Note

Quantitative Comparison of the Autofluorescence of Bacteria and Polystyrene Microspheres under Violet Wavelength Excitation for Verification of Fluorescence-based Bioaerosol Detector Results

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The autofluorescence intensity of bacteria and fungal spores was quantified by fluorescence microscopy in order to obtain the information for evaluating fluorescence-based bioaerosol detectors and was comparable to that of some types of polystyrene microspheres (PSMs). Although the intensity for microbes was distributed across a wide range over an order of magnitude in gray scale, it was in the intensity range of certain PSMs. Furthermore, some of those bacteria and PSMs were aerosolized in a test chamber and the fluorescence intensity was measured with a bioaerosol detector. Although there was a slight difference in the order of intensity from the results obtained by fluorescence microscopy, the fluorescence-based bioaerosol detector showed the intensity was in a comparable range.

Key words : Bioaerosol / Autofluorescence / Quantification / Polystyrene microsphere.

Microbial autofluorescence is a phenomenon that has been known since at least the first half of the 20th century (Darken, 1961). It is thought to be derived from nucleic acids, aromatic amino acids within proteins, NAD(P)H, flavins, chlorophyll, and fluorescent pigments of secondary metabolic products. In fermentation engineering, the microbial metabolic state can be monitored by measuring the autofluorescence intensity, particularly the fluorescence originating from NAD(P)H (Scheper et al., 1987; Scheper and Shügel, 1986). In diagnostic research, autofluorescence has been used in detection of the macrophages infected by Mycobacterium (Patiño et al., 2008). Recently, it has also been used in bioaerosol detection, leading to the development of fluorescence-based bioaerosol detectors (Hill et al., 1999; Pinnick et al., 1995).

Bioaerosols have been studied in the fields of environmental and industrial health, and in the quality control of pharmaceuticals and food products (Albrecht et al., 2007; Bernasconi et al., 2010; Pacheco and Pinto, 2010; Schlosser et al., 2009). In particular, bioaerosol monitoring is strictly required by regulations such as ISO 14644-1; it is important to monitor and control the air quality of the manufacturing environment in the pharmaceutical and food industries in order to avoid contamination of products by bioaerosols. Bioaerosol detection has been conventionally performed by the culture method, and various types of air samplers are commercially available for this purpose (Jensen et al., 1992). Recently, rapid methods for bioaerosol detection based on fluorescent dye staining, esterase activity, and autofluorescence of microbial cells as described above have been developed (Hernandez et al., 1999; Miller et al., 2009; Nocker and Camper, 2009; Smith et al., 2010).

The Model 3314 Ultraviolet Aerodynamic Particle Sizer® (UV-APS; TSI Inc., St. Paul, MN), BioLaz™ (Particle Measuring Systems, Inc., Boulder, CO), and IMD-A™ (Azbil BioVigilant, Inc., Tucson, AZ), etc. are commercially available fluorescence-based bioaerosol detectors. These detectors rely on particle detection based on elastic scattering or time-of-flight, and fluorescence detection by laser-induced fluorescence from bioaerosols. Thus, these detectors can simultaneously distinguish a bioaerosol from a non-biological particle using autofluorescence as a marker. Such detectors detect the total emission from a...
cell, not the emission at a specific wavelength of a targeted intracellular fluorophore.

Often when measuring biological samples, the target is fragile and may be a chaotic system of which many features remain unclear, so that the measured values are sometimes variable and in flux; thus, these measurements tend to be focused on the qualitative character behind the measured value itself rather than the quantitative performance. In that case, it is important to confirm whether we would reach the same conclusion by other methods based on different principles. The detection ability of fluorescence-based bioaerosol detectors has been compared with the culture method using air samplers (Brosseau et al., 2000), but there have been few reports validating bioaerosol detectors from a viewpoint of fluorescence sensitivity and in comparison with other fluorometric methods different from those used in the fluorescence-based bioaerosol detectors. We think the reason is that there are few methods for measuring the weak fluorescence from micron-size single particles and that almost no stable, artificial particle with weak fluorescence that can be used as a reference particle is currently known because the quantitative autofluorescence intensity of microorganisms is unclear. For this reason, only comparison with the culture method is possible at present; however, detectors should be evaluated not only by the comparison with the culture method, but also by other methods focusing on its fluorescence sensitivity. The purpose of this study is to demonstrate objective information of microbial autofluorescence intensity comparing with that of polystyrene microspheres (PSMs) in order to obtain reference particles with weak fluorescence that can be used in the verification of fluorescence-based bioaerosol detectors and other fluorescence-based microbial detection methods.

