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Short Communication

CRYOSECTIONING OF BIOFILMS FOR MICROSCOPIC EXAMINATION

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A method for rapid and minimally disruptive embedding and sectioning of bacterial biofilms has been developed and applied to binary population biofilms of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* grown on stainless steel surfaces in continuous flow annular reactors. Biofilms were cryoembedded using a commercial tissue embedding medium. Frozen embedded biofilms could be removed easily from the substratum by gently flexing the steel coupon. Microscopic examination of the substratum surface after biofilm removal indicated that less than a monolayer of attached cells remained. Five µm thick frozen sections were cut with a cryostat and examined by light or fluorescence microscopy. The cryoembedding technique preserved biofilm structural features including an irregular surface, water channels, local protrusions up to 500 µm thick, and a well-defined substratum interface. The method requires minimal sample processing without dehydration or prolonged fixation, and can be completed in less than 24 h.

KEYWORDS: biofilm, cryosectioning, microscopy.

INTRODUCTION

Most structural studies of microbial biofilms have relied on light and electron microscopy (Kinner *et al.*, 1983; Eighmy *et al.*, 1983; Robinson *et al.*, 1984; Costerton *et al.*, 1987; Lappin-Scott *et al.*, 1992; Ganczarczyk *et al.*, 1992; Stewart *et al.*, 1993). Problems associated with these techniques include disruption of biofilm structure during removal from the substratum, laborious preparations, 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 bacterial monolayer biofilms can also be accomplished easily by either light or fluorescence microscopy (Yu *et al.*, 1993; Yu & McFeters, 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.

The development of scanning confocal laser microscopy (SCLM) enables researchers to visualize reconstructed 2-D and 3-D images without physically disrupting the biofilm (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a; Caldwell *et al.*, 1992b). SCLM eliminates

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out-of-focus haze and allows horizontal and vertical optical sectioning (Lawrence *et al.*, 1991). The reconstruction of images is based on optical sections that can be applied nondestructively to living tissue in a matter of minutes. This technology involves relatively expensive instrumentation and has limited resolution when applied to thicker biofilms. These aspects restrict it from general application.

Cryoembedding and cryotomy (cryosectioning) of human and animal tissues for light and fluorescence microscopy are well-established histotechniques (Troyer, 1980; Bancroft, 1982; Carson, 1990; Elias, 1990). Cryosections, commonly known as frozen sections, have medical applications in the rapid diagnosis of pathological tissue lesions found during surgical applications (Sawady *et al.*, 1988). This technique has also been utilized with immunoperoxidase and immunofluorescent stainings of tissues for diagnostic and research purposes (Troyer, 1980; Elias, 1990). This report describes the application of cryoembedding and cryosectioning techniques to visualize the structure of bacterial biofilms.

MATERIALS AND METHODS

Binary population biofilms of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were grown on 316L stainless steel slides in an annular reactor (Siebel & Characklis, 1991). The annular reactor was a continuous flow stirred tank reactor in which 12 removable slides were positioned around the inside wall of the outer drum. Minimal salts medium (van der Wende, 1991) with 20 mg·l⁻¹ glucose and phosphate buffered water were used to grow the biofilms. The reactors were operated at a dilution rate of 3.2 h⁻¹ to ensure that the growth of the planktonic cells was negligible. The effluent was sampled daily and plated on R2A (Difco) agar to monitor the growth of biofilm bacteria and to check for contamination.

The preparation of biofilm samples is illustrated in Figure 1. Biofilms were collected by withdrawing slides from the reactor and placing them in staining container with the biofilm side up. Formalin (5%, final concentration) was added to fix the specimens which were then immediately stained with 1 µg·ml⁻¹ 4', 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Company). The liquid was removed from the staining container after 5 min. Cryoembedding was performed with Tissue-Tek® OCT compound (Miles Incorporated) by placing the slide on top of a dry ice slab (Callis *et al.*, 1991), and immediately dispensing a thick layer of OCT on top of the biofilm. Alternatively, the specimen was allowed to infiltrate under the OCT layer for 10 min before transferring the slide onto dry ice. The embedded biofilm was allowed to rapidly freeze until the specimen turned opaque white. The specimen was then separated from the substratum by gently bending the slide to remove the frozen sample. The embedded biofilm was then turned over the embed the side that was previously attached to the substratum. The whole process was performed on dry ice to prevent the samples from thawing. The second step of cryoembedding created a layered frozen matrix in which biofilm was completely surrounded by OCT. The specimen was then wrapped in aluminum foil and stored at -70°C before cryotomy.

