. Stewart, and G. McFeters, The study of microbial biofilms by classical fluorescence microscopy, pp. 411–429. *in* "Digital Image Analysis of Microbes: Imaging, Morphometry, Fluorometry and Motility Techniques and Applications" (M. Wilkins and F. Schut, eds.), p. 411. Wiley, Chichester, UK, 1998.
[12] H. Harmsen, H. Kengen, A. Akkermans, A. Stams, and W. de Vos, *Appl. Environ. Microbiol.* **62**, 1656 (1996).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) allows direct, nondestructive, and three-dimensional visualization of biofilm structure. When cells residing within these biofilms have been labeled or probed with fluorescent dyes, information on physiological activity at the intracellular and microniche level can also be ascertained. Several of the techniques described in the following sections have used CLSM to visualize the respective biofilm systems. Fluorescent microscopy can be used with these systems as well, but the biofilms will have to be embedded and cryosectioned.

It is not the objective of this article to discuss the application of CLSM to the visualization of biofilms. Lawrence *et al.*[13] have published an excellent introduction and review on using CLSM to study biofilms (see chapter 9 of this volume). Also, a compilation of other methods to visualize microbes has been recently published.[14] Questions and information about the use of CLSM and image analysis for the study of biofilms can be submitted to the Center for Biofilm Engineering at Montana State University at http://www.erc.montana.edu.

Autofluorescence Quenching

Autofluorescence from bacteria, algae, and abiotic materials (e.g., clay, soil) has emission spectra very similar to one or more of the commercially available labels and probes. Therefore, differentiation between labeled or probed bacterial cells and autofluorescing cells or debris may be practically impossible. Selecting the correct combination of microscope filters can partially reduce autofluorescence, especially if the autofluorescence occurs within a narrow wavelength. As an alternative, quenching solutions can be applied to the biofilms. Slavik[8] lists commonly used quenchers for types of labels and probes that have been used with planktonic cells, but does not address autofluorescence. However, some of the quenchers may be applicable to biofilm systems.

Huang *et al.*[15] evaluated sodium borohydride (50 m*M* after ethanol dehydration), Evans blue [0.5% (w/v) in phosphate-buffered saline and 0.1 *M* sodium azide], and crystal violet (2 mg/ml) as autofluorescence quenchers in industrial biofilm systems. These quenchers shifted the autofluorescence

[13] J. Lawrence, G. Wolfaardt, and T. Neu, The study of biofilms using confocal laser scanning microscopy, pp. 431–465. *In* "Digital Image Analysis of Microbes: Imaging, Morphometry, Fluorometry and Motility Techniques and Applications" (M. Wilkins and F. Schut, eds.), p. 431. Wiley, Chichester, UK, 1998.

[14] M. Wilkinson and F. Schut, eds., "Digital Image Analysis of Microbes: Imaging, Morphometry, Fluorometry and Motility Techniques and Applications." Wiley, Chichester, UK, 1998.

[15] C. Huang, G. McFeters, and P. Stewart, *Biofouling* **9**, 269 (1996).

emission spectra to green, bright red, and dark red, respectively. The selection of an appropriate quencher is dependent on emission spectra of the label or probe and quencher so as to optimize contrast. The use of quenchers in conjunction with optimized microscope filter combinations may minimize autofluorescence effectively.

Labeling and Probing Bacterial Biofilms

Total Cell Counts Using 4',6-Diamidino-2-phenylindole

DAPI is a DNA-specific probe that forms a fluorescent complex when bound in the minor groove of A-T-rich sequences.[16] The following version of this technique describes the staining of a bacterial biofilm with DAPI for total cell counts within the biofilm and a two-dimensional view of its structure.

