RAPID FLOW CYTOMETRIC DETECTION AND IDENTIFICATION OF MICROBIAL PARTICLES

Hazel M. Davey and Douglas B. Kell
Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed, Wales, U.K.

ABSTRACT

There is a continuing need to be able to effect the rapid estimation of the microbial load in a sample, sometimes merely in terms of the total numbers of cells ("detection") but often also with respect to different cell types ("identification"). This need is particularly acute when the presence of pathogenic organisms is suspected, and the background consists of complex, often non-biological particulates and other, non-pathogenic biomaterials. It is shown herein that the technique of flow cytometry together with careful selection of fluorescent stains may help to meet this need. Optical brighteners represent fluorescent stains with many desirable properties.

INTRODUCTION

Cytometry refers to the measurement of the physical and chemical characteristics of cells. By extension, flow cytometry refers to the technique where such measurements are made as the cells pass in a fluid stream through a measuring point surrounded by an array of detectors[1].

In a generalised flow cytometer[1,2], individual particles pass through a measuring point where they intersect a beam of light from a laser or mercury arc lamp. The sample is typically constrained to the centre of a much faster flowing sheath fluid by a process known as hydrodynamic focusing, allowing reliable delivery of the particles to the centre of the illumination zone at a rate of some 1000-3000 cells.s^{-1}. Appropriate detectors, which may be gated electronically, measure the magnitude of a pulse representing the extent of light scattered as the particle passes through the illumination zone. The magnitudes of these pulses are sorted electronically into "bins" or "channels", permitting the display of 2D- or 3D-histograms of number of cells *vs* channel number (*vs* channel number for a second or further parameter). Light scattered in the forward direction provides information about the size of the scattering particle while light scattered at wider angles depends to a larger extent on the internal structure of the cell[3]. In addition, cellular autofluorescence may be exploited[4] however, it is by the addition of appropriate fluorophores prior to analysis that the full potential of flow cytometry is realised.

A wide variety of fluorescent stains have been used in flow cytometric studies[5] and include those which bind to molecules such as DNA, RNA or protein, fluorogenic substrates for

measuring the activity of a given enzyme, indicators of intracellular pH, fluorescent stains which are taken up or excluded by cells in a manner which reflects the intactness of their membranes, and, increasingly, antibodies (or oligonucleotides) tagged with a fluorescent probe.

Until recently flow cytometry had been applied mainly to mammalian cells. Microorganisms, with their smaller size and relatively low concentrations of DNA and other cellular constituents had proved to be below the resolution of most commercial flow cytometers[6]. However, within the last 10-15 years, with increased instrument sensitivity and with the development of brighter fluorescent probes flow cytometry has been successfully applied to a number of microbiological problems[7-15], and recently a book devoted to the subject has been published[16].

While the majority of flow cytometric applications in microbiology have involved the study of axenic cultures some work has been done on the identification of organisms in mixtures[17-20]. Flow cytometry offers several advantages for this type of work, (i) cells are analysed individually, rather than as populations allowing the identification of different cell types, (ii) typical data acquisition rates are in excess of 1000 cells.s^{-1}, enabling reasonably rare events to be observed, (iii) depending on the instrument, typically 3 to 8 different parameters of each cell may be measured simultaneously, increasing the possibility of distinguishing between similar organisms, and (iv) if the presence of a target organism is indicated the sorting capabilities of the flow cytometer can be called upon to separate the suspect cell from the mixture for further confirmation of its identity.

One situation where it would be advantageous to determine whether a target organism was present would be in cases where the release of a biological warfare agent was suspected. In the work described below *Bacillus globigii* (*B. subtilis* var *niger*) spores have been used as a simulant of *B. anthracis*[21-23]. The flow cytometric characteristics of this organism have been studied using both light scattering measurements and by fluorescence measurements following the addition of fluorescent brighteners. Fluorescent brighteners are common additives to domestic washing powders where they increase the apparent "whiteness" of clothes by absorbing UV-light and emitting in the visible part of the spectrum. These stains are non-toxic and would be expected to bind to the surface of bacterial spores, thus overcoming problems that may be found with other stains which would require permeabilisation of the spore prior to staining.

