

## Flow Cytometric Analysis, Using Rhodamine 123, of *Micrococcus luteus* at Low Growth Rate in Chemostat Culture

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### Introduction

In microbiology it is often necessary to determine the number of viable cells in a sample or culture of interest. This is usually achieved by plating out the sample (diluted as required) on to an agar plate (Postgate 1969; Hattori 1988). There are several problems associated with this technique, the greatest of which is the length of time required to obtain the results. For some slowly growing organisms (e.g. *Mycobacteria*) it may take in excess of a week to determine how many cells were "viable" in the original sample, and even when the sample contains fast-growing organisms and the plates are incubated under optimal growth conditions a minimum of overnight growth is usually required before the resulting colonies can be counted. For some clinical specimens even an overnight incubation may be too long to be of use and consequently many alternatives to plate counts have been proposed in order to decrease the time required to determine numbers of viable cells (Harris and Kell 1985).

Many of these so-called rapid methods involve the use of dyes to stain the cells. These include the DNA stain acridine orange (McFeters et al. 1991) that is used on the (rather doubtful) assumption that nucleic acids are degraded rapidly following cell death, such that "viable" cells are stained differently from "dead" ones (see Back and Kroll 1991). Other viability stains such as methylene blue exploit the ability of the intact membrane of viable cells to exclude the dye (Jones 1987; Stoicheva et al. 1989). Dye exclusion and DNA-staining methods rely mainly on microscopic examination of the stained cells to assess viability, and for this reason they are slow to perform and are prone to subjective error.

Plate counts, although usually considered to be a measure of viability, actually indicate only how many of the cells can replicate under the conditions provided for growth, which are likely to differ from those in the original sample (Roszak and Colwell 1987). Viability staining meanwhile provides information on how many of the cells can exclude the dye (i.e. how many of the cells have intact cell membranes). However, there is abundant

evidence that cells may exist in a state intermediate between being unable to replicate on an agar plate, and being dead as judged by viability stains (Postgate 1976; Jones 1987; Roszak and Colwell 1987). With conventional viability staining, microscopic examination is used to determine whether the cells are alive. By this method a judgement of "alive" or "dead" is all that is possible.

An alternative method of determining the amount of dye taken up by each cell is the technique of flow cytometry (Shapiro 1988; Pollack and Ciancio 1990). As described by many other authors in this volume, and recently reviewed elsewhere (Kell et al. 1991), flow cytometry is a technique that enables measurements to be made very rapidly on a large number of cells. Since the measurements are made on individual cells rather than on populations, differences in dye uptake between cells can be assessed quantitatively. Using supra-vital stains, estimates of the "degree of viability" of individual cells can thus be made, allowing one to *quantify* the heterogeneity of the cell population (Kell et al. 1991).

In a flow cytometer particles pass rapidly in single file through an illumination zone, and appropriate detectors measure the amount of light scattered or, via suitable filters, the fluorescence of the particle (Shapiro 1988). The data are converted into pulses, the magnitude of which represent the amount of scattered light, and/or the fluorescence of each cell as it passes through the illumination zone. The pulses are "binned" into channels that increase in number with increasing levels of scattered light or fluorescence. The data are then usually plotted as a histogram on which the abscissa represents channel numbers and the ordinate represents the number of cells measured in each channel (Dean 1990).

In conventional flow cytometers the source of the illumination is a laser (Shapiro 1988). However, given that both the cell volume and the DNA content of bacteria are some 1000-fold less than those of higher eukaryotic cells, laser-based flow cytometers have until recently proved unsuitable for the study of microorganisms (Steen et al. 1990). In the Skatron Argus 100 flow cytometer (Boye et al. 1983; Steen et al. 1990; Boye and Løbner-Olesen 1991) a high-pressure mercury arc lamp is used as the excitation source and this has proved to be more suited to measurements of microorganisms.

It is well documented that the mitochondria of eukaryotic cells have the ability to concentrate "lipophilic" cations such as rhodamine 123 (e.g. Johnson et al. 1980, 1981; Chen et al. 1982; Chen 1988; Grogan and Collins 1990) in an uncoupler-sensitive fashion, and the staining of mitochondria with rhodamine 123, in conjunction with flow cytometry, has been used to study their activity (Darzynkiewicz et al. 1981; Iwagaki et al. 1990; Lizard et al. 1990). Viable bacteria also accumulate rhodamine 123 but non-viable ones do not, and under appropriate conditions the extent to which individual bacteria take up rhodamine 123 quantitatively reflects *the extent of* their viability (Kaprelyants and Kell 1992).

