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An optical microsensor to measure fluorescent light intensity in biofilms

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Abstract

We have developed an optical microsensor to quantify fluorescent light intensity distribution in biofilms. The optical system consisted of a beam splitter, light couplers, filters and a spectrophotometer able to accept the fiberoptic cable to measure fluorescent light intensity. The emitted light, fluorescence from the biofilm, was collected at the tip of the optical microsensor and was transferred to a spectrophotometer via a fiberoptic cable. The total fluorescent light intensity was evaluated from the emission spectrum by numerical integration. The newly developed fiberoptic microsensor was tested using a *Staphylococcus aureus* strain producing yellow fluorescent protein (YFP) grown as biofilm. We used a 405-nm violet laser diode for excitation, and measured the emission intensity between 480 nm and 540 nm. The optical microsensor that quantifies fluorescent light intensity is a promising tool in biofilm research which often requires detection and quantification of fluorescent light intensity distribution generated by various fluorescent proteins.

Keywords: Biofilm; Fluorescent light; *Staphylococcus aureus*; YFP; Fiber optics

1. Introduction

Detection and enumeration of microorganisms in various environments are important parts of microbiological investigations. Routine methods to identify microorganisms include activity measurements, and isolation and cultivation of pure cultures. Recently, specific markers, genes conferring specific phenotypes, have been used to detect and enumerate the microorganisms. In 1994, a novel marker system for the bacteria, the green fluorescent protein (GFP), became available (Chalfie et al., 1994). Following Chalfie et al. (1994), fluorescent proteins have been used as markers of microorganism in microbiological research (Zim-

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mer, 2002; Toomre and Manstein, 2001; Belmont, 2001; Wouters et al., 2001; Errampalli et al., 1999). Derivatives of GFP (i.e., yellow fluorescent protein, YFP) have been also used to detect microorganisms in environmental samples. The emitted light is the marker of the microorganisms; it can be used to find their location, quantify their numbers and monitor their activity. Often fluorescent light intensities (such as GFP or YFP) are used to quantify planktonic microbial growth (Wolf et al., 2003; Ju et al., 1995; Li et al., 1991).

When microorganisms grow in suspension, it is easy to quantify the fluorescent light intensity because the excitation and the emission of light can be directed to or be detected from different locations. However, when microorganisms grow as a biofilm, it is difficult to determine fluorescent light intensity because the intensity changes with distance in the biofilm and the excitation and emission must be done from the same location. To detect fluorescent light intensities in biofilms, confocal scanning laser microscopes (CSLMs) are routinely used. Although CSLMs are indispensable in biofilm research, they are expensive and require skilled operators. Because of physical constraints, biofilm samples are often removed from the reactors and then mounted on the microscope stage for CSLM analysis which may alter biofilm structure. The goal of this study was to develop an alternative measurement of fluorescent light intensity in biofilms, an optical microsensor. Such a sensor would alleviate some of the difficulties associated with using CSLM, would be portable, would be relatively easy to use and be more affordable than CSLMs.

In this study, we have constructed a novel fiber-optic microsensor with a tip diameter less than 10 μm to measure fluorescent light intensity distribution in biofilms. To demonstrate its utility, we tested the microsensor in a biofilm of a yellow fluorescent protein producing strain of *Staphylococcus aureus*. A 405-nm laser diode was used to generate excitation light and an optical spectrophotometer was used to measure the emitted light. This sensor had an immediate application in our work with biofilms and we pursued this to demonstrate its utility in the research on critical environmental conditions associated with toxic shock syndrome toxin-1 (TSST-1) expression in *S. aureus* biofilms. It is believed that dissolved oxygen and carbon dioxide concentrations play a critical role in TSST-1 production (Yarwood and Schlievert, 2000). However, to find the relationship between TSST-1, carbon dioxide and dissolved oxygen concentrations, it is critical to measure all three components at the same location in biofilms. Therefore, using the newly developed fiberoptic sensor, we measured transcriptional regulation of *tst* (the gene for TSST-1 production) using a *yfp* reporter which can be correlated to toxin concentration.

