Appl Microbiol Biotechnol (1992) 38:268-272

Applied and Microbiology Biotechnology

Rapid assessment of bacterial viability by flow cytometry

J. P. Diaper, K. Tither, C. Edwards

Department of Genetics and Microbiology, University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK

Received: 22 November 1991/Accepted: 6 July 1992

Abstract. The ability of a flow cytometer to rapidly assess microbial viability was investigated using three vital stains: rhodamine 123 (Rh123); 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and fluorescein diacetate (FDA). Rh123 was found to clearly differentiate viable from non-viable bacteria. The methodology for staining bacteria with this dye was optimised. Rh123 was shown to stain and discriminate several different species of viable bacteria although this was not universal. Viable cells of *Bacillus subtilis* were found to stain better with FDA than with Rh123. The results demonstrate the ability of flow cytometry to rapidly detect and estimate the viability of bacterial populations.

Introduction

A method for the rapid assessment of microbial viability is a major requirement in several areas of microbiology including public health, the biotechnology industry, the medical sector and food technology (Mason et al. 1986). Traditional methods employed to detect and enumerate viable bacteria such as growth on laboratory media require time and may underestimate the number of viable bacteria present because it is now believed that some bacteria enter a viable but non-culturable state (Roszak and Colwell 1987). Therefore rapid direct methods for the detection of viable bacteria are of increasing importance.

Flow cytometry measures physical or chemical characteristics of individual cells as they move in a fluid stream past optical or electronic sensors. The ability to rapidly and precisely detect, characterise and identify cells in a mixed population demonstrates its potential as a tool for the analysis of microbial populations (Shapiro 1990b). The staining of viable eukaryotic cells by fluorescent dyes and their detection by flow cytometry has been reported by several workers. These dyes include fluorescein diacetate (FDA), which is lipophilic and can pass into the cell through the membrane. Once inside the cell it is cleaved to fluorescein, which is then retained by cells with an intact membrane (Shapiro 1988). Cationic lipophilic dyes such as rhodamine 123 (Rh123; Darzynkiewicz 1982) and 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$; Shapiro 1988] have also been used to detect viable eukaryotic cells by flow cytometry. These dyes also pass readily through the cell membrane but are only retained in cells with a membrane potential.

Both Rh123 and FDA have been used to detect viable bacteria (Bercovier et al. 1987; Chrzanowski et al. 1984; Matsuyama 1984). However all these reports used microscopy to detect the stained bacteria. The use of a flow cytometer (FCM) to detect viable bacteria has been limited to a brief report by Resnick et al. (1985) describing Rh123 staining of *Mycobacterium smegmatis*.

The aim of this study was to asses the ability of FCM to detect and estimate the viability of a range of bacterial species using the three dyes most commonly used for eukaryotic cells.

Materials and methods

Bacterial strains and culture conditions. Cultures of *Bacillus subtilis*, *Aeromonas hydrophila*, *A. salmonicida*, *Escherichia coli*, *Pseudomonas fluorescens* and *Salmonella pullorum* were obtained from laboratory stocks. The Cowan 1 strain of *Staphylococcus aureus* was a gift from Dr. C. Duggleby (PHLS CAMR, Porton Down, Salisbury, UK).

All the bacterial strains used in this study were maintained on nutrient broth (Lab M) solidified with 1.5% purified agar (Lab M). For FCM analysis bacteria were grown to the late exponential phase of growth in nutrient broth that had been filtered by passage (three times) through a 0.22-μm Durapore membrane filter (Millipore). Colony-forming units (cfu) were counted after incubation of appropriately diluted culture samples on nutrient agar at 37° C for 24 h.

