J Fluoresc (2006) 16:733–737
DOI 10.1007/s10895-006-0129-1

SHORT COMMUNICATION

Flow Cytometry Sorting Protocol of *Bacillus* Spore Using Ultraviolet Laser and Autofluorescence as Main Sorting Criterion

Christian Laflamme · Daniel Verreault · Jim Ho · Caroline Duchaine

Received: 18 July 2006 / Accepted: 28 August 2006 / Published online: 22 September 2006
© Springer Science+Business Media, LLC 2006

Abstract The ultraviolet (UV) Fluorescent Aerodynamic Particle Sizer (FLAPS), a flow cytometer-like apparatus was developed by the Canadian Department of National Defence for real-time detection of autofluorescence of biological aerosol particles such as bacterial spores. The direct relation between autofluorescence intensity and viability has recently been reported and viable spore are more autofluorescent in UV (Laflamme, Frontiers in Bioscience). The goal of this manuscript is to describe a flow cytometry sorting protocol based on UV-induced autofluorescence. An EPICS® ELITE ESP flow cytometer equipped with a UV laser and cell sorter was used to mimic the optical properties of FLAPS and to study the two extremes of a spore population according to its autofluorescence (lower level of autofluorescence (LLA) and higher level of autofluorescence (HLA) spores). *Bacillus subtilis* var *niger* was used as a surrogate for *Bacillus anthracis* spores and sorted using autofluorescence intensity as the main criterion. The protocol developed in our laboratory to sort *Bacillus* spores according to their autofluorescence properties is described. Purity of each sorted population was greater than 95%. Using autofluorescence as the main criterion, we demonstrate that it is possible to separate two distinct spore populations.

C. Laflamme · D. Verreault · C. Duchaine (✉)
Centre de recherche, Hôpital Laval, Institut Universitaire de cardiologie et de pneumologie de l'Université Laval, 2725 Chemin Ste-Foy, Québec, Québec, Canada
e-mail: Caroline.Duchaine@bcm.ulaval.ca

J. Ho
Biological detection group, Defence R&D Canada Suffield, CFB Suffield, Ralston, Alberta, Canada G1V 4G5

C. Duchaine
Département de biochimie et de microbiologie, Université Laval, Québec, Québec, Canada

Keywords Flow cytometry sorting · Ultraviolet laser · Autofluorescence · *Bacillus* spores

Introduction

Canadian National Defence scientists recently developed an ultraviolet (UV) fluorescence aerodynamic particle sizer (UV-FLAPS), this flow-cytometer-like apparatus detecting the presence of airborne bacterial spores. Detection is based on the principle that spores are more fluorescent than non-biological background particles [1]. This apparatus was developed for real-time detection of airborne agents (particularly *Bacillus anthracis* spores, the etiologic agent of anthrax) used for bio-terrorism. This device samples air at a rate of 1000 liters per minute and uses UV (352 nm) and red diode (680 nm) laser beams [2]; particles are accelerated through a small orifice and transit time between the two halves of the split red diode beam is measured to determine particle size. On the other hand, the UV laser beam induces intrinsic fluorescence in biological particles that can be measured [2]. Several studies reported a direct relation between autofluorescence emission and the presence of living organisms [2–8]. Moreover, relationship between autofluorescence emission after UV excitation and viability status were recently documented [3, 9–11]. At this wavelength FLAPS is able to detect the presence of bio-active molecules including NADH or other flavinoid molecules that are markers of living biological materials [1, 2]. As a result, FLAPS can distinguish, in real time, airborne particles containing living organisms from all the other background particles. To demonstrate the relation between autofluorescence and viability, it was necessary to sort bacterial spores using optical properties similar to those used by the FLAPS method. A powerful advantage of flow cytometry is the ability to sort

events using pre-determined detection parameters. Indeed, this advantage has brought to biological science a new tool that permits isolation and analysis of distinct populations of particles in a heterogeneous sample [12, 13]. Most studies that involve cell sorting are performed using an argon laser beam that excites cells at 488 nm. In fact, the majority of the commercially available fluorescent cell markers are designed for this excitation threshold and most commercial flow cytometers are equipped with an argon laser. Other excitation sources including UV (between 300 nm and 400 nm) and far red (630 nm and more) are also useful. Use of UV laser (354 nm) methodology for cell sorting is not conventional but has been applied extensively for chromosome sorting applications [14]. Additionally, this technology is useful for sperm cells sorting (stained with Hoechst 33342) and separation of X and Y chromosome-bearing spermatozoa [15]. This procedure is increasingly developped in the bovine industry for gender selection [16] and for stem cells sorting [17].