A binary biofilm of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was grown in a continuous flow annular reactor containing 316L stainless-steel slides. The biofilm growth medium was minimal salts medium supplemented with 20 mg/liter glucose. Biofilm thickness ranged from 50 to 100 μm. A similar approach has been used to label bacteria in cooling tower water biofilms that were monolayers to several cells thick.[17]

1. Remove the biofilm sample from the growth medium and place it in a staining container, biofilm side up, containing formalin [5% (v/v), final concentration]. Incubate at room temperature for 5 min to fix the cells.
2. Immediately add DAPI (1 μg/ml, final concentration) and incubate at room temperature for 5 min.
3. Gently remove the solution from the staining container and transfer the biofilm sample to a dry ice slab, biofilm side up, and process for cryosectioning as described earlier.
4. Cells that have retained DAPI will fluoresce blue.

The fixation step may not be compatible with all labels and probes, as some fixatives may quench, dissolve, or release the intracellular label or probe. Controls to assess the compatibility of labels, probes, and fixatives should be performed on planktonic cells prior to their use in biofilm systems.

DAPI is used routinely for total cell counts in planktonic and biofilm systems. Additionally, it has also been used to indirectly determine viability based on the presence of an intact genome or nucleoid. Studies have shown

[16] J. Kapuscinski, *Biotech. Histochem.* **70**, 220 (1995).

[17] G. Wolfaardt, R. Archibald, and T. Cloete, *Biofouling* **4**, 265 (1991).

that labeling cells with DAPI may overestimate the number of nucleoid-containing bacteria due to nonspecific binding; this can be removed by gently rinsing.[18–21]

Physiological Activity in Biofilms

To date, there has been limited use of fluorescent labels and probes for the assessment of bacterial physiology within biofilms. The techniques in this section are not necessarily restricted in their application to the described biofilm systems. They should be used as general guidelines for the respective application of the labels and probes to any biofilm system. As discussed previously, the characteristics described in Table I should always be considered.

The following techniques represent a collection of methods for labeling and probing different types of biofilms. Each technique is introduced by a brief description of the conditions under which the biofilms were grown and their thicknesses. Also, references to using fluorescent or confocal laser scanning microscopy are included as described in the referenced literature. However, depending on the desired result, either technique may be used with any of the following labeling and probing techniques.

Assessing General Bacterial Activity.[11,22] *Klebsiella pneumoniae* biofilms were grown in a continuous flow annular reactor using minimal salts medium supplemented with 40 mg/liter glucose as the growth medium. Biofilms were grown on 316L stainless-steel slides at room temperature for 7 days. The biofilm thickness ranged from 50 to 150 μm.

1. Embed and cryosection the biofilm samples as described earlier.
2. Fix cryosections of biofilm in an acidic fixative [5 ml formalin, 2.5 ml glacial acetic acid, 42.5 ml 95% (v/v) ethanol] for 10 min at 4°.
3. Rinse the sections twice with 85% ethanol at 4° and allow to air dry.
4. Add 5 μl of 4 μg/ml acridine orange solution on each section and incubate for 1 min at room temperature in the dark.
5. Gently remove the excess acridine orange with a paper towel or pipe.
6. View using fluorescence microscopy. Cells within the biofilm that fluoresce orange are presumed to have an elevated RNA content and high activity. Cells that fluoresce green are presumed to have a reduced RNA content and low activity.

[18] U. Zweifel and A. Hagstrom, *Appl. Environ. Microbiol.* **61,** 2180 (1995).
[19] J. Choi, E. Sherr, and B. Sherr, *Limnol. Oceanogr.* **41,** 1161 (1996).
[20] M. Suzuki, E. Sherr, and B. Sherr, *Limnol. Oceanogr.* **38,** 1566 (1993).
[21] J. Vosjan and G. van Noort, *Aquat. Microb. Ecol.* **14,** 149 (1998).
[22] E. Wentland, P. Stewart, C. Huang, and G. McFeters, *Biotechnol. Prog.* **12,** 316 (1996).