Bacterial staining. The method used to stain bacteria with Rh123 (Sigma), unless otherwise stated, was essentially that of Matsuyama (1984). A 1-ml culture sample was washed and resuspended in 5 ml phosphate buffered saline (PBS), pH 7.3, containing 5 μg Rh123/ml. Gram-negative species were washed and resuspended in 5 ml TE Buffer (10 mM TRIS, 1 mM EDTA, pH 8.0) containing

Correspondence to: J. P. Diaper

5 µg Rh123/ml. Cells were then incubated for 30 min at 37° C, washed three times in PBS and immediately examined by FCM. Cells were stained with $DiOC_6(3)$ (Sigma) by the method of Shapiro (1990a) and the method used to stain the bacteria with FDA (Sigma) was that described by Fry (1990). Again Gram-negative bacteria were incubated in TE buffer instead of PBS. For all three vital stains examined, cells incubated with 20 µg/ml of the ionophore gramicidin S (Sigma), which was used to abolish the cell membrane potential, were used as negative controls. Numbers measured by FCM were also routinely compared to cfu on nutrient agar.

Flow cytometric analysis. Flow cytometry was performed using a Skatron Argus 100 instrument (Skatron Ltd, PO Box 34, Newmarket, Suffolk, UK). The sheath fluid pressure was set at 1.00 kPa/cm and the sample flow rate was set between 1 µl/min and 10 µl/min. Green fluorescence was detected using an excitation wavelength of 470–495 nm, band-stop of 510 nm and emission of 520–550 nm. Cellular fluorescence was detected using a logarithmic gain and a photomultiplier setting of 650 V. Cells with a fluorescent peak channel of greater than 50 were considered stained. Beads of 2 µm diameter (CV <2%; Polyscience, Warrington, Pa., USA) were used to focus the FCM before use.

Statistical analysis. Coefficients of variation (CV) for the fluorescent histogram distributions were calculated by the software supplied by the manufacturer. Analysis of variance and regression analysis was performed using the Minitab computer package (Ryan et al. 1976). All bacterial cell counts were transformed by $Log_{10}X$ to ensure normality of distribution and homogeneity of variances. Minimum significant difference was calculated by the Turkey-Kramer method (Sokal and Rohlf 1982).

Results and discussion

Flow cytometric analysis of viable bacteria using different fluorescent dyes

Figure 1 shows several examples of histograms of cellular fluorescence as measured by the FCM. Increasing fluorescence intensity is represented by increasing channel number on the x-axis. Viable *S. aureus* cells stained with Rh123 were readily detected by the FCM with a peak channel of 99 whereas cells treated with the ionophore gramicidin had a much reduced cellular fluorescence with a peak channel of 19 (Fig. 1a). Unstained cells gave similar results (not shown) to gramicidin-treated cells. Therefore the low levels of fluorescence observed were predominantly due to cellular autofluorescence. It has been demonstrated by other workers using fluorescent microscopy (Matsuyama 1984; Bercovier et al. 1987) that Rh123 can be used to stain viable *S. aureus* and that this staining was dependent on membrane potential (Matsuyama 1984). Our result confirms this and demonstrates the ability of the FCM to distinguish between viable and non-viable cells.

Viable *S. aureus* cells stained with $DiOC_6(3)$ were also readily detected by the FCM and a fluorescence distribution with a peak channel of 159 was recorded. Reduced uptake of the dye was again observed when the cells were treated with gramicidin, indicating that staining was dependent on the cell membrane potential (Fig. 1b). However, unlike the results observed using Rh123, gramicidin-treated cells exhibited a considerable fluorescence, resulting in a large overlap between the two distributions. This result demonstrates the problems associated with staining with cyanine dyes. These include the binding of the dye to hydrophobic regions of the cell increasing the fluorescence of the dye disproportionally to its concentration in the cell and fluorescent quenching of the dye by the viable cells (Shapiro 1988).

