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# AUTOFLUORESCENCE AS A VIABILITY MARKER FOR DETECTION OF BACTERIAL SPORES

**Christian Laflamme [1], Daniel Verreault [1], Sophie Lavigne [1], Luc Trudel[3], Jim Ho [2] and Caroline Duchaine [1,3]**

*[1] Centre de recherche, Hôpital Laval, Institut Universitaire de cardiologie et de pneumologie de l'Université Laval, Québec, Québec, Canada, [2] Biological detection group, Defence R & D Canada Suffield, CFB Suffield, Ralston, Alberta, Canada, [3] Département de biochimie et microbiologie, Faculté des sciences et de génie, Université Laval, Québec, Québec, Canada*

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## 1. ABSTRACT

Recent biological terrorism events have indicated that bacterial spores such as *Bacillus anthracis* are real threat agents. Real time detection of biological agents is possible with the use of an ultraviolet Fluorescent Aerodynamic Particle Sizer (FLAPS) that measures particles' intrinsic fluorescence. It is important to know whether intrinsic fluorescence could be used to estimate agents' viability. Two categories of *Bacillus* spore populations can be differentiated by the intensity of intrinsic fluorescence emitted by ultraviolet (UV) stimulation : autofluorescent and non-autofluorescent. This study was performed to determine whether intensity of autofluorescence correlates with spore viability. Spores were analyzed using flow cytometer (equipped with a cell sorter) to mimic optical properties of FLAPS. Autofluorescent and non-autofluorescent spores were sorted according to the intensity of autofluorescence emitted following UV stimulation. Culturability, membrane integrity, membrane potential and dipicolinic acid (DPA) content were assessed. Autofluorescent spores were 1.7 times more culturable than the corresponding non-autofluorescent population. Moreover, a small proportion of autofluorescent spores exhibited extracellular membrane damages. Autofluorescent spores also showed higher membrane potential activity and contained higher levels of DPA. In conclusion, this study documents that the overall viability potential of bacterial spores can be assessed by UV flow cytometry used in the FLAPS technology.

## 2. INTRODUCTION

There is worldwide concern regarding *Bacillus anthracis* spores aerosol in work places in North America as summarized by Jernigan *et al.* who reported 5 fatalities from anthrax related incidences (1). Detection and quantification of an aerosol threat is an extremely difficult task. The microbial content of outdoor air varies with respect to time of day and temperature along with seasonal and human activity. Significant changes in outdoor bacterial levels could be produced by the introduction of unusual biological agents. Detection of these new sources of microorganisms, particularly when pathogens are

suspected, should be carried out rapidly and in real time to allow authorities to react in a timely fashion to initiate protective and therapeutic measures.

Outdoor air contains both dead and living microorganisms. Since only viable organisms cause infections, knowing the viable fraction of a microbial burden would help to reduce false alarms and to detect potentially serious situations. Plate cultures are commonly used to determine presence of live (or culturable) microorganisms (2); however, they underestimate the real number of viable cells as only microorganisms that replicate under the provided growth conditions (i.e., nutritional factors, temperature, and other conditions) are screened for (3, 4). Moreover, most culture techniques require days to complete (from 1 to 60-70 days for certain fastidious microorganisms) and, given that no single condition allows growth of all microbial species, culture media and growth conditions selected will always be a compromise (5). Molecular biology methods, such as PCR can provide information on nucleic acid content of environmental sample containing microorganisms. However, this technique is of limited usefulness for determining bacterial viability since positive signals are obtained in the presence of extracellular DNA or from non-viable microorganisms.

New tools used in conjunction with cell culture to estimate airborne microbial burden and cell viability have not only helped to delineate the limits of classical approaches; they have also increased the speed of analysis. Fluorescent methods have recently been used in combination with culture to measure microorganism levels in various types of environments (6, 7).

### 2.1. Bacterial autofluorescence (intrinsic fluorescence)

Measurement and detection of biological aerosols in real time has recently been reviewed (8). The Fluorescent Aerodynamic Particle Sizer (FLAPS), a system based on particle sizing and intrinsic fluorescence intensity measurements using ultraviolet (UV) excitation, has also been described (9). This technology relies on detection of fluorescence emitted by biological particles such as spores from *Bacillus anthracis* (9). It is speculated that the FLAPS fluorescence signals can be directly or indirectly attributed to nicotinamide adenine dinucleotide (NADH) (10) and intracellular riboflavin contents (9). NADH is often associated with viability under UV excitation (350-360nm) and is present within most metabolically active prokaryotic and eukaryotic cells. Shorter excitation wavelengths (266 nm) may produce signals from amino acids (11, 12). In addition, flavin compounds (flavin mononucleotide and flavin adenine di-nucleotide) are potential fluorescent constituents that are present in all cells that exhibit energy metabolism (13). Given the role played by these molecules in living systems, measurement of intrinsic fluorescence in living cells should provide important information regarding cell viability.

