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22 Study of Biofouling Control with Fluorescent Probes and Image Analysis

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This chapter discusses the use of various fluorescent probes in biofilm study. As fluorescence microscopy and digital imaging technology progress rapidly due to the development of high-speed personal computer processors, it will no longer be necessary to rely on a bulky super computer to do the complicated algorithmic calculations. Micrographs can be digitized, enhanced, and visualized beyond the limit of the human eye. Digital image processing has brought light microscopy into three-dimensional resolution, which is crucial if biofilm researchers are to understand the spatial distribution and heterogeneity among biofilms. Current progress in biofouling control, with the combination of fluorochromes, microscopy and image analysis is reviewed here.

INTRODUCTION

The existence of biofilms was first recognized in soil (Söhngen, 1913), and later studied in aquatic environments (Henrici, 1933; Zobell, 1943; Mekalanos, 1992). Since then much progress has been made in understanding the ecology and physiology of adherent microorganisms (Characklis and Marshall, 1990; Lappin-Scott *et al.*, 1992). The adhesion event exerts a profound effect on bacteria. Attachment alters their physiological processes (Costerton *et al.*, 1987; Davies and McFeters, 1988; Kölbel-Boelke and Hirsch, 1989), their surface structures (Rosenberg *et al.*, 1967; Costerton *et al.*, 1981; Costerton and Lappin-Scott, 1989; Marshall, 1992; Wolfaardt and Cloete, 1992) and their relationships to the bulk fluid (Wolfaardt *et al.*, 1992; Lau and Liu, 1993). Both biofouling and microbiologically influenced corrosion are phenomena that are linked to the existence, properties and activities of biofilms. Biofilm processes are manifested in many forms and are studied by researchers from a wide variety of disciplines. There are numerous industrial environments where corrosion and biofouling processes are potentially troublesome, including cooling water systems, storage tanks, water and wastewater treatment facilities, filters, piping, and drinking water distribution systems (Flemming and Geesey, 1990).

Control of biofilm problems has usually been attempted by the application of biocides in water systems. It was initially assumed that the disinfection kinetics of attached bacteria would be similar to those of their planktonic counterparts. Many industrial systems have experienced the inevitable problems caused by biofilms, even in the presence of an effective disinfectant residual. It is now recognized that strategies to control attached bacteria must be based on data generated from biofilm studies. Difficulties associated with studying surface-associated cells have hindered work on characterizing the activities of adherent bacteria, compared to the progress made with free-living bacteria. For instance, it is necessary to remove the cells from the substratum prior to the enumeration of viable bacteria attached to surfaces. Differences in physiological activity between attached and free-living bacteria (Fletcher, 1984) may explain the diverse susceptibilities and growth requirements after cells have been removed from the substratum. In addition, enumeration of viable bacteria by plate count (PC) methods may not detect all viable cells, particularly those injured by environmental stress (Camper and McFeters, 1979; McFeters *et al.* 1982; Roszak and Colwell, 1987). Also, detached bacteria that are aggregated may be problematic in the plate counting technique.

The conventional approach to biofilm study involves physically removing samples from the substratum, followed by biological and chemical analyses. Using this approach, a great deal of information on biofilm heterogeneity has been revealed from years of research; however, spatial patterns were neglected in the process. However, recent advances in optic technologies have enabled researchers to conduct non-destructive study on the spatial distribution, thickness and the physiological activities of biofilms and their responses to biofouling control agents. Furthermore, digital image processing has given light microscopy a three-dimensional resolution, which has lead to major advances in biofilm study and biofouling control. With the aid of high speed CPU chips, complicated logarithm calculations can be achieved with personal computer within seconds.

The purpose of this chapter is to review progress in combining fluorescent probes, microscopy and image analysis in biofouling control.

FLUORESCENT PROBES

Accurate detection and enumeration of bacteria is an important task in many areas of microbiological investigation. However, most of the methods commonly used to count bacteria in samples taken from natural environments have limitations (Daley, 1982). One example is the widely acknowledged failure of bacteria to form colonies on specific media due to the taxonomic (Atlas, 1984) and physiological (Roszak and Colwell, 1987; McFeters, 1989) diversity of microorganisms in most environments. Viable counts generated with plate enumeration from aquatic environments yield as little as 1 % of the total microscopic counts for bacterial isolates from freshwater (Servais and Menon, 1991). Microscopic enumeration showing various physiological activities within a microbial population has significant advantages over conventional methods.