In order to determine the efficiency of biofilm removal by the cryoembedding technique, biofilm slides before and after cryoembedding were scraped, disaggregated and suspended in reagent-grade water. The suspension was then filtered onto a 25 mm diameter (0.2 µm pore size), black Nuclepore polycarbonate membrane (Costar). The total direct microscopic count procedure described by Hobbie *et al.* (1977) was used with minor modification. Because the biofilms were already stained with DAPI, staining with acridine orange was omitted.

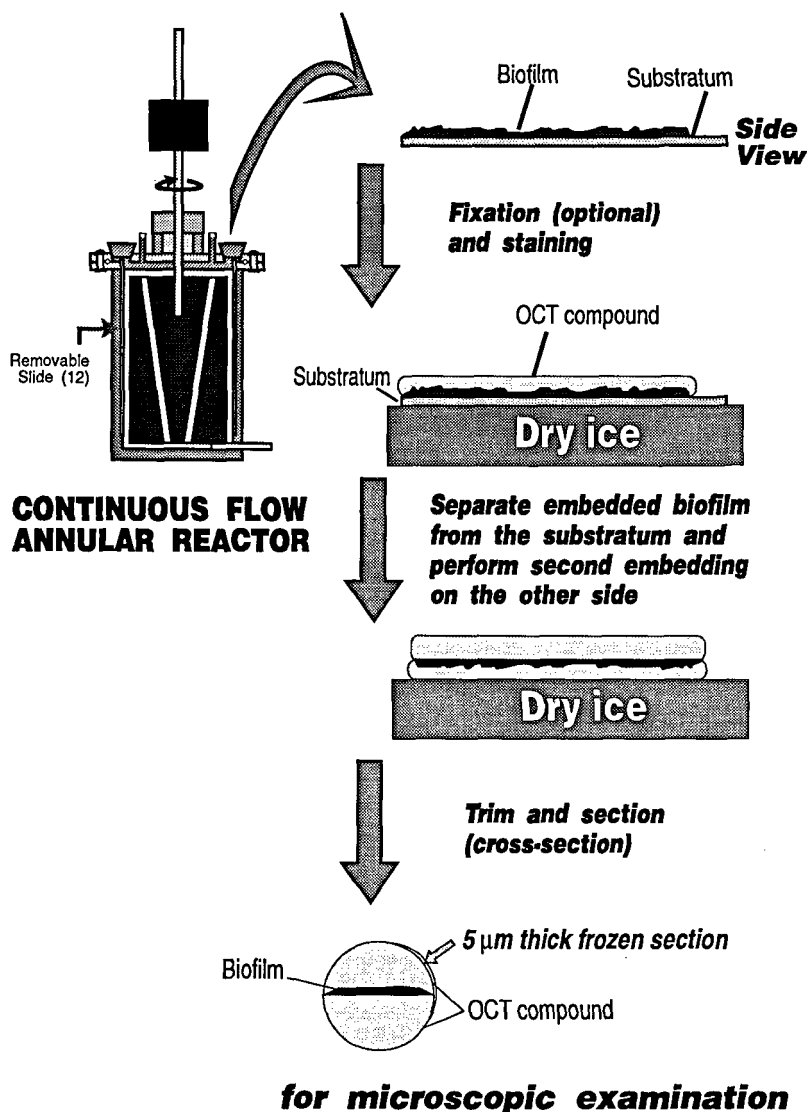


Fig. 1 Diagram of cryoembedding and cryosectioning procedures.

Frozen sections were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) operated at -19°C . The frozen specimen was placed in the cryostat for 15 min prior to sectioning to allow thermal equilibration. A small portion (2 cm long) of the matrix was bisected for mounting on a precooled (-19°C) specimen chuck. This was done by pouring a small amount of OCT on the chuck and pressing the specimen into the embedding medium. When the OCT solidified, the sample was fixed on the chuck and ready for sectioning. The specimen was oriented perpendicular to a disposable microtome blade (#815, Reichert-Jung, Leica). Trimming was necessary to create a smooth surface prior to cryosectioning. Each 5 μ m thick frozen section was collected on a glass slide for microscopic examination.