*Bacillus subtilis* var. *niger* (*B. globigii*) spores emit intrinsic fluorescence when excited by UV (9, 14); this fluorescence provides a means to distinguish biological material from inanimate particles. A casual relation between autofluorescence and culturability of a spore population (12 % autofluorescent and 17% culturable) has been suggested but the link between the two remains unclear. Techniques employed at the time did not permit unambiguous interpretation to relate fluorescence with viability or at least culturability (15).

### 2.2. Bacterial viability assays

Confirmation of bacterial viability is complex and requires several assays (7). Bacteria can be found in non-culturable states but may still exhibit viability properties (16). Culturability is the major marker of viability but it must be interpreted with caution. When microorganisms can be cultured, viability makes no doubt, while viability of non-culturable microorganisms could be assessed by approaches such as membrane integrity. Several viability assays based on dye fluorescence technology have recently become commercially available. One of these, the LIVE/DEAD *Baclight* technique is based on propidium iodide permeability and is effective for *Bacillus* spores (17, 18). Dipicolinic acid (DPA) can also be used as an efficient marker of spore viability (19). Additionally, the membrane potential assay, using 3,3$^{I}$-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), quickly predicts viability potential of *Bacillus* spores by the evaluation of germination (20).

The goal of this study was to establish a link between intrinsic fluorescence and viability in spores to better interpret the real time detection data from surveillance systems such as FLAPS technology. To reach our goal, spores were sorted according to their UV-induced autofluorescence by a flow cytometry system harboring fluorescence parameters similar to FLAPS' parameters. Viability assessments were performed on the two sorted populations representing strong-autofluorescent (referred to as autofluorescent for the remainder of this manuscript) and weak-autofluorescent (referred to as non-autofluorescent) spores.

## 3. MATERIAL AND METHODS

### 3.1. Bacterial strains

Powdered spores (*Bacillus subtilis* var *niger* [BG]) used in this study were provided by the U.S. Army, Dugway Proving Grounds, Utah. We performed quality control on this material by analyzing fatty acid (Microbial ID, Newark, DE) and partial 16S ribosomal DNA sequences (MIDI Labs, Newark, DE). All the following experiments were performed on powered spores suspended in phosphate buffer saline (PBS) at a concentration of $10^7$ spores/ml according to microscopic count.

3.2 Sample sonication

In order to disperse clumps, spore suspensions were sonicated for 5 to 10 minutes at room temperature using a sonication bath set at 42 kHz ± 6% (Fisher Scientific, Nepean, Ont, Canada). After sonication, monodisperse status was confirmed by microscopy.

### 3.3. Flow cytometry settings and sorting

Flow cytometric analysis of spores was done using an EPICS® ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL) with version 4.02 software. An air-

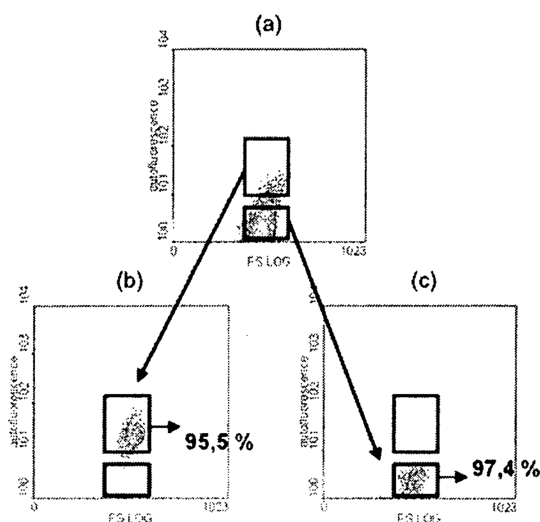


**Figure 1.** Cytofluorograph of Bg spore preparation following UV-flow cytometry. The sorting gates were arbitrarily placed on autofluorescent spores (gate A) and the non-autofluorescent population (gate B) (A). At the end of the sorting process, a control was done to evaluate the purity of autofluorescent (B) and non-autofluorescent sorted spores (C). The x-axis shows linear forward scatter while the y-axis represents autofluorescence intensity at 545 nm on a log scale.