An ideal fluorescent probe should allow the selective microscopic examination of particular activities or physiological properties of bacteria. Fluorochromes may bind

specifically to components of the cell envelope or contents, and some are modified by cellular activity. After binding or metabolism, the fluorochrome can be observed directly with epifluorescence microscopy using appropriate excitation and emission filters.

Acridine Orange

Acridine orange (AO), one of the nucleic acid intercalating dyes, was originally applied to stain bacterial cells for fluorescence microscopy (Rigler, 1973) in order to detect and enumerate bacteria. The method commonly employed by microbial ecologists is the AO direct count (AODC) technique, also known as the direct epifluorescent technique (DEFT). The unique metachromatic property of AO (Bitton *et al.*, 1993) led to the widespread application of AO to vital staining. AO fluoresces either green or red, depending on the nature of the binding reaction with nucleic acid. It was assumed that the color of AO-stained bacteria could be used to discriminate between active and inactive cells (van Es and Meyer-Reil, 1982; Lopez-Torres *et al.*, 1988; Menezes *et al.*, 1995), because AO bound to double stranded DNA fluoresces green while single stranded RNA-bound AO fluoresces orange. Actively metabolising cells would be expected to have higher RNA content and thus appear orange with AO staining. However, studies (McFeters *et al.*, 1991) have shown that the AO staining reaction may be suggestive of physiological activity only under defined conditions. Variables in staining and fixation procedures as well as uncertainties associated with mixed bacterial populations in environmental samples may produce results that are not consistent with the classical interpretation of this reaction. Hence, AO has limited application as a vital stain.

The direct microscopic enumeration method using AO (AODC) usually shows a reasonable correlation with viable counts when applied to exponentially growing cultures. However, AODC data can exceed PC enumeration by several orders of magnitude (Roszak and Colwell, 1987) when used to examine bacterial populations in natural environments. The direct viable count (DVC) method developed by Kogure *et al.* (1979; 1984), has been employed successfully in enumerating bacteria within environmental samples (Maki and Remsen, 1981; Xu *et al.*, 1982; Rollins and Colwell, 1986; Liebert and Barkay, 1988; Singh *et al.*, 1990). The DVC method was later applied as a direct *in situ* enumeration method for thin biofilms (Yu *et al.*, 1993), where the results indicated that this adaptation of the method can provide rapid (4h) as well as more accurate information regarding bacterial number and viability within biofilms.

However, using the DVC method on biofilm bacteria it would be difficult to enumerate the elongated cells without removing and dispersing the biofilm aggregate. Therefore, an *in situ* biofilm activity assessment might not be possible using the DVC method.

Fluorescein Diacetate

Fluorescein diacetate (FDA) is a fluorochrome conjugated to two acetate radicals. After FDA enters the cell membrane *via* active metabolism (Brunius, 1980), it is hydrolyzed by intracellular esterases then fluorescein is released from the compound. Fluorescein

has an absorbance at 490 nm, and fluoresces green when excited with blue light. The use of FDA has been suggested for the detection of microbial activity in bacterial suspensions (Jarnagin *et al.*, 1980; Chrzanowski *et al.*, 1984) and biofilms (Pawley, 1990; Safferman and Bishop, 1996; Battin, 1997). However, FDA hydrolysis might be limited to environments rich in eukaryotes and Gram-negative cells (Chrzanowski *et al.*, 1984). It has also been observed that fluorescein derived from cleaved FDA in bacteria tends to leak out under some physiological conditions.

Tetrazolium Salts

The reduction of tetrazolium salts to formazan has been used for many years in histo-, cyto-, and biochemical determinations of oxidase and dehydrogenase activities. Zimmermann *et al.* (1978) used the redox dye, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to study respiratory activity in aquatic bacteria. However, the reduced form of INT (INT-formazan) can only be observed within bacteria by light microscopy, which can not be applied to the study of biofilm bacteria on opaque substrata without removal of the cells. Although the combination of fluorescent-antibody (FA) and INT reduction has been applied successfully to the study of cellular activity (Baker and Mills, 1982), the tedious procedures of preparing and examining FA has limited this approach as a general application. Another redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has been applied successfully to the study of physiological activity within eukaryotic (Stellmach, 1984) and prokaryotic (Rodriguez *et al.*, 1992; Kaprelyants and Kell, 1993) cells. The *in situ* performance of CTC reduction was compared with the *in situ* DVC and PC methods in the determination of bacterial viability within pure culture biofilms. Both the CTC and DVC methods showed comparable numbers, and the results were two-fold higher than PC enumeration (Yu and McFeters, 1994a).