At present, all sorting protocols available for bacterial cells in the published literature use fluorescence staining of cells to provide an acceptable discrimination level between populations that need to be sorted. In this paper we describe a novel approach to sort unstained bacterial spores using UV-induced autofluorescence as the main sorting criterion. This approach mimicked FLAPS properties and used flow cytometry to analyze different spore populations.

Material and methods

Bacterial strain

A *Bacillus subtilis* var *niger* (BG) powdered spore preparation was used for these studies (provided by the U.S. Army, Dugway Proving Grounds, Utah); cell purity and identity controls were performed by analysis of fatty acid content (Microbial ID, Newark, DE) and partial 16S ribosomal DNA sequences (MIDI Labs, Newark, DE). All sorting experiments were performed using sonicated powdered spores suspended in phosphate buffer saline (PBS) at a concentration of 10^7 spores/ml.

Sonication

Spore suspensions were sonicated (42 kHz $\pm$ 6%; Fisher Scientific, Nepean, Ont, Canada) for 5 min at room temperature to disperse clumps; dispersal of spores was confirmed by light microscopy.

Flow cytometry settings

Analysis of spores was performed with an EPICS® ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL) using version 4.02 software. The UV light source (325 nm) was an air-cooled multimode 30 mW (20 mW output) HeCd laser (Omnichrome model 74) with a beam diameter and divergence of 1.2 mm and 0.5 mrad, respectively. The laser signal was condensed using a beam reducer (Beckman coulter). Autofluorescence signals collection was triggered through a 525 ± 25 nm bandpass filter as previously described [5] and data were collected by forward and side scatter gating. Data rates, for analysis and sorting, were set at less than 500 events per second; samples were allowed to run for approximately 1 min before data acquisition recorded in log scale in pulse amplitude.

Startup and pre-sorting preparation

At least 30 min was allowed to pre-heat the UV laser and the bimorph crystal (by increasing the drive to 92%) before analysis of the coefficient of variation (CV). Laser alignment was performed daily and coefficient of variation ($CV = 100 \times$ (standard deviation)/mean) less than 5 relative units was the maximum required for acceptability. After this time period, the camera was zoomed and panned to allow setup of the droplet break off using the frequency function; this step was critical for good stream deflection.

Sorting of spores according to autofluorescence

Filtered-sterilized ISOTON II (Beckman-Coulter, Miami, FL) was used as sheath fluid. Careful attention was paid to the bimorph crystal to prevent accumulation of salt deposits that interfere with cell sorting. A cleaning procedure using fresh tap was performed on a regular basis. Dry bimorph crystals without scratches, chips or cracks were used. A 100 μm orifice sort sense flow cell (sense-in-quartz) and 16×125 μm beam shaper were used. Moreover, a Quartz 197R high sensitivity forward scatter obscuration template (mask) was used since it is the smallest template available and it provides the best resolution. An ND1 filter, that transmits 1/10 of incident light, was positioned before the forward scatter detector. Sample and sheath pressures were set at 10.5 and 10.8 psi, respectively. The ELITE system uses a video camera to set up the cell sorting procedure (used per manufacturer's instructions) and converts the video image to determine correct drop delay settings. Cell sorting modes were set at "Pur1Rec2;" these settings improve purity and recovery using the extended sort mode of the instrument. The coincidence abort function was set to on. The threshold discriminator was set between 6 and 8 (full scale was 100) on forward scatter detector. Background noise was acceptable when fewer than 5 events per second were recorded. Typically, the bimorph drive frequency was set between 26 and 32 kHz at a point where stream separation was most stable. Drop delay was optimized using a 1 μm bead and verified