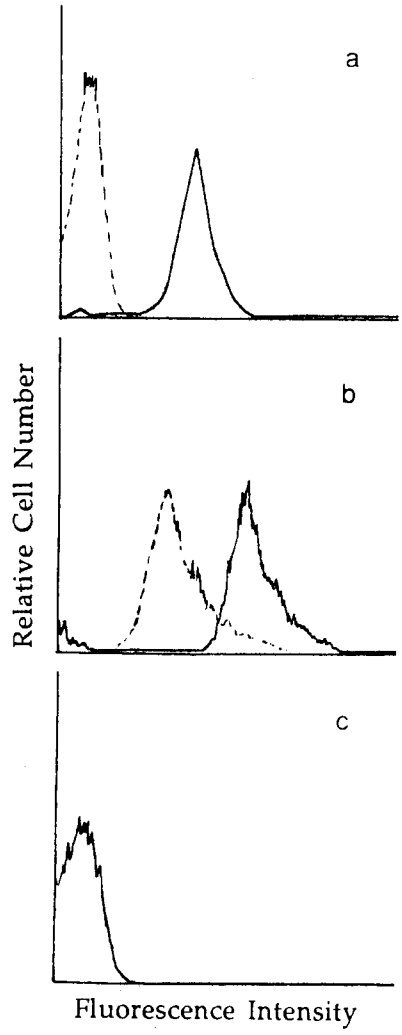

Fig. 1. Fluorescence histograms of *Staphylococcus aureus* stained with **a** rhodamine 123 (Rh123), **b** 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], **c** fluorescein diacetate (FDA). Fluorescence histograms of cells treated with gramicidin S are represented by a *broken line*

Fluorescein-stained *S. aureus* could not be detected by the FCM after incubation with FDA (Fig. 1c). This was despite the measurable esterase activity of *S. aureus*. Presumably the fluorescein was not retained by the cells long enough for them to be analysed by the FCM. The leakage of fluorescein out of viable cells has been reported by Lundgren (1981).

The ability of FCM to detect Gram-negative bacteria was demonstrated using *E. coli*. *E. coli* was incubated in an EDTA-containing buffer (TE buffer) to permeabilise the outer membrane before staining (Matsuyama 1984). The results (not shown) obtained when *E. coli* was stained with Rh123, $DiOC_6(3)$ and FDA were similar to those described for *S. aureus*.

Optimal conditions for staining bacteria with Rh123

S. aureus and *E. coli* were used as Gram-positive and Gram-negative model species to optimise conditions for staining with Rh123. Accumulation of Rh123 by both *S. aureus* and *E. coli* was dependent on its concentration (Fig. 2). The fluorescence intensity of viable *S. aureus* cells increased with increasing concentration of Rh123 up to 2 μg/ml. This was in contrast to gramicidin-treated cells the fluorescence of which remained constantly low up to 5 μg/ml. When *E. coli* was stained with different concentrations of Rh123 maximum fluorescence was achieved at a concentration of 4 μg/ml and again the fluorescence of gramicidin-treated cells remained low. Other workers (Bercovier et al. 1987; Matsuyama 1984; Resnick et al. 1985) have used 5 μg/ml of Rh123 to stain viable bacteria but concentrations as high as 10 μg/ml have been used to stain yeast cells (Skowronek et al. 1990) and mitochondria (Johnson et al. 1980). Our results suggest that at concentrations of Rh123 greater than 2 μg/ml fluorescent quenching of the dye in the *S. aureus* cells occurred. This may decrease the sensitivity of the stain to detect changes in membrane potential of *S. aureus*. However, since the cellular fluorescence of *E. coli* cells continued to increase at higher concentrations of Rh123 compared with the results for *S. aureus*, and for both species the fluorescence of gramicidin-treated bacteria remained low, to keep the protocol consistent a concentration of 5 μg Rh123/ml was selected to stain for viable bacteria.

The accumulation of Rh123 inside viable cells was found to be independent of temperature between 20° C and 37° C, and maximum cellular fluorescence was obtained within 5 min incubation (results not shown). The accumulation of Rh123 within mitochondria has also been shown by other workers (Darzynkiewicz et al. 1982) to be independent of temperature between 24° C and 37° C, and although Rh123 is not classed as a fast response dye for measuring changes in membrane potential, the typical accumulation of similar cationic lipophilic dyes occurs within seconds to minutes (Shapiro 1988).