Assessing Membrane Integrity Using LIVE/DEAD BacLight Viability Kit in Laboratory Biofilms.[7,23] The LIVE/DEAD *Bac*Light Viability kit (Molecular Probes, Inc.) uses the principle of dye exclusion to determine the integrity status of the bacterial membrane. The first label, SYTO 9, is a membrane-permeant, DNA-labeling dye that labels all cells and fluoresces green. The second label is propidium iodide, which also labels DNA, but is excluded from cells with intact membranes. Propidium iodide is able to enter cells with compromised membranes and competes with and quenches SYTO 9, making such cells fluoresce red.

Salmonella enteritidis was grown as a batch culture to mid exponential phase in tryptic soy broth (10%, v/v) at 21° and then used to inoculate modified flow cells. The dimensions of the chamber were 1.3 × 5 × 80 mm with a volume of approximately 0.5 ml. Biofilms were grown within the modified flow cells on glass coverslips for 72 hr at 21°. The biofilm thickness ranged from 3 to 9 μm.

1. Prepare a solution of the LIVE/DEAD *Bac*Light viability assay as described by the manufacturer.
2. Turn the flow to the flow cell off and add approximately 0.3 ml of the LIVE/DEAD *Bac*Light solution to the flow chamber.
3. Incubate for 15 min at 21° in the dark.
4. Reinitiate the flow to the flow cell to wash the unbound label from the system.
5. View the labeled biofilms using CLSM. Cells that fluoresce green have intact cell membranes, whereas cells that fluoresce red have compromised cell membranes.

Use of the LIVE/DEAD *Bac*Light viability assay has been used by numerous researchers to assess the status of membrane integrity in bacterial systems. There has been some question as to its ability to penetrate bacterial exopolymers.[24] However, Korber *et al.*[25] have shown that penetration of the labels is not inhibited in biofilms of *S. enteritidis*. Additionally, some cells may demonstrate both green and red fluorescence.[26,27] This dual fluo-

[23] D. Korber, A. Choi, G. Wolfaardt, S. Ingham, and D. Caldwell, *Appl. Environ. Microbiol.* **63,** 3352 (1997).
[24] D. Caldwell, G. Wolfaardt, D. Korber, and J. Lawrence, *Adv. Microb. Ecol.* **15,** 1 (1997).
[25] D. Korber, A. Choi, G. Wolfaardt, and D. Caldwell, *Appl. Environ. Microbiol.* **62,** 3939 (1996).
[26] S. Terzieva, J. Donnelly, V. Ulevicius, S. Grinshpun, K. Willeke, G. Stelma, and K. Brenner, *Appl. Environ. Microbiol.* **62,** 2264 (1996).
[27] J. Lisle, B. Pyle, and G. McFeters, *Let. Appl. Microbiol.* (1999).

rescence has not been addressed in the technical information that is supplied by the manufacturer, but has been discussed elsewhere.[27,28]

Assessing Respiratory Activity Using 5-Cyano-2,3-ditolyltetrazolium chloride (CTC) in Dechlorinated Drinking Water Biofilms.[29] CTC is a soluble nonfluorescent tetrazolium salt that forms intracellular, insoluble, red fluorescent formazan crystals on reduction.[30] The presence of intracellular CTC–formazan crystals indicates that the cell has an active respiratory system, as succinate dehydrogenase and NADPH dehydrogenase have been shown to be responsible for its reduction.

Treated drinking water was fed into a polymethylene methacrylate (PMMA) flow cell that contained removable coupons. The coupons were made of stainless-steel, copper, high-density polyethylene and PMMA. The dimensions of the chamber were 217 × 100 × 65 mm or approximately 1.4 liters. Biofilms of the uncharacterized natural population water were grown for 7 days at 12°. Biofilms were heterogeneous in their spatial distribution and were one to several cells thick.