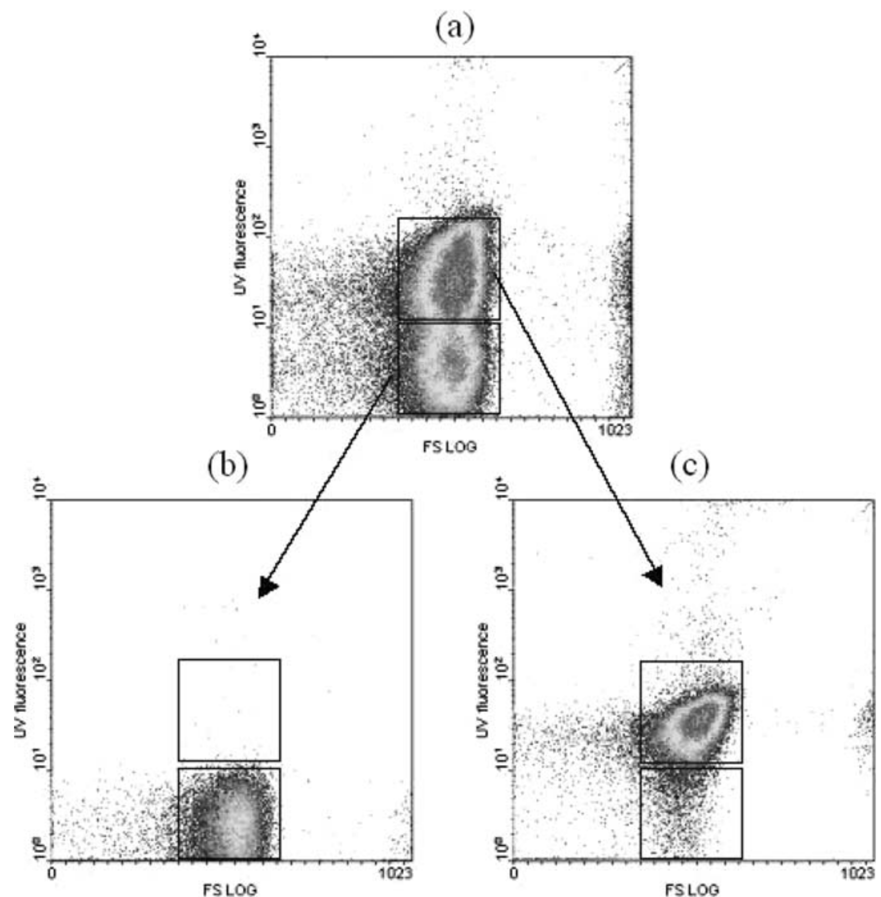

Fig. 1 Cytofluorograph of the sorting gates for UV-flow cytometry using one micrometer beads. The x-axis represents linear forward scatter and y-axis shows UV fluorescence intensity at 381 nm on a logarithmic scale. The gates were adjusted in order to surround the two beads populations (a). The non fluorescent beads (b) and fluorescent beads (c) were sorted in two different tubes

using FLOW-CHECK beads (Beckman-Coulter, Miami, FL) according to the manufacturer's instructions. Sorting gates were arbitrarily set to include higher and lower intensities of the autofluorescent spores. An equal number of spores (minimum 30000) were sorted in each population: lower level of autofluorescence (LLA) and higher level of autofluorescence (HLA).

Following sorting, purity checks were performed by analyzing a minimum of 10000 spores from the sorted LLA and HLA populations. 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. Percentage purity was calculated using the following equation:

$$\text{Purity} = \left(\frac{\text{Number of sorted spores analyzed that satisfy the sort bitmaps}}{\text{Total number of sorted spores analyzed}}\right) \times 100$$

Validation of the UV sorting protocol using one micrometer beads

In order to confirm the sorting protocol efficiency, non-fluorescent 1.0 microns beads (Molecular Probes, Eugene, OR) and 1.0 microns UV-fluorescent beads (Fluoresbrite® Polyfluor® 394 microsphere, Polysciences, Inc. Warrington, PA) were mixed together (1:1) and sorted according to the same protocol.

Results

Sorting using non-fluorescent and UV-fluorescent one micrometer beads

The mix of the two beads were sorted according to the fluorescence intensity. First of all, the mixed population was located by the flow cytometer with forward scatter and UV-fluorescence using 381 ± 25 nm bandpass filter (Fig. 1a). Using the same sorting protocol as for *Bacillus* spores, it was possible to sort the beads using their UV-fluorescence intensity as criteria (Fig. 1b,c). Since the protocol used (unstained one micron spores) is in the lower limit of detection of the flow cytometer, several noise events that affect the resolution of the plots are visible outside the analysis gates. (Fig. 1a). This noise is also present in the sorted populations (Fig. 1b,c). These noise events are present at low data rate when filtered buffer, that serves as diluent for samples, is analyzed (data not show).