ooled 20 mW HeCd laser (Melles Griot, Carlsbad, California) was the UV light source (325 nm). Autofluorescence signals were collected through a 525 ± 25nm bandpass filter as suggested by Aubin (21). Data were analyzed by forward gating and side scatter, data rates were set at less than 500 events per second. Samples were allowed to run for approximately 1 minute before acquiring a minimum of 5000 events. Sorting protocol was set as described (22). Sorting gates were set arbitrarily to include large and smaller intensity of autofluorescent spores (Figure 1a). An equal number of spores (minimum of 30,000) were sorted in each population (autofluorescent and non-autofluorescent). Following this step, purity controls were performed by sorting a minimum of 10,000 spores from the autofluorescent (Figure 1b, typical result) and non-autofluorescent populations (Figure 1c, typical result). The same gates and settings were used to confirm that both tubes contained two distinct spore populations based on autofluorescence. Nominal purity was above 95% for most assays (90% for DPA assays). Percentage purity was calculated using the following formula:

$$\text{Purity} = \left( \frac{\text{Number of spores in the sorting tube satisfying the sort bitmaps}}{\text{Total number of sorted spore in the collection tube}} \right) \times 100$$

### 3.4. Particle size analysis of spores

The *BRINKMANN PSA model 2010* (Galai Production Ltd., Migdal Haemek, Israel) was used for analysis of particle size. A liquid suspension of the particles to be analyzed was placed in a 1 cm cuvette with a magnetic stirrer. Analysis was initiated by running the included software.

### 3.5. Culturability of sorted spores: Plate count

The same number of autofluorescent and non-autofluorescent *Bacillus* spores were plated on tryptic soy agar (TSA, Difco, Detroit, Michigan) and incubated for 24 hours at 35°C. A 100 µl aliquot of sorted spores was plated directly on TSA (3000 spores). Serial dilutions of spore suspensions were done in triplicate and also plated to obtain 300, 30, and 3 spores per plate. A total of 5 independent sorting experiments (different days) were performed.

### 3.6. Membrane integrity testing of sorted spores and microscopic counts

Spore membrane integrity was assessed using LIVE/DEAD *Baclight* bacterial viability kit (Molecular Probes, Eugene, OR). Sorted autofluorescent and non-autofluorescent spores were incubated for 15 minutes in the presence of 0.9 mM of propidium iodide (PI, red dye) and 0.005 mM SYTO 9 (green dye). Under epifluorescence microscopy, spores permeable to PI appeared green and red while spores with intact membranes appeared green only. Using a predetermined volume (1 ml) of spores, stained samples were filtered (Nalgene) through a 0.22 um black polycarbonate NUCLEOPORE membrane (Millipore, Bedford, MA). After two washes with PBS, the filters were mounted between slide and coverslip using LIVE/DEAD *Baclight* mounting oil (Molecular Probes, Eugene, OR). Observations and picture capture were performed rapidly using a NIKON E-600 equipped with charge-coupled device (CCD) digital camera spot RT (Diagnostics Instrument, Iowa City, IA). Five microscope fields (representing 0.0096 $mm^2$) and 300 spores were counted per slide. Microscopic counts were determined from random fields on the photomicrograph using the formula:

Number of spores in the aliquot=Number of spore counted x Filtration area (201 $mm^2$)/Number of photomicrograph counted x photomicrograph area (0.0096 $mm^2$)

### 3.7. Membrane potential assay

This method, previously described (20) is briefly summarized here. $DiOC_6(3)$ (Ex: 484 nm, Em: 500 nm) was obtained from Molecular Probes (Eugene, Or). A stock solution of 20 uM was dissolved in dimethyl sulfoxyde (DMSO) and stored at –20°C. A total of $10^6$ autofluorescent and $10^6$ non-autofluorescent spores were placed in tryptic soy broth (TSB) immediately after sorting and incubated at 35°C for 0 and 15 minutes. Aliquots of culture (1ml) were incubated with 2.5 nM of $DiOC_6(3)$ for 2 minutes prior to flow cytometry analysis. The optimal dye concentration used in this study was established in setup experiments at 2.5 nM (data not shown). In most treatments, almost 100% of cells were stained. Differences were observed in intensity of staining by the mean fluorescence intensity. This procedure required analysis by a second flow cytometer. [EPICS® XL-MCL™ flow cytometer; Beckman-Coulter, Miami, FL, with acquisition software EXPO 32 (version 1.1c)], equipped with an air-cooled 15 mW argon laser (488 nm) light source. Fluorescence signals were collected through a 525 nm bandpass filter (FL1).