MATERIALS AND METHODS

Flow Cytometry

All flow cytometric analyses were performed using a Coulter Epics Elite flow cytometer (Coulter Electronics Ltd., Luton, U.K.) equipped with a HeCd laser (325 nm), an argon laser (488 nm) and a HeNe laser (633 nm). The flow cytometer was set up as described in the manufacturers manual. The sheath fluid was prepared using Millipore Milli-Q water filtered to 0.22 μm and contained 150 mM KCl and 10 mM HEPES. The pH was adjusted to 6.8 with KOH and the sheath fluid was then filtered to 0.1 μm using a Whatman WCN filter. The forward scatter signal from the argon laser was used to discriminate between the signal from the spores and the background noise. The wavelengths over which side scattering and fluorescence were collected together with the photomultiplier voltages used are given in the legends to the figures.

Fluorescent Brighteners

Three different fluorescent brighteners were studied in terms of their usefulness in staining *B. globigii* spores. Tinopal CBS-X and Tinopal SWN were obtained as a gift from Ciba Dyes and Chemicals Ltd., Macclesfield, U.K. and Calcofluor White M2R was purchased from Sigma, Poole, U.K. They were tested at various concentrations and the time-dependency of staining was determined by using the time parameter on the flow cytometer.

Sample Preparation

B. globigii spores were obtained form the CBDE, Porton Down, Salisbury, U.K. Two samples were received, a "wet" suspension of spores and a "dry" preparation. Prior to analysis the spores were suspended in sheath fluid to give a concentration of approximately $1x10^6$ spores.ml^{-1}. Unstained samples were run without further preparation. Stained samples were prepared as described in the legends to the figures.

RESULTS AND DISCUSSION

On analysis of the light scattering properties of the "wet" suspension of spores supplied by the CBDE it became apparent that there were two subpopulations present (see Figure 1), as well as a

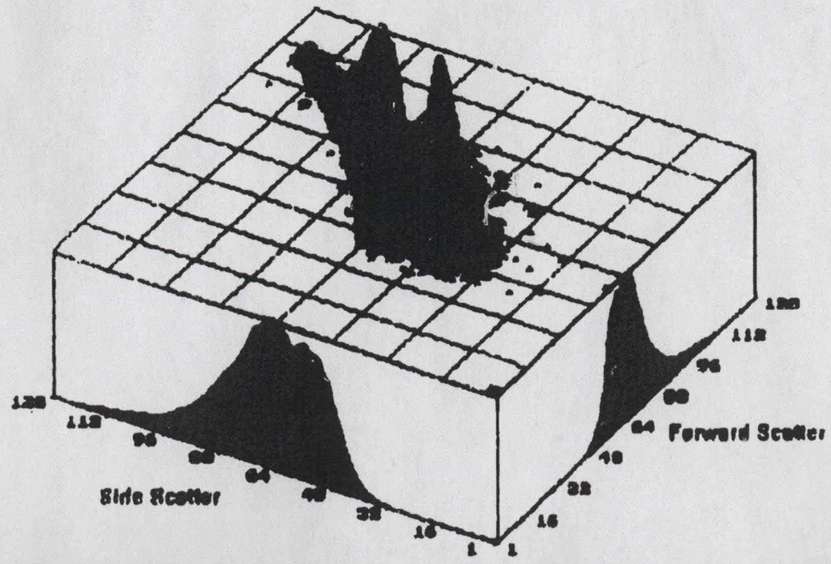


Figure 1 : Flow cytometric analysis of the light-scattering properties of the "wet" preparation of *Bacillus globigii* spores revealed that the sample contained two distinct populations. An argon laser (488 nm) was used as the excitation source and light scattering was collected in the forward direction and also at wide angles. In both cases the PMT voltage was set to 400 and the gain was logarithmic, with full scale representing 4 decades. Only in the case of the dual-parameter plot is it possible to resolve the two populations fully, thus demonstrating the special power of multiparameter analysis.

noticeable degree of clumping leading to the apparently large heterogeneity of the spore suspension. In order to determine whether the two subpopulations were real, or whether they were an artefact of the different alignment of the spores as they passed through the measuring point of the flow cytometer we decided to exploit the sorting capabilities of the Coulter Epics Elite to separate them prior to re-analysis of the separated samples by flow cytometry.