The sections were examined using an Olympus BH-2 microscope with epifluorescence illumination (100 watt mercury lamp). An Olympus U excitation filter cubic unit with excitation filter (UG-1), a dichroic mirror (DM 400) and a barrier filter (L420) were used for visualizing the sectioned biofilms.

RESULTS

Cryosectioning allowed visualization of cross-section images of biofilms with resolution to the cellular level. An example micrograph (Fig. 2) reveals the internal structure of a biofilm grown on a stainless steel substratum. The heterogeneous structure of the biofilm, which consists of microcolonies and water channel can be clearly seen in this specimen. Individual cells are easily resolved. There is some blurring of the image, presumably due to overlapping bacterial cells in the 5 μm thick section. The interface between the substratum and base of the biofilm is also well defined. The embedding technique yielded intact sections with equally good feature preservation for biofilm samples at variable thickness up to 500 μm . It should be noted that the thickness of the biofilm and the frozen section are different in orientation. The thickness of the biofilm is measured in the direction perpendicular to the substratum, whereas the 5 μm thick frozen slices were cross-sections of the embedded biofilm that were also perpendicular to the substratum. The thickness of the frozen sections was, therefore, in a direction parallel to the substratum (Fig. 1).

The cryoembedding technique cleanly removed the biofilm from the substratum. The average cell density on a slide before embedding was $3.72 \pm 1.70 \times 10^{11}$ cells·m⁻² (n = 3), when analyzed by scraping, disaggregation and total direct microscopic counting. After cryoembedding and removal the cell density was $1.66 \pm 1.41 \times 10^3$ cells·m⁻². Direct microscopic examination of slides before and after biofilm embedding and removal visually confirmed good removal efficiency (Fig. 3). When examined at high

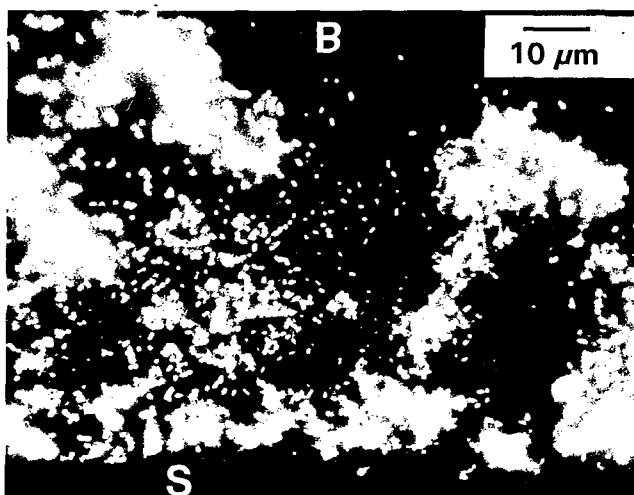


Fig. 2 Fluorescence micrograph of frozen section of a mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm grown on stainless steel. S = the location of the substratum and B = the location of the bulk fluid.

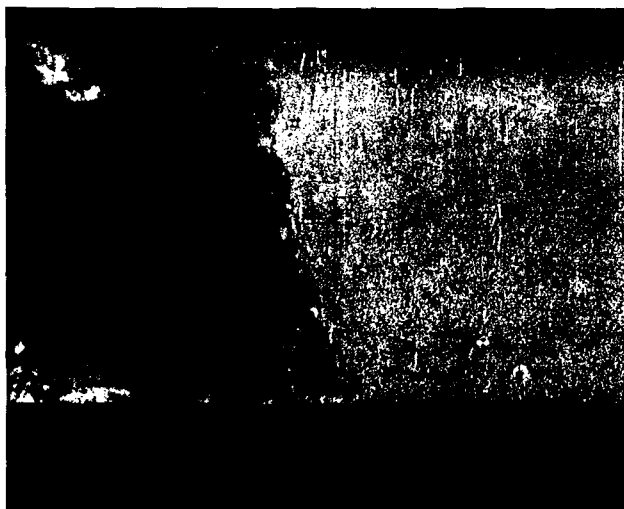


Fig. 3 Plan view of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm distribution on stainless steel slide before (left) and after (right) removal by cryoembedding. The width of the slide is 17 mm.

magnification, occasional cells or aggregates of cells were found on the substratum after embedding and removal. This indicates that while removal was good, it was not perfect.