### 3.8. Measurement of dipicolinic acid (DPA)

DPA measurements on spores were performed as previously described (19). A total of 1 x $10^7$ autofluorescent or non-autofluorescent sorted spores (purity

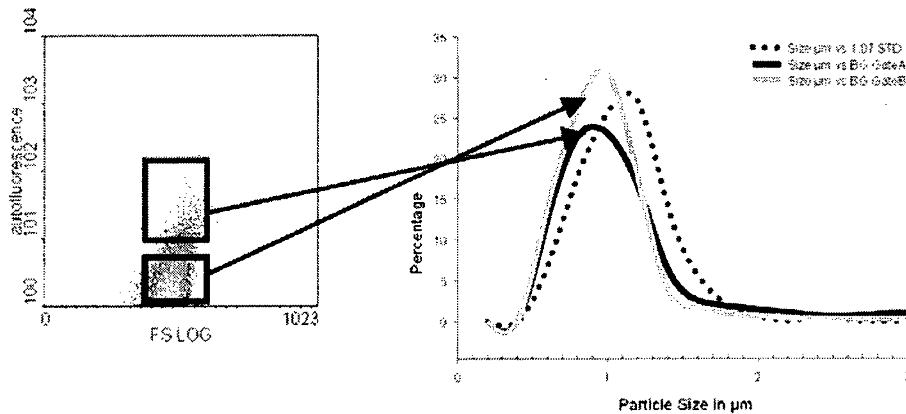


Figure 2. Particle size analysis (in um) of sorted spores. Autofluorescent BG sorted spores (grey) and non-autofluorescent sorted spores (light black) were analyzed on the basis of proportion versus size. Standard beads of 1.07 um (dark black) were analyzed at the same time.

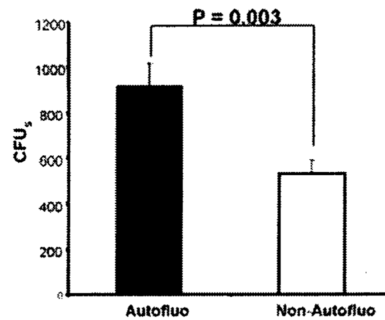


Figure 3. Number of $CFU_s$ ± SEM for an equal number of plated sorted spores. Autofluorescent and non-autofluorescent spores were plated in triplicate (for each experiment) on TSA.

over 90 %) were used for each test. Sorted spores were autoclaved at 121 °C, 15 psi for 20 minutes at liquid cycle and centrifuged (100,000 x g) at room temperature for 15 minutes (ODT Combi, Sorvall ultracentrifuge, Dupont). Supernatant was discarded and pellet resuspended in 4.5 ml of DPA buffer (40.4 mM TRIZMA-HCL, 10 mM TRIZMA base, 4%(v/v) alcool, pH 7.7) and 0.5 ml of stock solution of $TbCl_3$ (333.33 µM) and incubated, protected from light at room temperature for one hour. Stock solution of $TbCl_3$ was prepared in argon atmosphere using argon grade 5 ($H_20$ less than 100 ppm, BOC Gaz, Quebec, Qc) and Atmosbag (Aldrich, Milwaukee, Wi) as gas chamber. Terbium tri-chloride powder was dissolved in DPA buffer. Fluorescence intensity was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, Mississauga, ON) set at 275 nm excitation wavelength; fluorescence emission was collected at 545 nm and corresponds with the optimal fluorescence spectra of $[Tb(DPA)_3]^{3-}$ (23). The photomultiplier of the spectrophotometer was set at 900 V (determined using a standard curve) and 5 different measures were taken at each dosage.

### 3.9. Statistical analyses

Statistical analyses were performed using SAS software. Results are expressed as means ± SEM. Pertinent data were analyzed using paired t-tests. All reported *p*-values were considered significant at $p < 0.05$.

## 4. RESULTS

### 4.1. Flow cytometry sorting based on autofluorescence

Sorting gates were selected by visual inspection to delineate 10-14% of the fluorescent population (Figure 1a). Two groups of spores were sorted on the basis of intensity of autofluorescence. Under phase-contrast microscopy, no optical difference could be observed between autofluorescent (Figure 1b) and non-autofluorescent (Figure 1c) populations. Replicate experiments showed that the number of sorted spores was equal for both populations (p=0.83, Figure 1b,c). Moreover, when measured with a Brinkman particle sizer, sorted spores were of comparable size (Figure 2).