The CTC method has also been applied successfully to other biofilm research concerned with disinfection, culturability, and enzymatic removal (Stewart *et al.*, 1994; Yu and McFeters, 1994b; Pyle *et al.*, 1995; Johansson *et al.*, 1997; Kalmbach *et al.*, 1997).

Rhodamine 123

Rhodamine (Rh) 123 is a cationic fluorescent dye which is found to be concentrated in mitochondria by the relatively high negative potential across the energized mitochondrial membrane (Johnson *et al.*, 1981). Rh-123 is a proton motive force (PMF)-driven dye, which is only taken up by viable cells. In bacterial cells Rh 123 is accumulated in an uncoupler-sensitive fashion *via* transmembrane potential (Haugland, 1996). This fluorochrome has been utilized to assess the physiological states of *Micrococcus luteus* and *Escherichia coli* (Kaprelyants and Kell, 1992), *Salmonella typhimurium* (Mason *et al.*, 1995; Lopez-Amoros *et al.*, 1995) and *Aeromonas salmonicida* (Morgan *et al.*, 1993) by flow cytometry.

Rh 123 is not readily absorbed by Gram-negative bacteria because of a permeability limitation in their outer membrane (Nikaido and Vaara, 1985). However, treatment with Tris and EDTA at alkaline pH (Kaprelyants *et al.*, 1992) eliminates

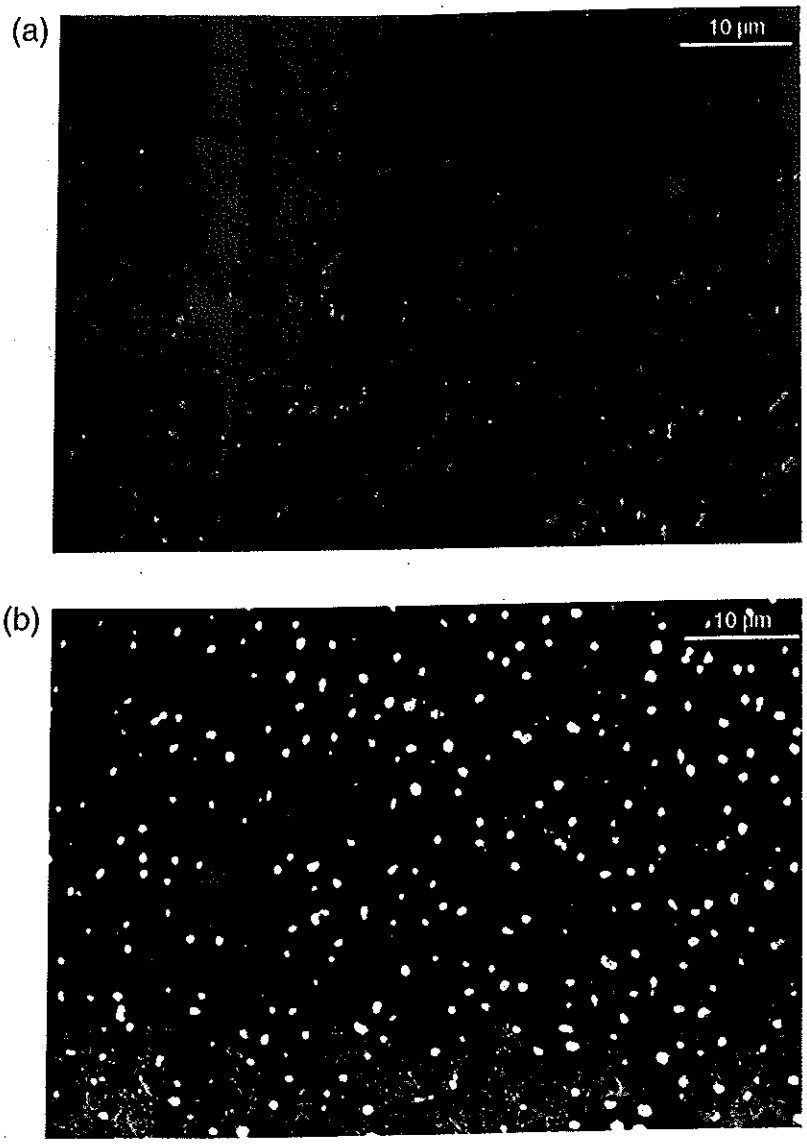

Figure 1. Epifluorescence micrographs of a *K. pneumoniae* biofilm on 316 stainless steel stained with (a) Rh 123 and (b) CTC formazan and DAPI (green), using a Leitz filter block H.