2. Materials and methods

2.1. Optical sensor

A schematic diagram of the optical microsensor is shown in Fig. 1A. A violet laser diode at 405 nm (5-mW CW power, single transverse mode; Nichia Chemical Industries) was used as a light source. The laser beam was reflected by a beam splitter (LWP-30-RS325-TP400-PW-1012-UV, CVI Laser) and focused onto the front end of the fiber by a lens. A single-molded glass aspheric lens (C230TM-A 350–600 nm 0.55 NA moderate size aspheric lens; Thorlab; Newton, NJ) was used to collimate the light from the laser diode without introducing aberrations. We used a multimode fiberoptic cable (140 μm/62.5 μm; Corning) to construct optical microsensor. The scanning electron microscopy (SEM) micrograph of the tip of the microsensor was shown in our previous publication related to fiberoptic microsensors to measure back-scattered light in biofilms (Beyenal et al., 2000). Tapered tips were formed using a variation on previously described techniques (Beyenal et al., 2000). The fiber tip was mechanically stripped, cleaned with isopropyl alcohol and cleaved. The fiber was held vertically in a precision linear positioner and was lowered into unstirred 37.5%-concentrated hydrofluoric acid. After etching the tip for 15–60 min at room temperature, the fiber was removed and rinsed in deionized water.

The fluorescent light from the biofilm was collected at the fiber tip and was passed through the beam splitter and through a long-pass filter with a cutoff wavelength at 450 nm (Colored Glass Filter, Rolyn Optics; Covina, CA) and collimated by same lens. The collimated light power was around 250 μW at the