Before determining the autofluorescence intensity, we confirmed the spectrum of the observed microbial emissions in our system. *Escherichia coli* ATCC 13706, *Micrococcus lylae* ATCC 27566, obtained from American Type Culture Collection (ATCC; Manassas, VA), and *Staphylococcus epidermidis* ATCC 12228, obtained from RIKEN BioResource Center (RIKEN BRC; Ibaraki, Japan), were cultivated in TSB, and *Corynebacterium afermentans* ATCC 51403, obtained from RIKEN BRC, was cultivated in R medium (Bacto peptone 10 g, glycerol 2 g, yeast extract 5 g, Tween 80 50 mg, malt extract 5 g, MgSO4 · 7H2O 1 g, casamino acids 5 g, beef extract 2 g, in 1 L of distilled water [DW], pH 7.2) at 35°C until reaching the stationary phase. The harvested cells were washed with sterilized DW three times, and then re-suspended in DW. *Aspergillus niger* ATCC 9142, obtained from ATCC, was cultivated on potato dextrose agar (PDA; ai-science co., LTD., Saitama) at 25°C for 7 d and the spores were recovered according to the method of the Japanese Industrial Standards (JIS Z 2911:2010). The spores were re-suspended in DW for experimental use. A commercial spore suspension of *Bacillus atrophaeus* ATCC 9372 (Ref: SUN-07; NAMSA, Toledo, OH) was used without any treatment. Because the bioaerosol detectors detect desiccated airborne cells, a spot of the cell suspension was dried on glass slide for observation.

The spectrum of the microbial emissions was confirmed with a fluorescence microscope BX51 (Olympus Corporation, Tokyo, Japan) on which a spectrometer USB4000B (Ocean Optics, Inc., Dunedin, FL) was mounted. Since commercially available bioaerosol detectors use 355 nm (UV-APS) or 405 nm wavelength (BioLaz™ and IMD-A™) for excitation, we chose 405 nm for excitation using a U-MNV2 mirror unit (BP400-410, BA455, DM455) on the microscope. Differential spectra before and after fluorescence degradation by exposure to the excitation light for 60–120 s were calculated to subtract effects of scattering and reflection. Results are shown in Fig. 1. The main peak of the bacterial autofluorescence spectrum was around 500 nm, whereas that of *A. niger* was somewhat longer.

We next investigated the distribution of the microbial autofluorescence intensity (unpublished data). Glass slides were spotted with the diluted cell suspension to disperse cells and then dried. The fluorescence microscope image was captured by a CCD camera with a U-MNV2 mirror unit. A bright-field image was photographed simultaneously. The obtained fluorescence image was converted to gray scale and the cells were extracted by Image-Pro Plus software.

![FIG. 1. Spectra of microbial autofluorescence obtained by fluorescence microscopy under violet wavelength excitation (404.5 nm). Intensity was re-scaled to compare the spectral peak of each bacterium, although quantitative information was lost in this process. Spectra of *E. coli* (←), *S. epidermidis* (→), *B. atrophaeus* spore (←), *M. lylae* (→), *C. afermentans* (←), and *A. niger* spore (→) are indicated.](image-url)
cytometer calibration was generally too high for microorganisms (data not shown). The degree of polymerization and the autofluorescence intensity of polystyrene are generally correlated probably because of the amount of π-electron systems, and actually the autofluorescence intensity and weight-averaged molecular weight (Mw) showed a completely linear relationship in the PSMs of Thermo Fisher Scientific products. These PSMs were defined by a Mw range of 300,000 to 700,000 as the result of gel permeation chromatography (unpublished data). This indicates that approximate a 1 to 3-μm PSM with a Mw of this range can be a candidate used as a reference particle for microbial autofluorescence. Thus, those PSMs can be used as reference particles, and they can be substituted for microbial cells as weak intensity fluorescence particles when evaluating fluorescence-based microbial detection; however, this also means that PSMs are not always suitable as reference non-fluorescence particles.

Furthermore, we actually investigated the fluorescence intensity of bioaerosols and aerosolized PSMs using a fluorescence-based bioaerosol detector in order to compare the results with those obtained by fluorescence microscopy. The experiment was performed using IMD-A™ and a 3-m³ test chamber, according to a previous report (Hasegawa et al.,...

![FIG. 2](image)

**FIG. 2.** Distribution of the autofluorescence intensity of PSMs (a) and microorganisms (b). (a): PSMs of 0.5- (---), 1.0- (⋅⋅⋅), 3.0- (—), and 5.0-μm (→) were from Thermo Fisher Scientific and 1.0- (--) and, 5.0-μm (→) polystyrene, and 5.0-μm PS/S/2% DVB (→) microspheres were from Bangs Laboratories. (b): E. coli (---), C. alfermentans (--), M. lylae (--), S. epidermidis (→), B. atrophaeus spore (⋅⋅⋅), and A. niger spore (→). Frequency was normalized to reach a maximum of 1.0 in order to compare distributions on the same scale.

(Media Cybernetics, Inc., Bethesda, MD). The intensity of a single cell was calculated as the summation of the gray level of pixels located within the area designated as a cell by the software algorithm. If multiple cells were clumped together and recognized as a single cell by the software, the cell number was counted in the bright field image of the same field of view and the total intensity of the congregation was divided by the cell number to obtain the average intensity of a single cell. Values were corrected to obtain intensity per 1-s exposure. In this way, the distribution of the autofluorescence intensity of a single cell in the stationary phase was determined.