A sort protocol for the *B. globigii* was created in the Elite software, instructing the flow cytometer to place events from Region 1 (R1 in Figure 2A) into one collection tube and events from R2 (Figure 2A) into a second tube. When enough sample had been processed to give at least 0.5 ml in each collection tube the two subsamples were passed in turn through the flow cytometer. If the two subpopulations were an artefact one would expect to obtain similar results from the two subsamples; however as can be seen in Figure 2B,C the two populations have been

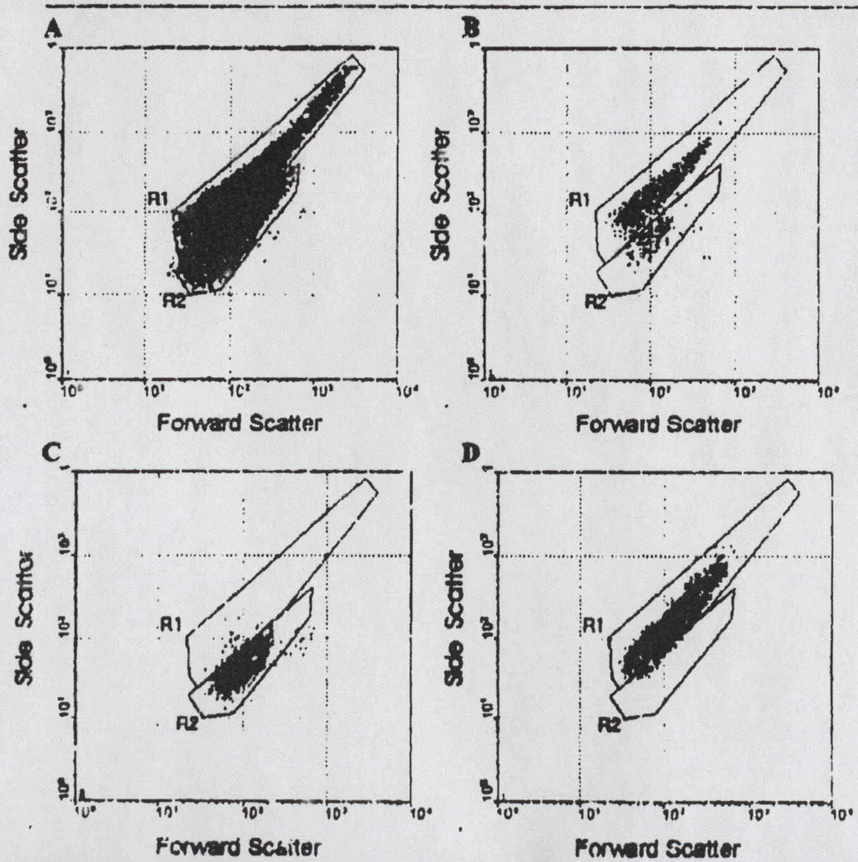


Figure 2 : Flow cytometric analysis of the *B. globigii* spores. The flow cytometer was set up as described in the legend to Figure 1. A) The "wet" preparation of spores consists of two subpopulations. These were sorted by flow cytometry based on the regions (R1 and R2) shown in the figure. B) and C) are the flow cytometric traces produced by the resulting sorted subsamples from R1 and R2 respectively. D) The "dry" preparation of spores supplied by the CBDE have characteristics very similar to the major subpopulation (R1) of the "wet" population

successfully separated. Analysis of the "dry" *B. globigii* sample revealed that it contained only one population, which was similar in flow cytometric terms to the major population (R1) in the "wet" sample (Figure 2D).

When one's aim is to distinguish between different organisms the presence of two distinct subpopulations within one nominally homogenous organism standard is far from desirable and so the "dry" preparation of spores was chosen for use with the fluorescent brighteners. Of the three fluorescent brighteners studied Tinopal CBS-X was the most effective in staining the spores (Table 1). It was found that a concentration of 40 $\mu g.ml^{-1}$ (mol. weight = 562.5) is sufficient to effect maximum staining of the spores but that at this concentration and in the absence of congeners ~20 minutes are required for maximum staining to be achieved. The extent of staining of *B. globigii* spores is illustrated in Figure 3A,B.