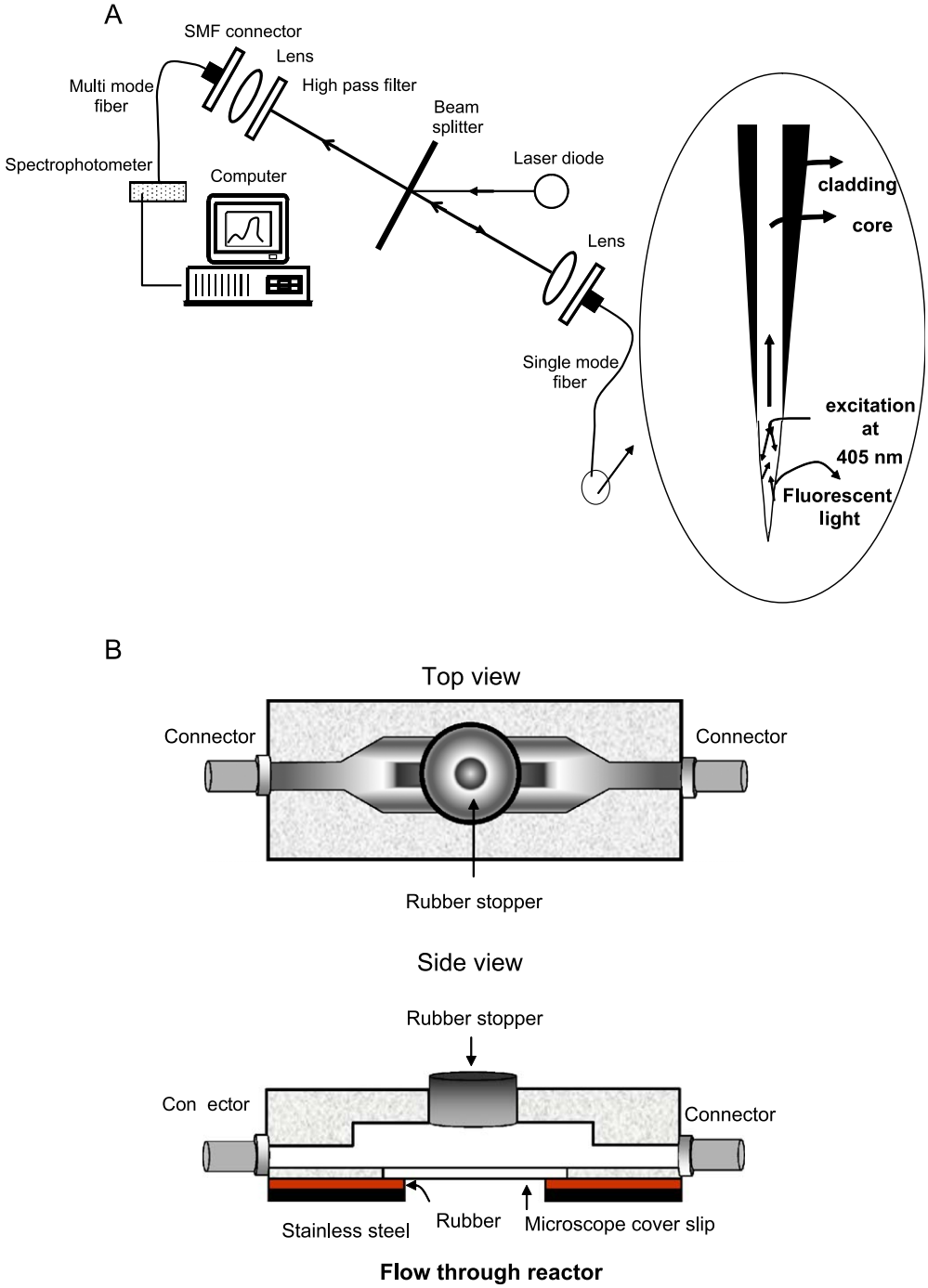

Fig. 1. (A) Schematic diagram of the optical microsensor and (B) the flow through reactor used to grow biofilms.

tip of the microsensor with 50% coupling efficiency, and we set the laser power to 500 μW during measurements. The fluorescence signal was detected by fiber-optic spectrometer (SD2000 by Ocean Optics; Dunedin, FL). The spectrophotometer was connected to a computer for automatic data collection. The sensitivity of the spectrophotometer, estimated by the vendor, was 86 photons/count.

The detection limit of fiberoptic sensors used to measure fluorescent light intensity depends on the characteristics of the fiberoptic cable (single-mode versus multimode, tip diameter of the sensor), characteristics of the spectrophotometer (signal to noise ratio, sensitivity), characteristics of the sample (transparent versus opaque, auto fluorescence of the sample, nonspecific hybridization of the fluorescent probes), intensity of the incident light, quality of the optics used to build the optical system (type of material and diameter of the fiberoptic cable, quality of the beam splitter) and on the operational parameters of the optical system (time selected to integrate the photon count). Therefore, the detection limit of the sensor is affected by many factors which may vary from one system to another, and from one sample to another. To simplify matters, in our measurements we referred to the specification of the spectrophotometer provided by the vendor to address the detection limit in our system and to select the operational parameters for our system. According to the vendor, the lowest count of photons that our spectrophotometer could register was 40 photon counts. Knowing that, we used 3-s integration time which was sufficiently long to register a strong signal in the presence of the species expressing YFP (Fig. 4) or a very weak signal, below the detection limit, in the absence of YFP (Fig. 3). However, the 3-s integration time which worked well in our system, may not necessarily be the best choice when using optical systems made of different components than those we used. The integration time used for the photon count should be long enough to give a meaningful readout and to assure satisfactory signal to noise ratio.

The spectra were analyzed using a custom-written software using Ocean Optics software and Resource library. The custom-written software calculates the light intensity for a given range of wavelengths. For our application, we measured total light intensities from 480 to 540 nm. The measured florescent light intensities were normalized by dividing each measured value by the maximum value (measured from the profiles) and presented as relative fluorescent light intensity.