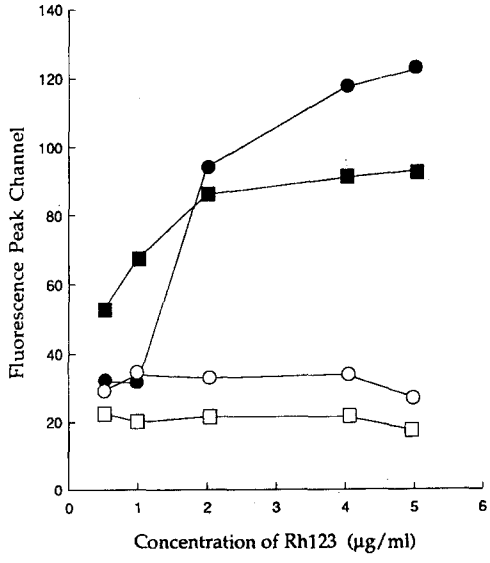

Fig. 2. Effect of Rh123 concentration on cellular fluorescence intensity: ■, *S. aureus*; □, *S. aureus* treated with gramicidin S; ●, *Escherichia coli*; ○, *E. coli* treated with gramicidin S. Increasing fluorescence intensity is represented by increasing peak channel number

Discrimination of dead and viable cells using Rh123

Formaldehyde-fixed and viable cells of *S. aureus* were mixed in a ratio of approximately 2:3 and stained with Rh123. Two distinct populations were detected by the FCM (Fig. 3). The fluorescence of formaldehyde-fixed cells was higher than that of gramicidin-treated cells but it was still lower than that of viable cells. The ratio of numbers within each histogram was the same (2:3) as the dead:live ratio determined by total direct counts and cfu. Similar results were also obtained for *E. coli* (not shown).

Comparison of cfu with viable numbers obtained using the FCM

The ability of the FCM to estimate numbers of viable cells was investigated using known dilutions of *S. aureus* cells. As can be seen from Fig. 4, *S. aureus* numbers estimated by the FCM were not significantly different from those estimated from cfu ($P > 0.05$). In our experiments Rh123 counts never exceeded those determined from cfu and no evidence was obtained for viable but non-culturable cells (Roszak and Colwell 1987). For example, numbers of *S. aureus* after starvation in filtered lakewater for 8 days showed no significant difference ($P > 0.05$) when estimated from cfu or by Rh123 staining allied to flow cytometry. Other workers have demonstrated the ability of FCM to estimate populations of bacteria stained with ethidium bromide (Patchett et al. 1990; Pinder et al. 1990). These reports how-

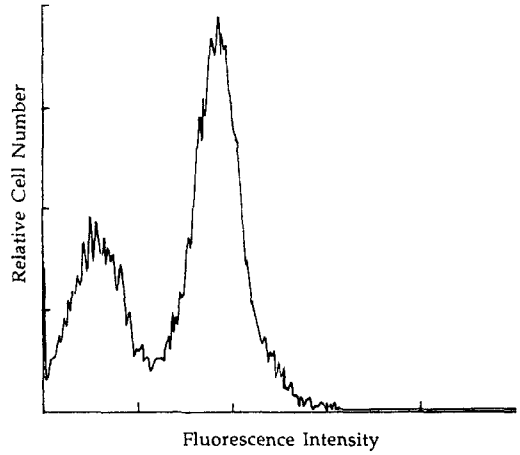

Fig. 3. Fluorescence histograms of an *S. aureus* mixed culture of viable cells (3.3×10^8 ml^{-1}) and formaldehyde-fixed cells (2×10^8 ml^{-1}) stained with Rh123

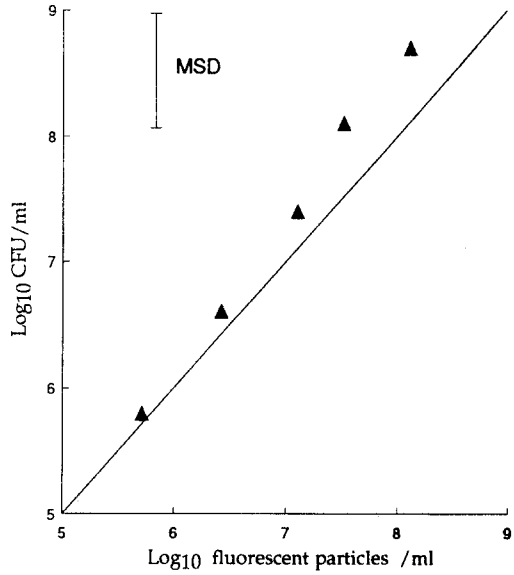

Fig. 4. Relationship between viable plate counts and counts of Rh123 stained cells by flow cytometry. The ideal relationship is represented by the *line*; ▲ represent actual values; MSD, minimum significant difference

ever did not use vital stains and therefore could only demonstrate the use of the FCM to estimate the total number of bacteria. This is in contrast with our results, which demonstrate the potential of FCM to estimate the number of viable cells within a sample.