this barrier and achieves optimal staining within 2h. Both the Rh 123 and the CTC methods gave comparable enumeration results on biofilm bacteria (Yu *et al.*, 1994a). Figure 1 is an example of Rh 123 and CTC staining of a monolayer *Klebsiella pneumoniae* biofilm. Active biofilm bacteria appear green under epifluorescence microscopy using a Leitz filter block H (Figure 1a). The "H" filter block has a combination of excitation filter (BP420-490), dichromatic mirror (RKP 455) and suppression filter (LP515). Figure 1b shows good color contrast for both DAPI-stained green non-respiring cells and respiring cells that contain red CTC-formazan crystals.

Figure 2 *Bifidobacterium sp.* stained with the *Bac*Light probe. When incubated with the SYTO 9 and propidium iodide nucleic acid stains, live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red. (Image contributed by Bruce Roth and Paul Millard, Molecular Probes Incorporated.)

Live/Dead Viability Assay

The Live/Dead *Bac*Light viability assay (Molecular Probes Incoporated, Eugene, Oregon) has been successfully applied to study the susceptibility of bacterial biofilms (Korber *et al.*, 1997; Wood *et al.*, 1998) treated with antimicrobials. The *Bac*Light probe distinguishes live bacteria with intact plasma membranes from dead cells with compromised membranes (Haugland, 1996). Figure 2 shows *Bifidobacterium sp.* stained with the SYTO 9 and propidium iodide, where live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red. Two different nucleic acid stains are included; live bacteria fluoresce green by taking up the stain SYTO 9, and propidium iodine causes the dead cells to show red fluorescence. The *Bac*light assay also allows the end-user to vary the ratio of SYTO 9 and propidium iodine to give balanced staining of most samples. The bacterial fluorescence at each emission wavelength can be calibrated for quantitative assays using equipment such as a fluorometer, fluorescence microplate reader or flow cytometer.

Other dual staining approaches to distinguish viable from non-viable bacteria are the oxonol dye and calcolfluor white (Mason *et al.*, 1995) combination. The dye bis(1,3-dibutylbarbituric acid) trinmethine oxonol ($DiBAC_4(3)$) is a lipophilic anion that responds by decreasing fluorescence with increasing membrane potential. Calcafluor white (CFW) is the disodium salt of 4′,4′-bis(4 anilino-bis-diethyl amino-s-triazin-2-ylamino)-2,2′-stilbene dissulphonic acid); it is used as fluorescence brighteners in the dye industry. The viable cell can exclude CFW whereas non-viable cells have bright fluorescence.

Other Vital Stains

There are several proprietary fluorescent probes that have been applied to industrial cooling water biofilm study without revealing detailed mechanisms. The proprietary stain PRB is reported to be visualized under both light and epifluorescence microscopy (Chalut *et al.*, 1994). Active cells stained pink with a dark spot; inactive cells do not have a spot under bright field illumination. Both active and inactive cells appear red under epifluorescence illumination (Kogure *et al.*, 1980). Fluorochrome APY is reported to stain RNA but not DNA (Chalut *et al.*, 1994). Cells killed with chemical biocide would not be stained or else appeared distorted (Kogure *et al.*, 1980). Another fluorochrome CEB is specific for bacterial exopolymeric substances (EPS) (Chalut *et al.*, 1994). The slime layer containing EPS fluoresces neon blue, whereas the bacterial cells are yellow under the epifluorescence microscope (Chalut *et al.*, 1994).

Fluorochromes also have various applications in fluorescence conjugates and oligonucleotide probes. These research topics are not covered in this chapter.

IMAGE ANALYSIS

Image analysis is the process of manipulation by converting images through digital processing with computer, then analyzing with customized software (Figure 3). The process involves multidisciplinary elements of optics, electronics, mathematics and computer science. Image analysis contains two main components, *viz.* image acquisition and object analysis.