2.2. Growing biofilms

The utility of the microsensors were demonstrated by measuring fluorescent light intensity profiles in biofilms of *S. aureus* which produced yellow fluorescent protein (YFP). The biofilm was grown in a growth medium made of 1% glucose, 10 μg/ml chloramphenicol, 1/50 Luria–Bertani (LB) medium and 0.200 mM of dissolved oxygen. The reactor (Fig. 1B) was inoculated with a 24-h-old culture of the microorganism, and the biofilm was grown statically for 24 h. Flow of the growth media at 30 ml/h was then initiated and continued for 3 days in a one-pass flow-through flat plate reactor at 37 °C. The reactor was placed in a custom-designed temperature-controlled chamber and was mounted on an Olympus CK2 inverted microscope. The biofilm structure was monitored through a transparent window (cover slip) in the bottom of the reactor (Fig. 1B). This arrangement also allowed for the monitoring of the position of the tip of the microsensor when fluorescent light intensity profiles were measured.

2.3. Bacterial strains, plasmids and construction

The strains and plasmids used are described in Table 1. Planktonic cultures of either *S. aureus* or *Escherichia coli* were grown in Luria–Bertani (LB) broth or plated on LB agar with the appropriate antibiotics for plasmid selection or maintenance (chloramphenicol, 10 μg/ml; ampicillin, 100 μg/ml) and were incubated at 37 °C. For flow-cell experiments to test strains, we used a medium containing 2% LB and 0.1% glucose.

In this work, we used two strains of *S. aureus* biofilms: (1) AMD238 which does not produce YFP and (2) AMD226 which produces YFP when TSST-1 is produced. We used AMD238 for control experiments and for measuring fluorescent light intensity profiles in AMD226 biofilms.

The PCR primers used are listed in Table 1. Generally, we used *E. coli* throughout plasmid

Table 1
Strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant characteristics	Source or reference
S. aureus		
MN8	*tstH*$^{+}$, clinical isolate from nonmenstrual TSS case	(Schlievert and Blomster, 1983)
AMD226	MN8 containing *p*DB48 plasmid (*tst-yfp*)	
AMD238	MN8 containing *p*DB39 plasmid (control)	
AMD265	MN8 containing *p*DB59 plasmid (RNAIII-*yfp*)	
*p*CR2.1-TOPO	TA cloning vector	Invitrogen
*p*KEN-GFPmut2	gfp_{mut2}	(Cormack et al., 1996)
*p*RSET-10B	yfp_{10B}	amino acid substitutions
*p*DB39	*p*UC18/*p*C194 shuttle vector containing *Bam*H1–*Bgl*II yfp_{10B} fragment from *p*DB44	This study
*p*DB44	pCR2.1–TOPO containing yfp_{10B} PCR-amplified from *Bam*H1-digested *p*RSET-10B, yfp_{10B} contains *gene10* RBS (O93 and O97)	This study
*p*DB48	PCR-amplified (O102 and O103) *tst* promoter region from MN8 cloned into *Bam*HI-digested *p*DB39, RNAIII-yfp_{10B}, Em^{R}	
*p*DB59	PCR-amplified (O110 and O111) *agr* P2–P3 region from MN8 cloned into *Bam*HI-digested *p*DB39, RNAIII–yfp_{10B}, Em^{R}	This study
Primer (5′ –3′)		
O93	ATGGGAAGCTTCGTGGATCCTCTAGATTTAAG	
O94	GTTCTTCTCCTTTACTCATATGTATATCTCC	
O97	CGCTGGCAGATCTTTATTATTTGTATAGTTCATCCATGCC	
O102	TGTCGACGGACGTTTCAGCGTAAAAAAC	
O103	TGGATCCGTAGCGATTGTCGCAAGCAAC	
O110	GGATCCACCACTCTCCTCACTGTTATTATACGA	
O111	AGATCTTTTTCCATCACATCTCTGTGATCTAGT	

construction. The plasmids were used to transform *S. aureus* RN4220, and RN4220-modified plasmids were used to transform *S. aureus* MN8. In all cases, *S. aureus* was transformed by electroporation (Schenk and Laddaga, 1992).