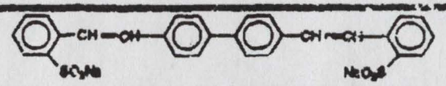
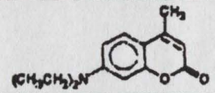
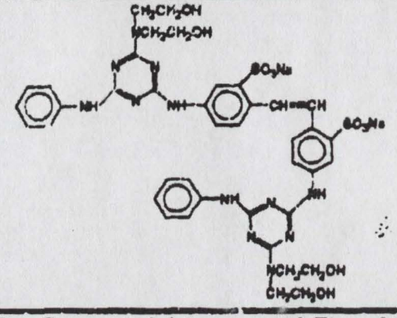
Fluorescent Brightener	Ionic Character	Chemical Structure	Extent of Staining
Tinopal CBS-X	Strongly Anionic		+++
Tinopal SWN	Weakly cationic		-
Calcofluor White (Tinopal LPW)	Anionic		+

Table 1 : Characteristics and structures of the fluorescent brighteners used. Tinopal CBS-X was most effective in staining the *B. globigii* spores.

CONCLUSIONS

It is often desirable to detect target microorganisms against a high biological background, not least when the presence of a pathogen is suspected. Modern flow cytometers are sufficiently sensitive to detect such organisms and as outlined above offer considerable advantages over other techniques. The value of the multiparameter nature of flow cytometric data in

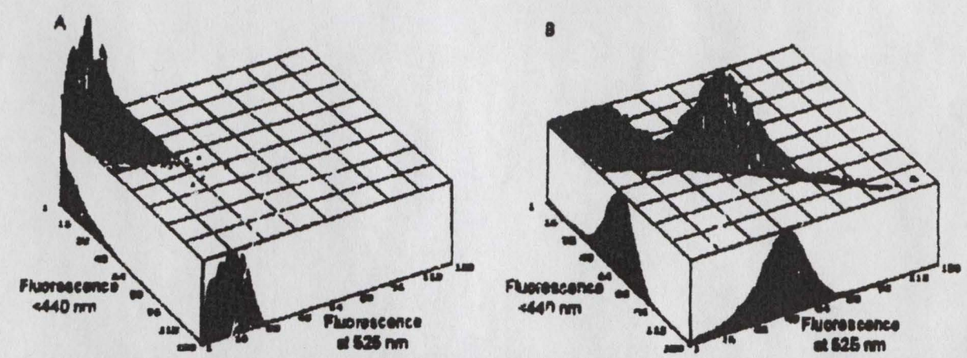


Figure 3 : *B. globigii* spores, A unstained, and B stained with 40 $\mu g.ml^{-1}$ Tinopal CBS-X. In A there is little detectable fluorescence at 525 nm and the emission collected below 440 nm is a result of side scattering and possibly autofluorescence of the spores. In B an increase in fluorescence at both wavelengths can be seen. Fluorescence below 440 nm was collected on PMT1 *via* a 440 dichroic lens, while fluorescence at 525 nm was collected on PMT3 using a 550 dichroic lens and a 525 nm band pass filter. The voltage for PMT1 was set to 460 and for PMT3 a voltage of 610 was used. All gains were logarithmic.

distinguishing subpopulations in a nominally homogenous sample (Figure 1) may easily be extended to distinguishing between different organisms in a mixture. The more parameters one measures the more likely it is that one will be able reliably to identify a target microorganism in such a mixture. The capability of flow cytometers to sort cells according to their flow cytometric characteristics is illustrated (Figure 2); this may prove to be very useful in confirming the identity of a suspect organism. The full potential of flow cytometric analysis is realised when fluorescent stains are added to the sample. For the purpose of staining spores an ideal stain is one which is non-toxic and binds to the surface, avoiding the requirement for permeabilisation. It is shown that the fluorescent brightener Tinopal CBS-X is an effective stain for *B. globigii* spores (Figure 3).

ACKNOWLEDGEMENTS

The research reported herein has been sponsored by the United States Army through its European Research Office.