1. Remove the coupons from the flow cell, submerge in a mixture of R2A medium (50%, v/v) and CTC (final concentration, 5.0 m*M*), and incubate for 1 hr at room temperature.
2. Gently rinse the coupons and air dry.
3. Counterstain the cells by submerging the coupon in DAPI (2.0 μg/ml) for 20 min at room temperature.
4. Gently remove the excess DAPI with a paper towel or pipette.
5. Using fluorescence microscopy, cells in the CTC/DAPI-probed biofilm will fluoresce red if they have reduced CTC, within an intracellular background blue fluorescence.

CTC has been the most frequently used fluorescent probe to assess physiological activity in biofilms. However, several factors should be considered prior to its use and interpretation of the results. Several studies have addressed the effects of nutrient addition or removal on the efficiency of CTC reduction.[31–36] Additionally, inorganic constituents (i.e., phosphates)

[28] P. Millard and B. Roth, *Biotech. Int.* **1,** 291 (1997).
[29] G. Schaule, H. Flemming, and H. Ridgeway, *Appl. Environ. Microbiol.* **59,** 3850 (1993).
[30] J. Smith and G. McFeters, *J. Microbiol. Methods* **29,** 161 (1997).
[31] G. Rodriguez, D. Phipps, K. Ishiguro, and H. Ridgway, *Appl. Environ. Microbiol.* **58,** 1801 (1992).
[32] L. Gribbon and M. Barer, *Appl. Environ. Microbiol.* **61,** 3379 (1995).
[33] A. Braux, J. Minet, Z. Tamanai-Shacoori, G. Riou, and M. Cormier, *J. Microbiol. Methods* **31,** 1 (1997).
[34] F. Joux, P. Lebaron, and M. Troussellier, *FEMS Microbiol. Ecol.* **22,** 65 (1997).
[35] J. Coallier, M. Prévost, and A. Rompré, *Can. J. Microbiol.* **40,** 830 (1994).
[36] M. Prévost, A. Rompré, J. Coallier, P. Servais, P. Laurent, B. Clément, and P. LaFrance, *Water Res.* **32,** 1393 (1998).

and pH values >6.8 have been shown to affect CTC reduction negatively.[30,37,38] CTC has also been found to be toxic at concentrations of ≥5.0 μ*M*.[39] All of these studies assessed CTC reduction using planktonic cells.

Assessing Respiratory Activity Using CTC in Industrial Biofilms.[15] Biofilms were grown under the following conditions: (a) simulated cooling tower water (mixed culture of bacteria, algae, and 5 g of fresh soil in dechlorinated tap water), bentonite/kaolinite (50 mg/liter) was added as a slurry after 1–2 months of biofilm growth, and polyvinyl chloride (PVC) was used as the biofilm growth substratum. The biofilms were grown for approximately 13 months at 30° and pH 8.0. Biofilm thickness ranged from 2 to 5 mm. (b) Artificial seawater was fed continuously into an outdoor recirculating cooling tower. Biofilms were grown for 2 months on silicone tubing placed in the effluent line at 25–30°and pH 8.0. Biofilm thickness was less than 1 mm. (c) Biofilms from paper mill water (wood fibers, alum, rosin size, starch, and a polymer retention aid) were grown on the clear leg wall of a flotation device in an acid fine paper machine. Biofilms were grown for 3–4 weeks at 43–49° and pH 4.9. Biofilms were removed with a razor blade by gently scraping and lifting the biofilms from the substratum and immediately placing them on stainless-steel slides. Biofilm thickness ranged from 3 to 5 mm. (d) Polycarbonate coupons were placed in a treated wastewater effluent ditch. Biofilms were grown for 6 months at 5–25° and pH 7.0. Biofilm thickness ranged from 1 to 3 mm.

1. Collect biofilm coupons, slides, or samples and submerge in CTC (final concentration, 1.3 m*M*) for 2 hr at room temperature.
2. Fix the biofilms by submerging the coupon, slide, or sample in formaldehyde (5% v/v) for 5 min at room temperature.
3. Embed and cryosection the fixed biofilm as described earlier and view under fluorescence microscopy.