DISCUSSION

The non-destructive structural analysis of biofilms has only recently been practical with the development of SCLM techniques. Preparation of samples for electron microscopy tends to cause morphological changes due to shrinkage during fixation and processing (Woldringh *et al.*, 1977). In addition, there have not been good procedures for cleanly separating biofilm from the supporting substratum. The cryoembedding and cryosectioning results described in this study demonstrated the application of a simple histological technique for the imaging of biofilm cross-sections with minimal disruption. The frozen sections of biofilm provided images of biofilm bacteria was maintained in a fully hydrated state during the cryoembedding procedure. The cell boundaries were clearly resolved without the shrinkage problems encountered in electron microscopy (Woldringh *et al.*, 1977). A major advantage of the cryosectioning technique is its ability to allow examination of the biofilm regardless of the type of substratum employed. The residual water left in slide mounted frozen sections tended to quench DAPI fluorescence and resulted in excessive bleaching. Therefore, overnight air drying is recommended before examining biofilm frozen sections by fluorescence microscopy.

The OCT compound used in this study to provide support for the biofilm during quick freezing and sectioning is a mixture of water soluble polymers, including polyvinyl alcohol and polyethylene glycol. It was originally developed to surround and support tissues during cryoembedding and cryosectioning. OCT maintained the intact structure of biofilm with minimal sample fixation and preparation. Quick freezing using dry ice minimized ice crystal formation in the specimen and allowed the embedded biofilm to be

separated easily from the substratum. Liquid nitrogen cooled isopentane was not recommended for this quick freezing process because the lower temperature (-150°C) creates difficulties during cryosectioning resulting in split sections which are hard to collect on microscope slides (Carson, 1990; Elias, 1990).

The timing of OCT addition was critical to ensure appropriate cryoembedding and eventually allowed a clean separation between the frozen biofilm and the substratum. The high viscosity of OCT tended to dislodge some of the biofilms when placed directly on unfrozen biofilms. In order to retain biofilm integrity, the slides were first placed on dry ice and OCT immediately added to the biofilm. This permitted an initial freezing of biofilm components (*i.e.* water molecules, exopolymers, and bacteria), which stabilized and strengthened the biofilm structure from artifacts caused by forces associated with the addition of OCT. However, if biofilms stayed in contact with dry ice too long before covering with OCT, the interface between OCT and biofilm became unstable and poor removal of biofilms resulted. Simultaneous embedding and freezing preserved biofilm structure while permitting effective removal from the substratum (Fig. 3).

Sometimes, a visible gap was seen between the two layers of OCT. This indicates a poor binding of OCT to the sample, which causes sections to split (Callis *et al.*, 1991). This could also be due to excessive water in the samples resulting in ice layers that shatter during sectioning (Troyer, 1980). The existence of inorganic corrosion products in certain biofilms might destroy knife edges, and could also result in difficulties in sectioning samples.

Difference embedding techniques have been applied to biofilms for measurement and evaluation of morphological parameters. Embedding media including paraffin (Li & Ganczarczyk, 1990), plastic resin (glycol methacrylate) (Stewart *et al.*, 1993; Ganczarczyk *et al.*, 1992) and agar (Ganczarczyk *et al.*, 1992) have been utilized. The cryoembedding technique described here involves less sample processing and is more rapid than any of these procedures. The whole process could be completed in less than 24 h. The cryoembedding procedure should be compatible with specialized staining or labeling techniques, such as double staining, 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 described here offers an alternative minimally disruptive approach for biofilm microscopy. Individual cells, microcolonies, void areas for biofilm microscopy. Individual cells, microcolonies, void areas and biofilm thickness were observed through the application of this technique. It is a simple, relatively rapid technique which required minimal sample preparation, and can be applied in different areas of biofilm research at reasonable expense.

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