Applicability of FCM to determine bacterial viability

Table 1 shows the ability of Rh123 to stain other bacteria. Although it was possible to stain several different bacterial species with Rh123 it is not an universal vital stain (Table 1). It was not possible to distinguish viable cells of *B. subtilis* or *A. salmonicida* using Rh123. Additionally, Rh123 did not appear to stain 100% of the cells. In the case of *E. coli*, *S. aureus* and *Salmonella pullorum*, 90% or more of the population detected by the FCM could be stained, however less than 10% of *P. fluorescens* cells detected by the FCM were stained with

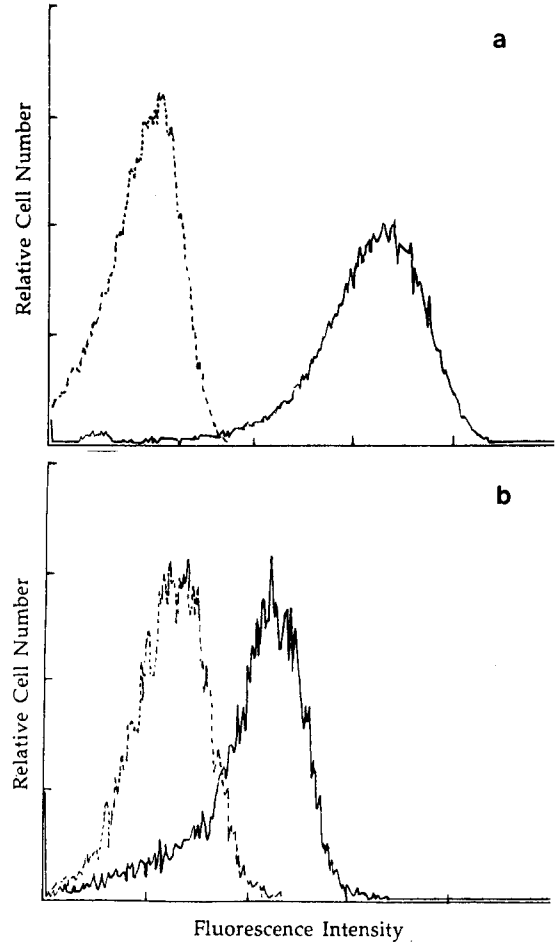

Fig. 5. Fluorescent histograms of **a** *B. subtilis* stained with FDA; **b** *P. fluorescens* stained with $DiOC_6(3)$. Fluorescent histograms of cells treated with gramicidin S are represented by a *broken line*

Rh123. Similar variations in the ability of Rh123 to stain different bacteria have also been reported by Matsuyama (1984). This possibly reflects differences in the cell surface physiology of the bacteria used and demonstrates the limitations of Rh123 as a vital stain. For example aeromonads are known to have unusual surface layers, comprising an A-protein, which have also been implicated with virulence (Adams et al. 1988).

Table 1. Staining of bacteria with Rh123

Bacteria	Stained cells (%)[a]	Fluorescence peak channel number	CV of stained cells (%)	Fluorescence peak channel of gramicidin-treated cells
Staphylococcus aureus	96	100	13.5	19
Escherichia coli	91	128	9.6	28
Bacillus subtilis	0	40	nd	26
Salmonella pullorum	89	110	10	19
Aeromonas hydrophila	77	113	13.6	20
A. salmonicida	0	12	nd	15
Pseudomonas fluorescens	7	128	14	28

Bacteria were stained with Rh123 as described in Materials and methods; Gram-negative bacteria were incubated in TRIS-EDTA buffer as opposed to phosphate-buffered saline: CV, coefficient of variation; nd, not determined