The reporter plasmid *p*DB48 contains a fusion of the *tst* promoter region of *S. aureus* strain MN8 with yfp_{10B} (a gene coding for a bright variant of GFP). The *tst* promoter fragment extends 293 bp upstream of the start codon. The yfp_{10B} gene contained a T7 *gene10* Shine–Delgarno sequence (SD), created as follows: The *gene10* SD from *p*KEN was PCR-amplified using primers O93 and O94. This PCR product was then used as a primer in a second PCR reaction, together with primers O93 and O97, and *p*RSET-10B as template, to amplify yfp_{10B}. The fragment was cloned into a *p*UC18/*p*C194 shuttle vector to form *p*DB39. The *tst* or the RNAIII promoters were placed into *p*DB39 to create *p*DB48 and *p*DB59.

2.4. Measurements in biofilms

The fiberoptic microsensor was mounted on a micromanipulator (Model M3301L, World Precision Instruments; New Haven, CT) equipped with a stepper motor (Model 18503, Oriel; Stratford, CT) controlled by the Oriel Model 20010 electronic interface. Microsensors were introduced from the top of the reactor, perpendicular to the biofilm. The micropositioner controlled the microsensor movement through a computer-interface controller (CTC-283-3, Micro Kinetics), with a positioning precision of ± 0.1 μm. Custom software was used to control and to coordinate the microsensor movement and the data acquisition. Details of the measurement technique have been previously described (Beyenal et al., 2000).

The microsensors in the biofilm were observed via an inverted microscope; the sensor was introduced from the top and its position was monitored through the bottom. The fiber tip was stepped into the biofilm

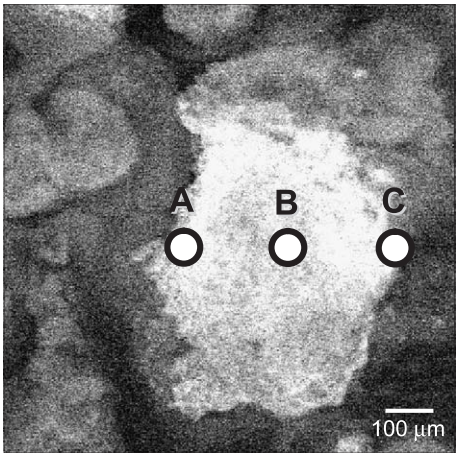

Fig. 2. CSLM image of the *Staphylococcus aureus* biofilm with marked measurement locations. Bright areas represent cell cluster and dark areas interstitial void. Locations A and C are located near the edge, while B is located in the middle of the cell cluster.

in 20 μm increments, and the data were recorded. The sampling average time was approximately 3 s, and multiple data points were collected at each vertical position. The measurements were performed in a cell cluster marked at locations shown in Fig. 2.

The measurements were repeated three times using three different biofilms, and all profiles of light intensity exhibited the same trend. Light intensity profiles in each AMD238 biofilm looked like the profile shown in Fig. 3, with undetectable YFP. Light intensity profiles in each AMD226 biofilm looked like the profile shown in Fig. 4, with peak in light intensity

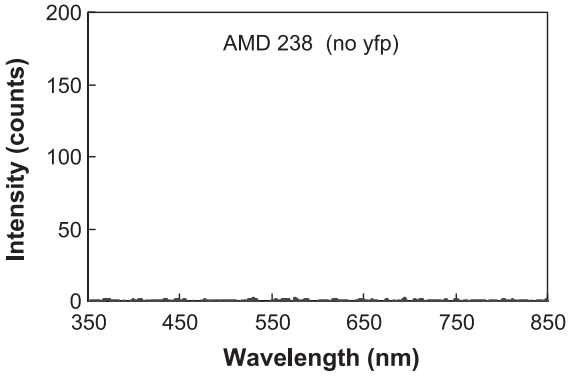

Fig. 3. Emission spectrum measured in AMD238 biofilm. The spectrum was recorded in the middle of a large cell cluster (similar to that shown in Fig. 2).