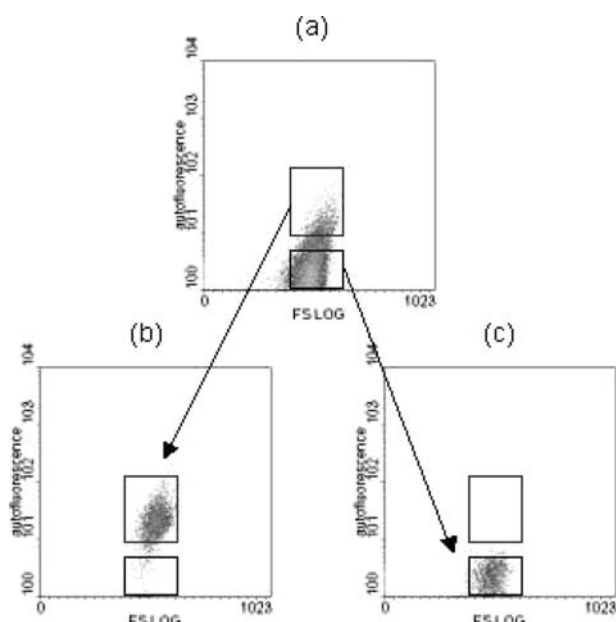

Fig. 2 Cytofluorograph of the sorting gate for UV-flow cytometry. The x-axis represent linear forward scatter and y-axis show autofluorescence intensity at 525 nm on a logarithmic scale. The gates were rationally placed on HLA spores (gate 1) and the LLA population (gate 2) (a). A control was done to evaluate the purity of HLA (b) and LLA sorted spores (c)

Sorting based on autofluorescence

Sonicated spore samples were used to set the sorting gates. This adjustment, done by visual inspection, separated the brightest of the population identified as HLA; these cells were within the second log scale range of the autofluorescence window. Cells within the first log scale population were identified as LLA (Fig. 2a). As such, two cell populations, HLA and LLA, were sorted based on the intensity of their autofluorescence after UV excitation; theoretical purity was >95%. No optical differences were observed between HLA (Fig. 2b) and LLA (Fig. 2c) populations under phase contrast microscopy and sorted spores were of comparable in number and in size ($p = 0.83$, 11). However, as reported, the HLA are significantly more viable than LLA (11).

Discussion

This study demonstrates, for the first time, successful and efficient sorting of *Bacillus* spores using UV-induced intrinsic fluorescence intensity as the primary criterion. To the authors' knowledge, use of flow cytometry for sorting of unstained bacteria or spores has not been previously reported. These findings provide an opening for future studies on the relations between natural autofluorescence and other biological or physiological characteristics. The criterion of autofluorescence could have implications in several areas of investigation including biological weapons detection using FLAPS, wastewater treatment, bioreactor studies, and evaluation of indoor air quality in homes and buildings. In this study *Bacillus* spores with an average particle size of 0.8 μm were used [18]. For this reason, the flow cytometer must be set up to provide the best resolution and highest sensitivity with no significant increase in CV. Different strategies have been used to reach these objectives; an important consideration involves the optical properties of the laser beam that passes through the flow cell. A smaller and more intense beam at the level of the flow cell increases both sensitivity and resolution. We used a multimode UV laser in these studies that produces spots that are not radially symmetric. Positioning a beam reducer in front of the laser markedly condenses the laser beam. After passing through the beam reducer, the UV signal is deflected at 90° before passing through the beam shaper; choice of the beam shaper is critical. The best solution for our study was a beam width of 16×125 μm to provide a lower CV and higher sensitivity using calibration beads. In accordance with the manufacturer's suggestion, we used a orifice size of 76 μm instead of a 100 μm flow cell due to the small particle size of the samples. The 76 μm flow cell was tested using different combinations of beam shapers and masks; no significant difference was observed for the two different orifice sizes. As a result, we decided to use the 100 μm orifice size as it also meets the other application needs (i.e., eukaryotic cell studies) and does not need to be changed for each application. The laser beam is projected to an obscuration template, also known as mask. When an event passes through the laser beam, light is scattered in all directions. The flow cytometer used in this study collects scattered light in two directions: forward and side scatter. The mask can affect the nature of the projected light and consequently, affects both resolution and sensitivity. With regard to the beam shaper, several masks were tested with the same optical objective to obtain the better compromise between high sensitivity and resolution, low CV and low background noise. Finally, a neutral density filter (ND1), which transmits 1/10 of the incident light, was used to reduce background noise for forward scatter.