Thicker biofilms may be difficult to section due to abiotic material adsorbed to and embedded within the biofilm matrices. Also, the presence of autofluorescing bacteria and algae may make the assessment of intracellular CTC reduction difficult. Autofluorescence will increase as the thickness of the biofilm sections increases. Methods to reduce autofluorescence in biofilms have been described previously in this article.

Assessing Alkaline Phosphatase Activity in Phosphate-Starved Biofilms.[40] Biofilms of *K. pneumoniae or P. aeruginosa* were grown in a defined minimal medium with 1 g/liter of Na_2HPO_4 and 0.1 g/liter of glucose fo

[37] B. Pyle, S. Broadaway, and G. McFeters, *Appl. Environ. Microbiol.* **61,** 4304 (1995).
[38] J. Smith and G. McFeters, *J. Appl. Bacteriol.* **80,** 209 (1996).
[39] S. Ullrich, B. Karrasch, H. Hoppe, K. Jeskulke, and M. Mehrens, *Appl. Environ. Microbiol.* **62,** 4587 (1996).
[40] C. Huang, K. Xu, G. McFeters, and P. Stewart, *Appl. Environ. Microbiol.* **64,** 1526 (1998

96 hr at room temperature on 316L stainless-steel slides. Prior to the alkaline phosphatase assay, the phosphate concentration was lowered to 0.01 g/liter of Na_2HPO_4. Samples were collected prior to and after 8, 12, 24, and 36 hr of exposure to the low phosphate medium. The biofilm thicknesses ranged from 60 to 110 μm. A similar approach has been used to assess the effect of oxygen availability on the spatial heterogeneity in *P. aeruginosa* biofilms.[41]

1. Prepare the ELF-97 phosphatase substrate solution (Molecular Probes, Inc.) as described by the manufacturer.
2. Submerge the biofilm sample slide in the ELF-97 solution for 30 min at 37° in the dark.
3. To counterstain the sample, submerge the slide in propidium iodide (10 mg/liter) (Molecular Probes, Inc.) for 5 min at room temperature.
4. Fix the labeled biofilm sample with formaldehyde (1% v/v) for 5 min at room temperature.
5. Embed and cryosection the fixed biofilm sample as described earlier and view under fluorescence microscopy.
6. Areas of the biofilm that fluoresce yellow–green have active alkaline phosphatase activity. If the sample has been counterstained, cells in areas with no alkaline phosphatase activity will fluoresce red.

Assessing Respiratory Activity Using CTC and Membrane Potential Using Rhodamine 123 (Rh123) in Disinfected Biofilms.[42] Rh123 is an anionic, lipophilic, fluorescent probe that is distributed across membranes having a membrane potential.[7,8] Cells that have lost their membrane potential are unable to distribute and accumulate Rh123 and, as a result, are nonfluorescent.

Klebsiella pneumoniae cultures were grown in tryptic soy broth (TSB) (10% v/v) for 24 hr at 35°. A subsample of this culture (1%) was used to inoculate another TSB (10% v/v) culture and was incubated for 24 hr at 35° before being used as the inoculum for the biofilm reactor. The biofilms were grown in batch reactors on 316L stainless-steel slides using TSB (10% v/v) as the growth medium. Biofilms were grown for 36 hr at 25°. The thickness of the biofilm was not given. However, the probed samples were assessed directly using fluorescence microscopy, suggesting that the biofilms were monolayers or only a few cells thick prior to disinfection. This probing technique requires at least three biofilm sample slides for probing with CTC and Rh123 and total cell counts (DAPI).

[41] K. Xu, P. Stewart, F. Xia, C. Huang, and G. McFeters, *Appl. Environ. Microbiol.* **64,** 4035 (1998).
[42] F. Yu and G. McFeters, *Appl. Environ. Microbiol.* **60,** 2462 (1994).