### 4.2. Culturability of autofluorescent and non-autofluorescent spore populations

Autofluorescent populations produced higher plate counts than non-autofluorescent populations (Figure 3, $p$=0.003). There were 1.7 times more colony-forming units ($CFU_s$) in the autofluorescent spore populations.

4.3 Membrane permeability of sorted spores

Figure 4 shows that non-autofluorescent spores were more permeable to propidium iodide (17.4%) than autofluorescent spores (3.4%, $p$= 0.01).

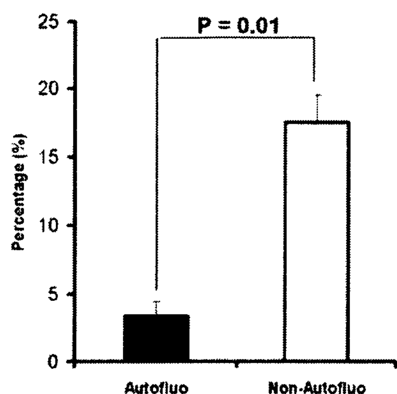


**Figure 4.** Proportion of autofluorescent and non-autofluorescent spores with permeabilized membranes using LIVE/DEAD *Baclight* bacterial viability kit. For each assay, a minimum of 5 fields were analyzed.

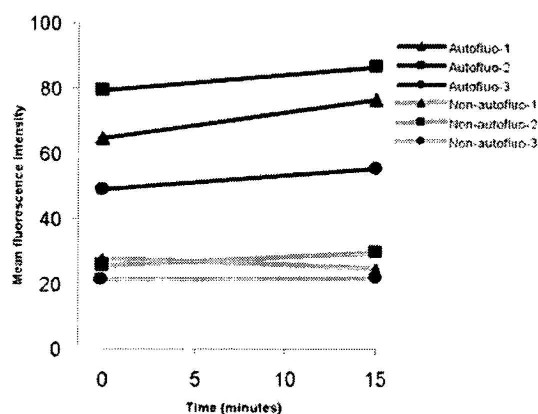


**Figure 5.** Flow cytometry measurement of membrane potential over time using $DiOC_6(3)$ staining. BG spores were cultured in TSB and harvested at 0 and 15 min and then stained with $DiOC_6(3)$. Mean fluorescence intensity was determined using a 525 nm bandpass filter (FL1 channel). A minimum of three records (independent experiments) per harvesting time were obtained.

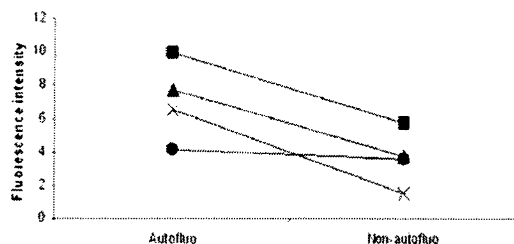


**Figure 6.** Quantification of $[Tb(DPA)_3]^{3-}$ fluorescence intensity per $10^6$ sorted spores for autofluorescent and non autofluorescent populations. For each assay, a minimum of 5 records of fluorescence were obtained.

### 4.4 Membrane potential of sorted spores

Autofluorescent spores showed greater membrane potential activity, after the sorting process (at time zero) compared to the non-autofluorescent spores (Figure 5). Moreover, only autofluorescent spores showed a significant increase ($p$= 0.04) in membrane potential after 15 minutes incubation in TSB (Figure 5).

### 4.5. DPA quantification of sorted spores

Autofluorescent spores contained higher levels of DPA than non-autofluorescent spores. Figure 6 shows that autofluorescent spores released in average 7.05 relative fluorescence units per $10^6$ sorted spores compared to 3.63 per $10^6$ non-autofluorescent spores (n = 4, $p$ = 0.01). The 95 % confidence interval was calculated to be 0.25, 6.56, (n = 4).