An additional parameter that merits consideration is sample and sheath pressure. At lower sheath pressures, the sample spend more time (not evaluated in these experiment) within the beam, thereby increasing sample illumination and increases sensitivity. This was helpful for our experiments as autofluorescence is not affected by photo-bleaching or quenching of fluorescence.

In conclusion, this study documents that it is possible to sort small diameter particles such as *Bacillus* spores using UV-induced autofluorescence as the principle criterion. Our UV-based sorting protocol would be a useful tool for in depth study of biomolecules involved in spore viability and survival.

Acknowledgements These studies were undertaken under a Department of National Defence (DND) contract W7702-00R802/001/EDM: Dr. J. Ho was the scientific authority acting for DND. Initial ideas for these studies originated from previous work performed at DND. The authors are thankful to Dr. André Darveau and Maurice Dufour for their insightful review of the manuscript. Christian Laflamme received a Natural Sciences and Engineering Research Council of Canada (NSERC)/Institut de recherche Robert-Sauvé en santé et sécurité du travail (IRSST) studentship. Dr. C. Duchaine acknowledges an Institut de Recherche Robert-Sauvé en Santé et Sécurité du Travail/Canadian Institutes of Health Research Scholarship.

References

1. Hairston PP, Ho J, Quant FR (1997) Design of an instrument for real-time detection of bioaerosols using simultaneous measurement of particle aerodynamic size and intrinsic fluorescence. J Aerosol Sci 28:471–482
2. Ho J, Spence M, Hairston P (2000) Measurement of biological aerosol with a fluorescent aerodynamic particle sizer (FLAPS): correlation of optical data with biological data. In: Stopa PJ, Bartoszcze MA (Eds). Rapid methods for analysis of biological materials in the environment. Kluwer Academic Publishers, Netherlands p 177–201
3. Horvath JJ, Glazier SA, Spangler CJ (1993) In situ fluorescence cell mass measurements of Saccharomyces cerevisiae using cellular tryptophan. Biotechnol Prog 9:666–670
4. Benson RC, Meyer RA, Zaruba ME, McKhann GM (1979) Cellular autofluorescence—Is it due to flavins? J Histochem Cytochem 27:44–48
5. Aubin JE (1979) Autofluorescence of viable cultured mammalian cells. J Histochem Cytochem 27:36-43
6. Pouneva I (1997) Evaluation of algal culture viability and physiological state by fluorescencent microscopic methods. Bulg J Plant Physiol 23:67–76
7. Shimazaki J, Laing RA, Tsubota K, Kenyon KR (1996) Non-invasive assessment of the donor corneal endothelial using ocular redox fluorometry. Br J Opthalmol 80:69–73
8. Caiola MG, Ocampo-Friedmann R, Friedmann EI (1993) Cytology of long-term desiccation in the desert cyanobacterium Chroococcidiopsis (Chroococcales). Phycologia 32:315–322
9. Tokunaga Y, Ozaki N, Wakashiro S, Ikai I, Morimoto T, Shimahara Y, Kamiyama Y, Yamaoka Y, Ozawa K, Nakase Y (1987) Fluorometric study for the noninvasive determination of cellular viability in perfused rat liver. Transplantation 44:701–706
10. Eng J, Lynch RM, Balaban RS (1989) Nicotiamide adenine dinucleotide fluorescence spectroscopy and imaging in isolated cardiac myocytes. Biophy J 55:621–630
11. Laflamme C, Verreault D, Lavigne S, Trudel L, Ho J, Duchaine C (2005) Autofluorescence as a viability marker for detection of bacterial spores. Front Biosci 10:1647–1653
12. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA (2002) The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clin Chem 48:1819–1827
13. Melamed MR (2001) A brief history of flow cytometry and sorting. In: Methods in cell biology. vol 63, p 3–17
14. Ibrahim SF, Van Den Engh G (2004) High-speed chromosome sorting. Chromosome Res 12:5–14
15. Johnson LA, Flook JP, Look MV (1987) Flow cytometry of X and Y chromosome-bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. Gamete Res 17:203–212
16. Garner DL (2001) Sex-Sorting mammalian sperm: concept to application in animals. J Androl 22:519–26
17. Goodell MA, Brose K, Paradis G, Stewart Conner A, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J Exp Med 183:1797–1806
18. Sneath PHA (1986) Bergey's manual of systematic bacteriology. vol 2. Baltimore: Williams & Wilkins