The autofluorescence intensity of polystyrene microspheres (PSMs) was also determined. PSMs 3500A, 5100A, 4203A, and 4205A (Thermo Fisher Scientific, Inc., Waltham, MA) were used as 0.5-, 1-, 3-, and 5-μm particles, respectively. Other PSMs, PS04N, PS05N (Bangs Laboratories, Inc., Fishers, IN), were also used as 1-, and 5-μm particles, respectively. Styrene/divinyl benzene copolymer (P([S/2% DVB]) microsphere PS06N (Bangs Laboratories, Inc.) was also used as other 5-μm particles. These PSMs were washed with DW three times to remove surfactants and preservatives. As the results, the microbial intensity was found to be similar to the autofluorescence intensity of some kinds of PSMs (Fig. 2).

The intensity of fluorescence microspheres for flow
Bacterial cells and PSMs were measured after aerosolization in the chamber. PSMs of 1.0-, 3.0- and 5.0-μm of Thermo Fisher Scientific products were chosen for the measurement because the intensity of those microspheres were located on the lower and the higher side of the intensity range of microorganisms according to Fig. 2. Thus, we will be able to evaluate whether the dynamic range of the apparatus is suitable for the microbial fluorescence intensity.

These PSMs also had complete correlation between the fluorescence intensity and Mw of their styrene polymer chains as described above. Such characteristics that can be explained according to the theory without any significant exceptional features, such as crosslink among polymer chains that influences the correlation, seem more suitable for reference particles. The 5.0-μm P(S/2% DVB) of Bangs Laboratories product was additionally measured. The results are shown in Table 1. The fluorescence intensity of these PSMs was also found to be comparable to that of bacterial in a fluorescence-based bioaerosol detector, although there was a slight difference in intensity order. Excitation and emission detection are statically done in microscopy, while a bioaerosol detector excites a particle and detects its emission in a moment of the movement of a particle in the flow of air. It is likely that variation in the features of different measurement principles influences the delicate order of intensity when dealing with such weak fluorescence. Thus, it should be considered that there may be a slight bias in measuring apparatuses when validating results by other methods.

In this study, we compared the microbial autofluorescence intensity to that of appropriately sized PSMs. Quantification of bacterial autofluorescence intensity has recently been mentioned in the use of high-sensitivity flow cytometry, where it was considered as producing background noise in high-sensitivity analysis using fluorescence dye (Yang et al., 2012). We have added information about artificial particles as comparable reference particles with regard to microbial autofluorescence intensity. Artificial particles have been used as reference particles in the experiments of aerosol or bioaerosol detection. However, they could not be actively used as reference fluorescence particles in the case of weak emission without clearly showing that their intensity is comparable to that of microbial autofluorescence. Our information will facilitate evaluation of whether the results of a fluorescence-based bioaerosol detector in microbial detection are reasonable from the viewpoint of fluorescence sensitivity.

Verification of fluorescence-based bioaerosol detection has been performed by comparison with the culture method using a test chamber (Agranovski et al., 2003a and 2003b; Brosseau et al., 2000). However, the quantitative evaluation of the cell number is difficult in the culture method because the capture efficiency of air samplers is different among them (Jensen et al., 1992; Yao and Mainelis, 2006), and also impaction stress (Stewart et al., 1995), and variation in cultural efficiency even in the same medium influence bacterial growth (Shintani and Sasaki, 2001). Furthermore, the method of sample preparation has not been standardized although it influences apparent culturability (Hasegawa et al., 2011). It would be impossible to clear all of these problems completely, we are required to verify the apparatuses by multiple experiments based on different principles, and to judge their performance comprehensively. In this case, a fluorescence-based microbial detection method would be able to be verified by comparison with the culture method, and also by the fluorescence sensitivity of the device to the intensity of the microbial autofluorescence.

On the other hand, the information we have about microbial autofluorescence has been obtained from cells cultivated in nutrient-rich media under optimal conditions in the laboratory, and some of this has not yet been elucidated in molecular biology. Future studies should try to investigate autofluorescence profiles and metabolic states of naturally occurring bioaerosols in desiccated, nutrient-poor conditions with biochemical data.

**Note**

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**TABLE 1.** The order of fluorescence intensity according to a bioaerosol detector.

| Sample          | Relative Intensity |
|-----------------|--------------------|
| *E. coli*       | 0.68               |
| *B. atrophaeus* | 0.71               |
| P(S/2% DVB)     | 0.74               |
| PSM1            | 0.82               |
| *S. epidermidis*| 0.90               |
| PSM3            | 1.00               |
| PSM5            | 7.71               |

a: Polystyrene microspheres are indicated by abbreviation. P(S/2% DVB): Styrene/divinyl benzene copolymer microsphere was from Bangs Laboratories. PSM1, PSM3 and PSM5: 1.0-, 3.0-, and 5.0-μm polystyrene microspheres were from Thermo Fisher Scientific, respectively.

b: Relative intensity was calculated as the average intensity against that of PSM3.
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