Image Acquisition

An image under the microscope is an object with two-dimensional spatial representation. The analog image can be acquired using a camera and processed by a frame grabber within the computer, then converted into a digital form. The digitized image is usually represented as a mathematical expression of $f(x,y)$, which is composed of numerous small rectangular picture elements called pixels. The dynamic range of pixel intensities varies, and the quality of the digitized form is dependent on this. For an 8-bit image, each pixel will have grey levels ranging from 0 to 255, which is an indication of the signal intensity or brightness. For a modern 32-bit operating system, such as Microsoft Windows® 98, the range will be from 0 to 1023.

Video Camera

A video camera, which is different from conventional still frame single-lens reflex film cameras, produces a continuous analog output of video images. These output signals conform to a specific video standard that varies around the world. The three main video types are 525-line NTSC (National Television System Committee) in the United States, 625-line PAL (Phase Alternating Line) in Europe and 625-line SECAM (Sequential Color a Memoire) in France. These standards vary in field rate (Hz), format ($H \times V$) and pixel rate (MHz). The most recent video cameras have replaced the tube

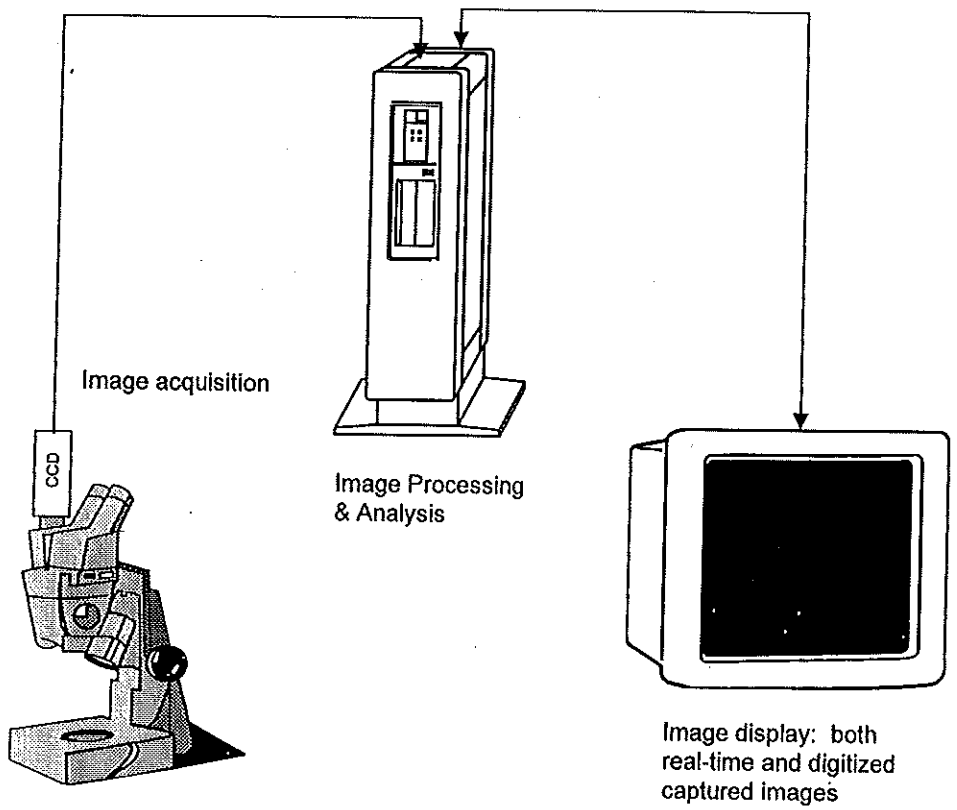

Figure 3 An image analysis system.

with a solid-state image sensor, called a charge-coupled device (CCD), that changes light energy into electrical pulses that can be recorded on videotape. The clarity and resolution of the video images depend on the number of pixels that the CCD can create. CCD devices can be divided into two groups, *viz.* video camcorder and scientific cameras. Scientific CCD cameras employ full frame CCD elements, the readout is processed through an on-chip pre-amplifier and an off-chip analog-to-digital converter to deliver a single high-quality digital image.

The CCD sensor is a monochrome imaging device. One way to record a color image is reconfiguration through three single color filters; a more advanced technology employs three CCDs to separate three color channels red, green and blue.

There is no standard for scientific cameras. They come with different options including pixel sizes, image dimensions, readout rate, integration time, cooling, quality grade and dynamic range (Castleman, 1996). These options determine the quality of image outputs. Since the computer and software can regulate shutter control, the common bleaching problem with fluorescence microscopy can be reduced to a minimum. It is not unusual for the captured images show higher resolution due to the image enhancement features of high-end cameras.