On average, larger cells may be expected to accumulate more molecules of rhodamine 123 than do smaller cells, but since flow cytometry allows collection of both fluorescence (rhodamine 123 uptake) and forward light scattering (cell size) from each cell, the data can thus be plotted as a dual-

parameter histogram, enabling one to take size differences between cells into account when interpreting the data.

In contrast to some of the other viability stains (e.g. acridine orange), the uptake of rhodamine 123 not only does not require the use of fixatives to permeabilize the cell, but the concentrative uptake is dependent on an intact and energized cytoplasmic membrane; thus living cells can be used for staining purposes. This has the great advantage that, following staining of the cells, further physiological studies may be conducted if required. Provided that at the concentrations used the dye does not affect the viability of the cells in any way (which we show herein to be true), rhodamine 123 may be expected to be a good probe for assessing the energetic status and/or the viability of gram-positive cells.

## Materials and Methods

### Organism

*Micrococcus luteus* (Fleming Strain 2665) was used throughout. It was maintained on nutrient agar slopes at 4 °C. The culture was resuscitated in rich liquid medium and streaked onto an agar plate to ensure that the culture was axenic.

### Media

The growth medium (lactate minimal medium) used for all continuous culture work was prepared as described in Kaprelyants and Kell (1992). This medium contained (in mg/l):  $\text{NH}_4\text{Cl}$  4000,  $\text{KH}_2\text{PO}_4$  1400, biotin 5, L-methionine 20, thiamine 40, inosine 1000,  $\text{MgSO}_4$  70,  $\text{CuSO}_4$  0.024,  $\text{MnCl}_2$  0.5,  $\text{FeSO}_4$  1,  $\text{Na}_2\text{MoO}_4$  0.025,  $\text{ZnSO}_4$  0.05, and lithium L-lactate 1000. The pH was adjusted to 7.5 with NaOH prior to autoclaving (121 °C, 25 min).

For batch culture a "rich" medium containing 1.3% (w/v) nutrient broth E, pH 7.4 (Lab M), was used.

### Carbon-Limited Continuous Culture

Aerobic continuous culture was carried out in an LH Fermentation (Maidenhead, Berks) 500 series fermentor with a working volume of 500 ml. The temperature was controlled at 30 °C. The dilution rate was set using a Pharmacia (Bromma, Sweden) Model P1 peristaltic pump connected to a tube that passed below the surface of the broth, ensuring pulse-free addition of fresh medium. The dilution rate was 0.01/h and at least five volume changes were allowed to elapse prior to the removal of samples for flow cytometry.

## Flow Cytometry

Flow cytometric experiments were carried out as described previously (Davey et al. 1990; Kaprelyants and Kell 1992) using a Skatron Argus 100 flow cytometer (Skatron Ltd, PO Box 34, Newmarket, Suffolk). The instrument was set up as described in the manual with an additional  $0.1\text{ }\mu\text{m}$  filter placed in the sheath fluid line; sodium azide ( $1\text{ mmol/l}$  final concentration) was added to the filtered sheath fluid. The sample flow rate was set at  $0.5$  or  $1\text{ }\mu\text{l/min}$  and a sheath fluid pressure of  $1.5\text{--}2\text{ kPa/cm}^2$  was maintained.

In the present work data were collected from the forward angle (LS1) detector and from a fluorescence detector (FL1) designed for use with fluorescein isothiocyanate (FITC filter). The optical characteristics of this filter are: excitation  $470\text{--}495\text{ nm}$ , band-stop  $510\text{ nm}$ , emission  $520\text{--}550\text{ nm}$ ; these are suitable for use with rhodamine 123.

The flow cytometer was run under the control of a Viglen IHHDE micro-computer (IBM-PC-AT-compatible, 80286 processor, EGA screen), with software supplied by Skatron. This provides data files in a standard format (Murphy and Chused 1984). The photomultiplier voltages were set at  $500$  and  $650\text{ V}$  for the light scattering and fluorescence channels, respectively, and all measurements were gated by the light scattering channel. The forward light scatter and rhodamine fluorescence measured for each cell were "binned" to give histograms of counts versus channel number. An in-house file conversion program written in Microsoft Quick BASIC v4.5 was used to import the data into the spreadsheet program FLOWTOVP. WKS (Davey et al. 1990). Assessment of peak channel numbers was performed on the raw data, whilst data displayed were first subjected to a three-point smoothing routine.