A similar approach has been used with a *K. pneumoniae* and *P. aeruginosa* biofilm system.[43] In this study, biofilms were grown in a continuous flow annular reactor using a defined minimal medium supplemented with 20 mg/liter glucose. Biofilms were grown for 7–10 days at room temperature and ranged in thickness from 60 to 125 μm prior to disinfection. The biofilms were cryosectioned.

1. Gently rinse biofilm sample slides in sterile distilled water and then transfer to an acid-washed batch reactor containing chlorine demand free phosphate-buffered water and a magnetic stirring bar.
2. Add the disinfectants, chlorine (0.25 mg/liter, pH 7.2) or monochloramine (1.0 mg/liter, pH 9.0), to the stirred reactors. Record the temperature of the reaction system.
3. After a predetermined exposure time, remove the biofilm samples and submerge them in sodium thiosulfate [final concentration, 0.01% (w/v)] to neutralize the disinfectant.
4. For CTC probing, gently rinse the neutralized biofilm sample and then submerge it in CTC (1.3 m*M*) for 2 hr at 35°.
5. For Rh123 probing, gently rinse the neutralized biofilm sample and submerge in Rh123 [final concentration, 5 μg/ml in permeabilizing solution (50 m*M* Tris–HCl, 5 m*M* disodium EDTA, pH 8.0)] for 1 hr at 35°.
6. For total cell counts, gently rinse the neutralized biofilm sample and submerge it in DAPI and incubate as described earlier. DAPI can be used as a counterstain with CTC-probed biofilms. However, DAPI is not compatible with Rh123 and cannot be used as a counterstain. A separate biofilm sample must be labeled with DAPI, counted, and then compared to Rh123 data.
7. Using fluorescence microscopy, cells will fluoresce red within intracellular background blue fluorescence that has reduced CTC. Cells that have not reduced CTC will fluoresce blue. Cells that have accumulated Rh123 will fluoresce green.

Interpretation of Results

The term "viability" is commonly used to describe a wide range of physiological states and activities, as measured by a variety of methods in planktonic and biofilm systems. It is not surprising, therefore, that microbiologists frequently fail to agree on a unified definition for bacterial viabil

[43] C. Huang, F. Yu, G. McFeters, and P. Stewart, *Appl. Environ. Microbiol.* **61,** 2252 (19

other than demonstrable growth.[44–46] Hence, the interpretation and comparison of results from studies assessing viability is problematic. A contributing factor to this problem is the incorrect assumption that data from methods assessing different aspects of bacterial physiology (e.g., culturability and membrane potential) are equivalent. Often a single aspect of physiology is measured and conclusions are drawn as to the overall viability of the cell. A more sensible approach is to assess multiple indices of physiological activity, such as respiratory enzyme activity, membrane integrity, and membrane potential. This approach allows a more comprehensive assessment of which aspects of cellular physiology are affected by a given environmental stressor or treatment.

The application of fluorescent labels and probes to the assessment of physiological activity makes the multiple indices approach practical because of their sensitivity and ability to provide rapid results. Also, fluorescent labels and probes have the potential to provide information pertaining to the intracellular site and degree of lethal and sublethal injury,[27,47] unlike culture-based techniques.

[44] D. Roszak and R. Colwell, *Microbiol. Rev.* **51,** 365 (1987).
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[14] Deconvolution Fluorescence Microscopy for Observation and Analysis of Membrane Biofilm Architecture

By DON PHIPPS, GRISEL RODRIGUEZ, and HARRY RIDGWAY

Deconvolution Fluorescence Microscopy for Observation and Analysis of Membrane Biofilms

The ability of nutrients, biocidal agents, cleaning compounds, and other substances to penetrate sensitive targets within the biofilm matrix is controlled by biofilm composition and architecture. Consequently, detailed knowledge of the microscale morphology of membrane biofilms is essential for understanding the mechanisms of water and solute transport phenomena in separation membranes. Using scanning laser confocal microscopy,

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