Image Analysis

Image analysis is accomplished by computer software to give quantitative data, such as the number of cells within a microscopic image, or the diameter or length of a selected bacterium. The output is the numerical data of the digitized image. The rapid development of the personal computer (PC) has allowed scientists today to process and analyze complicated images using a PC with specialized image processing boards. There are many sophisticated image processing packages in the market, which can be used without computer programming. Software such as IBAS (Kontron, Germany), Quantimet (Cambridge, UK), Visilog (Noesis, Canada) and Image-Pro Plus (Silver Spring, MD, USA) often provide similar functionality as image processing libraries, but in a much more user-friendly style.

The processed image in consecutive pixels can be stored as bytes in binary files. For example a 512 × 512 pixel image, stored at one byte per pixel, will take up 262 kilobytes (KB) of disk space. The most common output portable file formats are GIF (Graphics Interchangeable Format), TIFF (Tag Image File Format), JPEG (Joint Photographic Expert Group), BMP (standard Windows bitmap image), PCX (PC Paintbrush® file format), PDF (Adobe Acrobat® file format) and PICT (Macintosh graphics format). Of these formats, only TIFF images can be extended to include three dimensions.

BIOFOULING STUDY WITH IMAGE ANALYSIS

A great deal of work on biofilms has been done by removing bacteria from substrata and replacing them on glass slides for examination. Most structural studies have relied on light and electron microscopy (Kinner *et al.*, 1983; Robinson *et al.*, 1984; Costerton *et al.*, 1987; Eighmy *et al.*, 1983; Ganczarczyk *et al.*, 1992; Lappin-Scott *et al.*, 1992; Stewart *et al.*, 1993). Problems associated with these techniques include disruption of biofilm structure during removal from the substratum, laborious preparation, and extensive sample processing that may introduce artifacts. Light microscopy used in combination with computer-enhanced microscopy is an effective tool, but it is best applied during the early phases of biofilm development (Lawrence *et al.*, 1989). Visualization of monolayer bacterial biofilms can also be accomplished easily by either light or fluorescence microscopy (Yu *et al.*, 1993; 1994). Due to the resolution limits of optical microscopy, studies on thicker biofilms require mechanical removal of biofilms from the substratum prior to further analysis or optical sectioning by scanning laser confocal microscopy (SLCM).

Biofilms are highly heterogeneous in structure and physiological activity. Without understanding the spatial structure, composition and metabolic activity within biofilms, it is difficult to understand and control the processes of biofouling.

Scanning Laser Confocal Microscopy (SLCM)

The concept of confocal microscopy was first filed as a US patent in 1957 by Minsky (1961). In Minsky's embodiment of the confocal microscope, the conventional

condenser is replaced by a lens identical to the objective lens. The field of illumination is limited by a pinhole, positioned on the microscope axis (Pawley, 1990). The pinhole blocks the light from the out-of-focus planes above and below the plane of focus, which makes only the light from the plane of focus visible. This pinhole based confocal microscopy is applicable to both trans-illuminating and epi-illuminating modes. With the development of lasers in microscopy, the biological application of SLCM technology (Carlsson *et al.*, 1985; Amos and White, 1987; Carlsson and Åslund, 1987) was first published in 1985 and later introduced commercially by Srasstro, Biorad, Olympus, Zeiss and Leitz in the late 1980's. SLCM is also referred to as laser scanning confocal microscopy (LSCM), confocal laser scanning microscopy (CLSM) and scanning confocal laser microscopy (SCLM) in various publications.

SLCM technology has permitted major advances in biofilm research. The SLCM eliminates out-of-focus haze and allows horizontal and vertical optical sectioning. The reconstruction of images is based on optical sections that can be applied nondestructively to specimens in a matter of minutes. The systems are equipped with highly sophisticated image analysis capabilities that enable researchers to visualize reconstructed 2-D and 3-D images without physically disrupting the biofilm (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a; 1992b, Dalton *et al.*, 1994; de Beer *et al.*, 1994; Korber *et al.*, 1994; Stoodley *et al.*, 1994; Wolfaardt *et al.*, 1994; de Beer and Stoodley, 1995; Stewart *et al.*, 1995; Doolittle *et al.*, 1996, Müller *et al.*, 1996; Sanford *et al.*, 1996; Swope and Flickinger, 1996; de Beer *et al.*, 1997; Jayaraman *et al.*, 1997; Korber *et al.*, 1997; Neu and Lawrence, 1997; Okabe *et al.*, 1997; Lawrence *et al.*, 1998; Wolfaardt *et al.*, 1998). However, this technology involves relatively expensive instrumentation and has limited resolution when applied to thicker biofilms. These aspects restrict it from general application.