[a] Stained cells were defined as having a fluorescence peak channel number of greater than 50

Although viable *B. subtilis* and *P. fluorescens* cells could not be stained using Rh123, *B. subtilis* cells could be stained using FDA and *P. fluorescens* cells could be distinguished from gramicidin-treated cells using $DiOC_6(3)$ (Fig. 5). The staining of viable *B. subtilis* and other bacteria with FDA has been reported by other workers (Chrzanowski et al. 1984) but in our experiments, even though all the strains tested (except the *E. coli* strain) possessed esterase activity, only *B. subtilis* retained fluorescein long enough for the cells to be analysed by the FCM. The staining of *P. fluorescens* with $DiOC_6(3)$ was similar to that observed with both *S. aureus* and *E. coli*, therefore difficulties may be experienced in distinguishing living from dead cells in a mixed population. However since Rh123 failed to stain all but a small proportion of the cells, $DiOC_6(3)$ would appear to be the only vital stain tested that could be used to detect viable *P. fluorescens* cells by FCM.

In conclusion, fluorescent vital stains in conjunction with FCM can be used to rapidly detect and estimate the number of viable bacteria in a sample. The most useful stain tested was Rh123, but this was not found to be a universal vital stain. The choice of stain used depends on the bacteria being studied.

Acknowledgement. We would like to thank the Natural Environment Research Council (NERC) for funding this work (Grant no. GR3/7596).

References

Adams A, Bundy A, Thompson K, Horne MT (1988) The association between virulence and cell surface characteristics of *Aeromonas salmonicida*. Aquaculture 69:1–14

Bercovier H, Resnick M, Kornitzer D, Levy L (1987) Rapid method for testing drug-susceptibility of *Mycobacteria* spp. and Gram-positive bacteria using rhodamine 123 and fluorescein diacetate. J Microbiol Methods 7:139–142

Chrzanowski TH, Crotty RD, Hubbard JG, Welch RP (1984) Applicability of the fluorescein diacetate method of detecting active bacteria in freshwater. Microb Ecol 10:179–185

Darzynkiewicz Z, Traganos F, Staiano-Coico L, Kapuscinski J, Melamed MR (1982) Interactions of rhodamine 123 with living cells studied by flow cytometry. Cancer Res 42:799–806

Fry JC (1990) Direct methods and biomass estimation. Methods Microbiol 22:41–85

Johnson LV, Walsh ML, Chen LB (1980) Localisation of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci USA 77:990–994

Lundgren B (1981) Fluorescein diacetate as a stain of metabolically active bacteria in soil. Oikos 36:17–22

Mason CA, Hamer G, Bryers JD (1986) The death and lysis of microorganisms in environmental processes. FEMS Microbiol Rev 39:373–401

Matsuyama T (1984) Staining of bacteria with rhodamine 123. FEMS Microbiol Lett 21:153–157

Patchett RA, Back JP, Kroll RG (1990) Enumeration of bacteria by use of a commercial flow cytometer. J Appl Bacteriol 69:xxiii

Pinder AC, Purdy PW, Poulter SAG, Clark DC (1990) Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. J Appl Bacteriol 69:92–100

Resnick M, Schuldiner S, Bercovier H (1985) Bacterial membrane potential analyzed by spectrofluorocytometry. Curr Microbiol 12:183–186

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379

Ryan TA, Jointer BL, Ryan BI (1976) Minitab student handbook. Duxbury Press, North Scituate

Shapiro HM (1988) Practical flow cytometry. Liss, New York

Shapiro HM (1990a) Cell membrane potential analysis. Methods Cell Biol 33:25–35

Shapiro HM (1990b) Flow cytometry in laboratory microbiology: new directions. ASM News 56:584–588

Skowronek P, Krummeck G, Haferkamp O, Rodel G (1990) Flow cytometry as a tool to discriminate respiratory-competent and respiratory-deficient yeast cells. Curr Genet 18:265–26

Sokal RR, Rohlf FJ (1982) Biometry 2edn. Freeman and Co, New York