REFERENCES

1. Shapiro, H.M. (1988). Practical Flow Cytometry (2nd Edition). New York: Alan R. Liss Inc.
2. Melamed, M.R., Lindmo, T., and Mendelsohn, M.L. (1990). Flow Cytometry and Sorting (2nd Edition). New York: Wiley-Liss Inc.
3. Salzman, G.C., Singham, S.B., Johnston, R.G., and Bohren, C.F. (1990). Light Scattering and Cytometry. In Flow Cytometry and Sorting (2nd Edition), ed. M.R. Melamed, T. Lindmo, and M.L. Mendelsohn. 81-107. New York: Wiley-Liss Inc.
4. Cunningham, A. (1993). Analysis of Microalgae and Cyanobacteria by Flow Cytometry. In Flow Cytometry in Microbiology, ed. D. Lloyd. 131-142. London: Springer Verlag.
5. Waggoner, A.S. (1990). Fluorescent Probes for Cytometry. In Flow Cytometry and Sorting (2nd Edition), ed. M.R. Melamed, T. Lindmo, and M.L. Mendelsohn. 209-225. New York: Wiley-Liss Inc.

6. Steen, H.B., Skarstad, K., and Boye, E. (1990) DNA Measurements of Bacteria. In Flow Cytometry, ed. Z. Darzynkiewicz and H.A. Crissman. 519-525. London: Academic Press.
7. Betz, J.W., Aretz, W., and Hartel, W. (1984). Use of Flow Cytometry in Industrial Microbiology for Strain Improvement Programs. *Cytometry* 5, 145-150.
8. Frelat, G., Laplace-Builhe, C., and Grunwald, D. (1989). Microbial Analysis by Flow Cytometry: Present and Future. In Flow Cytometry: Advanced Research and Clinical Applications (Volume II), ed. A. Yen. 256-279. Boca Raton FL.: CRC Press.
9. Boye, E. and Løbner-Olesen, A. (1990). Flow Cytometry: Illuminating Microbiology. *The New Biologist* 2, 119-125.
10. Shapiro, H.M. (1990). Flow Cytometry in Laboratory Microbiology: New Directions. *ASM News* 56, 584-588.
11. Kell, D.B., Ryder, H.M., Kaprelyants, A.S., and Westerhoff, H.V. (1991). Quantifying Heterogeneity: Flow Cytometry of Bacterial Cultures. *Ant. van Leeuwenhoek* 60, 145-158.
12. Kaprelyants, A.S. and Kell, D.B. (1992). Rapid Assessment of Bacterial Viability and Vitality using Rhodamine 123 and Flow Cytometry. *J. Appl. Bacteriol.* 72, 410-422.
13. Davey, H.M., Davey, C.L., and Kell, D.B. (1993). On the Determination of the Size of Microbial Cells Using Flow Cytometry. In Flow cytometry in Microbiology, ed. D. Lloyd. 49-65. London: Springer-Verlag.
14. Davey, H.M., Kaprelyants, A.S., and Kell, D.B. (1993). Flow Cytometric Analysis, using Rhodamine 123, of *Micrococcus luteus* at Low Growth Rate in Chemostat Culture. In Flow cytometry in Microbiology, ed. D. Lloyd. 83-93. London: Springer-Verlag.
15. Fouchet, P., Jayat, C., Héchard, Y., Ratinaud, M.-H., and Frelat, G. (1993). Recent Advances of Flow Cytometry in Fundamental and Applied Microbiology. *Biol. Cell.* 78, 95-109.
16. Lloyd, D. (1993). Flow Cytometry in Microbiology. London: Springer-Verlag.
17. Balfoort, H.W., Snoek, J., Smits, J.R.M., Breedveld, L.W., Hofstraat, J.W., and Ringelberg, J. (1992). Automatic Identification of Algae: Neural Network Analysis of Flow Cytometric Data. *J. Plankton Research* 14, 575-589.
18. Boddy, L. and Morris, C.W. (1993). Analysis of Flow Cytometry Data - A Neural Network Approach. *Binary* 5, 17-22.
19. Boddy, L. and Morris, C.W. (1993). Neural Network Analysis of Flow Cytometry Data. In Flow cytometry in Microbiology, ed. D. Lloyd. 159-169. London: Springer-Verlag.
20. Boddy, L., Morris, C.W., Wilkins, M.F., Tarran, G.A., and Burkill, P.H. (1994). Neural Network Analysis of Flow Cytometric Data for 40 Marine Phytoplankton Species. *Cytometry* 15, 283-293.
21. Dando, M. (1994). Biological Warfare in the 21st Century. London: Brassey's.
22. Stevenson, R. (1994) Chemistry Aids the Germ War Watchdogs. *Chemistry in Britain* 693-696
23. CBDE (1994) Protecting by Scientific Understanding. London: MoD.