Cryoembedding and Cryosectioning

Cryoembedding and cryosectioning of human and animal tissues for light and fluorescence microscopy are well-established histological techniques (Troyer, 1980; Bancroft, 1982; Carson, 1990; Elias, 1990). Cryosectioning techniques have been utilized successfully in biofilm research to visualize the structure of bacterial biofilms(Yu *et al.*, 1994). Cryoembedding is performed with Tissue-Tek® OCT compound (Miles Incorporated, Elkhart, IN, USA) by placing the biofilm coupon on top of a dry ice slab. The embedded biofilm within the frozen OCT block can then be sectioned with a cryostat to yield 5 μm slices of biofilm cross-section.

Different embedding techniques have been applied to biofilms for measurement and evaluation of morphological parameters. Embedding media including paraffin (Lappin-Scott and Ganczarczyk, 1990), plastic resin (glycol methacrylate) (Ganczarczyk *et al.*, 1992; Stewart *et al.*, 1993) and agar (Ganczarczyk *et al.*, 1992) have been utilized. When combined with image analysis, the thin (2.5 μm) sections reveal quantitative data on biofilm heterogeneity such as thickness and size (Stewart *et al.*, 1993).

The cryoembedding technique involves less sample processing and is more rapid than any of other procedures. The whole process can be completed in less than 24 h. This method preserves the biofilm with minimal preparative artifacts. The advantage of the approach is that the biofilm cryosections can be examined with conventional