## 5. DISCUSSION

The present study documents that the intensity of autofluorescence signal of spores directly correlates with several important viability criteria including culturability, membrane integrity, membrane potential during germination and cellular DPA content. The phenomena explaining the observed intrinsic fluorescence after excitation using a light source at more than 325 nm is not clear. Plausible explanations are discussed below. We used a UV excitation source at 325nm while fluorescence emission was collected at 525 ± 25nm. These settings were chosen in accordance with and to mimic the optical features of the FLAPS technology. This combination of optical parameters could collect fluorescence emission of at least two biomolecules associated with viability: NADH and riboflavin. Several publications describe intrinsic fluorescence in vegetative bacterial cells at excitation levels of 340-360 nm. In most cases, these properties are used in batch fermenters to monitor cell biomass. This experimental approach using NADH spectra for autofluorescence signal detection was determined from different studies in various fields. In prokaryotic cells, intrinsic fluorescence has been used to characterize (24) and identify (25) bacteria. Autofluorescence and viability were compared in young and old cultures (up to five years) of two *Cyanobacteria* strains. In old cultures, cell viability and autofluorescence were lower compared to young cultures (26). A recent a study showed that glucose and sorbitol metabolism could also be measured by changes in NADH fluorescence in *Streptococcus mutans* (27). These studies might help to understand the relationship between NADH, viability and autofluorescence but data were obtained using vegetative forms of bacteria or eukaryotic cells and spore autofluorescence was only studied in previous Defence Research and Development Canada (DRDC) projects. Among these findings, it has been reported that FLAPS measurement was a function of live biological aerosols; gated fluorescent particles correlated with the levels of culturable particles measured by slit samplers and expressed as Agent Containing Particles per Litre of Air (ACPLA) (8).

In eukaryotic cells, Horvath and coworkers showed that it is possible to use autofluorescence intensity to evaluate cell mass in yeast (12). Similarly, autofluorescence has been successfully used to evaluate donor corneal endothelial viability. An increase in yellow-

green autofluorescence from reduced pyridine nucleotide and oxidized flavoproteins correlates with healthy cornea (28). Another study using Jurkat cells showed a major early decrease in blue autofluorescence presumably due to oxidation of NADH before the onset of apoptosis (29). Eng and co-workers documented that NADH fluorescence was related to viability of cardiac myocytes (30). Tokunaga *et al.* showed a positive correlation between NADH concentration and viability of rat liver cells (31).

It is difficult to prove that NADH contributes to the signal observed in spores since minute concentrations of this molecule have been measured chemically in this dormant form of bacteria (32). Improvement of methods that allow measurements of NADH in single spores are necessary to assess involvement of this biomolecule in UV induced autofluorescence. Attention should also be paid to measurement of riboflavin levels in spores. Since NADH measurement was performed in batches of spores (32) and such NADH is a labile molecule that may be altered during the fastidious extraction procedure, it is highly likely that NADH concentration in spores was underestimated.

An important parameter to verify was whether the cell sorter placed an equal number of spores in the corresponding tubes. If this was not the case plate count result could be misleading. The technique used to enumerate spore concentrations was adapted from Hobbie and coworkers (33). The protocol used in the present study comprises the advantage of counting live and dead spores without discrimination and differentiated spores from debris. Using this method we confirmed that the number of sorted spores was equal in both sample tubes and that very little cell debris were present.

Biological aerosols, especially those of bacterial spores, are currently an urgent threat in both military and civilian settings. Results of this study using intrinsic fluorescence could be applied to field trials to evaluate viability potential of spores. Moreover, these findings should help improve efficiency of surveillance systems such as FLAPS technology along with other laser-based detection methods.

We showed that the intensity of intrinsic fluorescence during UV excitation is a predictive viability marker for BG spores. These observations could be extended to other *Bacillus* spores and spores of other genera. Future work should include other excitation/emission combinations (for example the yellow-green fluorescence region) associated with other bacterial biomolecules (FMN, FAD, lipofuscin, etc.). The observations presented here confirm a previous suggestion (8) that live spores can be discriminated from other aerosol particulate materials using intrinsic fluorescence as a sorting criterion. In this paper, it was speculated that NADH was most likely the molecule responsible for the emission signals. Experiments are currently being performed to examine this hypothesis and to study the potential contribution of other fluorescent biomolecules that may be present in significant concentrations in spores.

## 6. ACKNOWLEDGMENTS

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**Send correspondence to:**Caroline Duchaine, Ph.D., Centre de recherche,Hôpital Laval, 2725 Chemin Ste-Foy, Ste-Foy (Quebec), Canada, G1V 4G5, Tel: 418 656-8711 ext. 5837, Fax: 418 656-4509, E-mail: Caroline.Duchaine@bcm.ulaval.ca

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