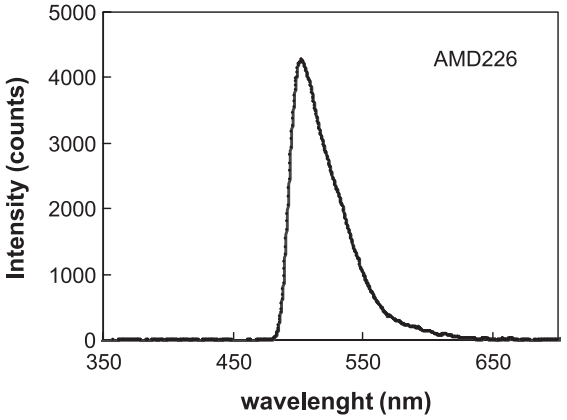

Fig. 4. Emission spectrum measured in AMD226 biofilm. The spectrum was measured in the middle of a large cell cluster (similar to that shown in Fig. 2).

in the middle of the biofilm. Additionally, all light intensity profiles measured in AMD226 biofilm showed maxima at the same wavelength, although the heights of the peaks varied from one biofilm to another. To determine if the fiberoptic sensor measured light intensity reproducibly, we repeatedly measured light intensity at a selected location in a biofilm. The results were almost identical and the sensor showed no visible drift in the measured response during the time of the measurements.

3. Results and discussion

The emission spectrum in Fig. 3 shows no detectable fluorescent light from the non-*yfp*-producing strain of *S. aureus* (AMD238), as expected, because AMD238 contains the plasmid *p*DB39 which does not have a functional promoter in front of *yfp*. Fig. 3 shows also that there was no detectable autofluorescence light (at 405-nm excitation) in AMD238 biofilms.

Fig. 4 shows the fluorescent light spectrum from AMD226 biofilms with a peak at 505 nm. Because most of the fluorescent light emitted was between 480 and 540 nm, we calculated total fluorescent light intensity between these wavelengths for the measurements in the biofilms.

The fiberoptic YFP sensor was constructed using a laser diode with peak emission at 405 nm. Report-

edly, *yfp* excitation occurs between 350 and 530 nm, with the peak between 500 nm and 520 nm (http://www.bdbiosciences.com/clontech/archive/OCT99UPD/RFP.shlml) which positions the excitation peak of YFP rather far from the emission peak of the laser diode we used. The main reason we decided to use the peak emission at 405 nm was availability of laser diodes which are not available at every wavelength. An alternative to using the laser diode was to use a laser at 488 nm which had peak emission spectra closer to the generally accepted position of the peak YFP excitation. However, lasers have much wider beams which are difficult to couple to the end of the fiberoptic cable. Therefore, using a laser diode was critical because the laser diode provide a very small beam size which increased light coupling efficiency and light intensity at the fiber tip. Since we demonstrated that the 405-nm laser diode worked well (Figs. 3 and 4), although it was working of the edge of the YFP excitation spectrum rather than at its peak, we used it and benefited from its use by separating excitation and emission spectra. Using any device with an excitation peak around 500 nm would have positioned the emission peak very close to the excitation peak of YFP (between 520 and 535 nm) which could potentially create analytical problems. When the excitation and emission peaks are overlapping, the sensitivity of the measurements is lowered. The nature and magnitude of this problem can be deduced from inspecting the YFP emission spectrum in Fig. 4, which is rather wide and does not cover the peak excitation at 405 nm. Therefore, using an excitation wavelength which peak at 405 nm, which is far away from the peak of the emission spectrum, helps separating the emission and excitation spectra.