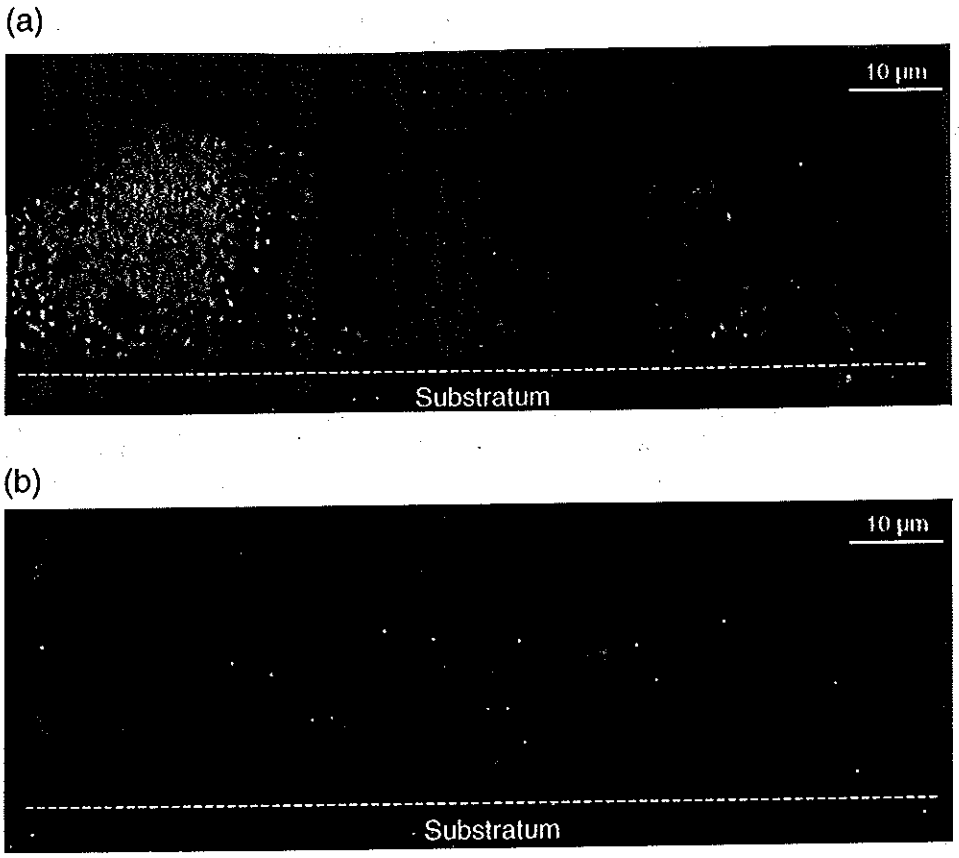

Figure 4 Epifluorescence micrographs of a frozen sections of a mixed *K. pneumoniae* and *P. aeruginosa* biofilm grown on 314 stainless steel and treated with monochloramine (4 mg l^{-1}). Disinfection intervals are (a) 30 min and (b) 60 min. The dashed lines indicate the position of the substratum. Biofilm bacteria were stained with CTC (red) and DAPI (green).

light and epifluorescence microscopy. The cryoembedding procedure is compatible with specialized staining or labeling techniques, such as fluorochromes (Yu *et al.*, 1994), immunofluorescence staining, oligonucleotide probing and radioisotope labeling. Fixatives and stains may also be applied to frozen sections after air drying (Bancroft, 1982; Elias, 1990). The cryosectioning technique offers an alternative, minimally disruptive approach to the study of biofilms by microscopy. Individual cells, microcolonies, void areas and biofilms thickness can be quantitatively determined by image analysis software (Murga *et al.*, 1995; Huang *et al.*, 1996; Wentland *et al.*, 1996). Figure 4 shows cryosections of a mixed *K. pneumoniae* and *Pseudomonas aeruginosa* biofilm. The biofilm bacteria showed decreased respiratory activity and thickness after treating with 4 mg l^{-1} of monochloramine. This approach also provides an unique opportunity to study the spatial response of biofilm bacteria to antimicrobial agents, and enables the mechanisms explaining their comparative resistance to disinfection

to be addressed in a way that has not been possible using traditional techniques (Huang *et al.*, 1995; McFeters *et al.*, 1995).

In a study comparing scanning electron microscopy (SEM), SLCM and cryosectioning techniques to visualize biofilm structural heterogeneity (Stewart *et al.*, 1995), SLCM and cryosectioning were shown to be superior to SEM in their ability to image the biofilm interior, and in their potential to provide quantitative information.

Digital Confocal Microscopy

Digital confocal microscopy (DCM) is a recent advancement in which digital signal processing (DSP) chips and software technology are combined to produce an image processing system capable of removing out-of-focus haze. The pinhole based hardware approach produces the analog confocal microscopy. Analog confocal microscopy, such as SLCM, produces sharp and clear images with a theoretical 1.4x improvement in resolution. However, the pinhole blocks 85% to 99% of the light, which limits its application under low-light conditions. In addition, SLCM systems are delicate and complex instruments that are often subject to alignment and laser stability problems (Beckwith and Margerum, 1997). The drawbacks of SLCM include the costs of the instrument and additional laser light sources.

DCM employs deconvolution algorithms to calculate and remove out-of-focus haze images captured with a standard research microscope and video camera. The process can be processed quickly with a PC under either IBM or Macintosh platforms. With a Z-axis stage motor and controller, DCM can de-blur a stack of microscope images and reconstruct a 3-D image of high quality (Kesterson and Richardson, 1991). The results are comparable to those produced by SLCM, but at a much more affordable cost.

The deconvolution approach of DCM is versatile. It can be used with a wide variety of specimens, such as paper fibers, computer chips and sandstone (Brading *et al.*, 1996). It is also valuable in applications that require a specific wavelength of light not available with the SLCM system, such as ultraviolet. It is gaining popularity in biological and microbiological research (Gorby, 1994; Richardson, 1997; Kunkier *et al.*, 1998) for visualizing structures.

With the availability of DSP chips, faster PC processors and image processing software, the DCM approach provides a powerful and useful tool with great versatility. However, DCM technology can only be used for epifluorescence applications in visualizing biofilm spatial distribution due to the difficulties involved with optical sectioning under bright ground illumination. The biofilm researcher may benefit from DCM technology since it is an affordable alternative to image analysis or SLCM. However, during recent years, with the increasing numbers of manufacturers of SLCM systems and image analysis software, these have become highly competitive.

In the future, biofilm research is moving towards the use of biosensors, chemical sensors, physical sensors and controllers that are characterized by merging concepts in classical disciplines like chemistry, biology, physics, medicine, computer and electrical engineering. The rapid development of these technologies will help in the better understanding of biofilms, and may lead to more effective biofouling control.

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