The relative fluorescent light intensity profiles in AMD226 biofilm at the marked location in Fig. 2 are shown in Fig. 5. Maximum fluorescent light intensity was observed for the location C which was close to the edge of the cell cluster. The fluorescent light intensities reached maxima around 150 μm from the bottom at each location. Correlation of *tstH-yfp* reporter expression and production of the TSST-1 protein has been confirmed (Yarwood, personal communication). Thus, we expected that these relative fluorescent light intensity profiles measured in biofilms should correlate with TSST-1 concentration as well. TSST-1 can be produced under critical concentrations of carbon dioxide and

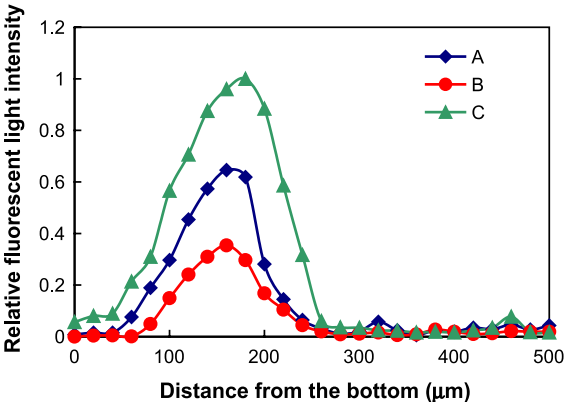

Fig. 5. Relative fluorescent light intensity profiles measured in AMD226 biofilm. The profiles were taken at the marked locations in Fig. 2. The legends A (♦), B (●) and C (▲) refer to the measurement locations shown in Fig. 2. Fluorescent light intensities were normalized by dividing each data value by the maximum value.

dissolved oxygen concentrations (Yarwood and Schlievert, 2000). The decrease in fluorescent light intensities near the bottom of the biofilm may have been caused by the lack of oxygen that has been shown essential for folding of the *yfp* chromophore. We expect higher oxygen concentration at locations C and A because they are close to adjacent interstitial voids. Combined measurements of dissolved oxygen, carbon dioxide concentrations and fluorescent light intensity profiles can be used to determine the critical conditions for TSST-1 production. This will be one of future applications of our fluorescent light intensity measuring microsensor.

3.1. Potential applications of the microsensor

The developed optical microsensor was tested using a biofilm containing *S. aureus* expressing YFP. However, because the excitation and emission profiles of *yfp* and *gfp* are within the bandwidth of the instrument, the sensor can be used as well to detect GFP distribution in biofilms. Because the detected fluorescent light can be quantified as an intensity spectrum, we believe it can be used to quantify different fluorescent proteins simultaneously. The only disadvantage we see, when comparing to CSLM, is that CSLM focuses on a focal plane and shows the distribution of the fluorescent protein over a certain area, while optical microsensors produce

vertical profiles of the fluorescent light over a small volume.

There are many potential applications of the newly developed optical microsensor. For example, fluorescent protein producing microorganisms are common in environmental research, e.g., to quantify mineral–microorganism interactions. In addition, for other applications, the laser diode we used can be replaced with a different diode. It is possible to replace the optical filters and the beam splitter to match the measured fluorescent light and its source proteins. Our study introduces fluorescent light intensity measurement in biofilms with a fiberoptic microsensor, tip diameter less than 10 μm, which deliver excitation light and collect emission light at the same location.

4. Conclusions

An optical microsensor was designed and applied to measure fluorescent light intensity profiles and was tested in *S. aureus* biofilms. The sensor was sensitive enough to detect the fluorescence produced by the proteins expressed by the microorganisms, and was small enough to be used for probing biofilms without damaging their structure.

Acknowledgements

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