Monodisperse ( $\text{cv} < 2\%$ ) latex particles were obtained from the Sigma Chemical Company, Poole, Dorset and from Dyno Particles A/S, PO Box 160, Lillestrøm, Norway.

## Preparation of Samples for Flow Cytometry

Samples were removed from the chemostat and placed, in  $1\text{ ml}$  aliquots, into Eppendorf tubes. Rhodamine 123 (Sigma) was dissolved in absolute ethanol at a concentration of  $1\text{ mmol/l}$ . From this stock solution several dilutions (in ethanol) were made. The amount of rhodamine solution added to each aliquot of cells was kept constant at  $1\text{ }\mu\text{l}$ ; the addition of  $1\text{ }\mu\text{l}$  ethanol to  $1\text{ ml}$  of cells (i.e.  $0.1\%$  ethanol) was observed to have no effect on cell viability (data not shown). Cell samples were incubated with the rhodamine 123 for  $30\text{--}40\text{ min}$  at room temperature prior to analysis by flow cytometry.

## Cell Viability Measurements

Standard pour plates consisting of  $1.3\%$  Nutrient Broth E (Lab M) solidified with  $1.5\%$  purified agar (Lab M) were used to assess cell viability. Serial dilutions of the cell samples from the chemostat were made in sterile



phosphate buffer (pH 7.4) at room temperature. Two replicate plates at a suitable dilution ( $10^{-6}$  of original sample) were incubated at 30°C for 4 days and counted manually. To determine whether the addition of rhodamine 123 at the highest concentration used for flow cytometry had any effect on the viability of the cells, rhodamine 123 ( $1\mu\text{mol/l}$  final concentration) was added to an identical diluted sample of cells from the chemostat prior to their being plated out. Rich medium was used in the preparation of these plates, but as shown earlier (Kaprelyants and Kell 1992) there was no difference in viabilities seen between these plates and plates consisting of lactate minimal medium solidified with agar.

### Total Cell Counts

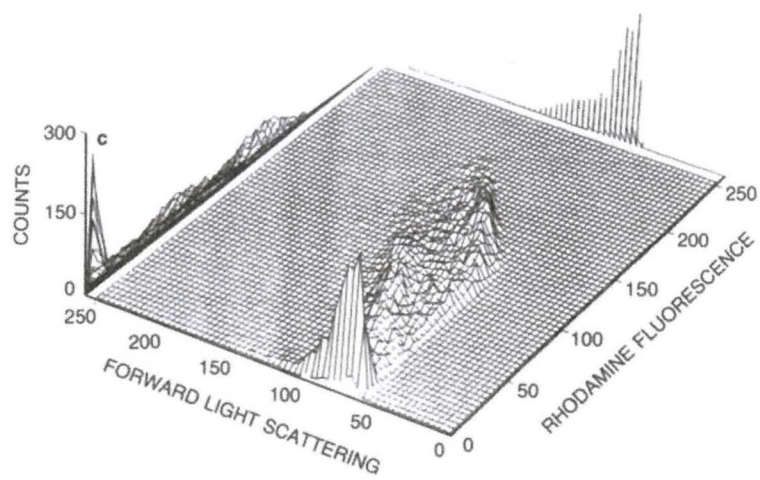
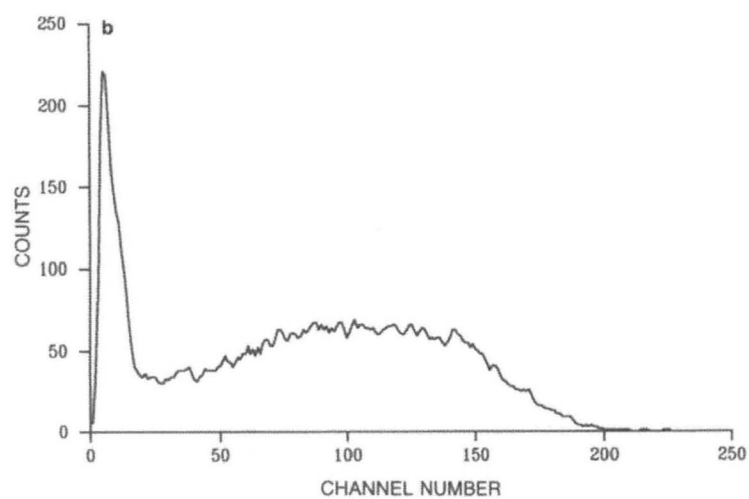
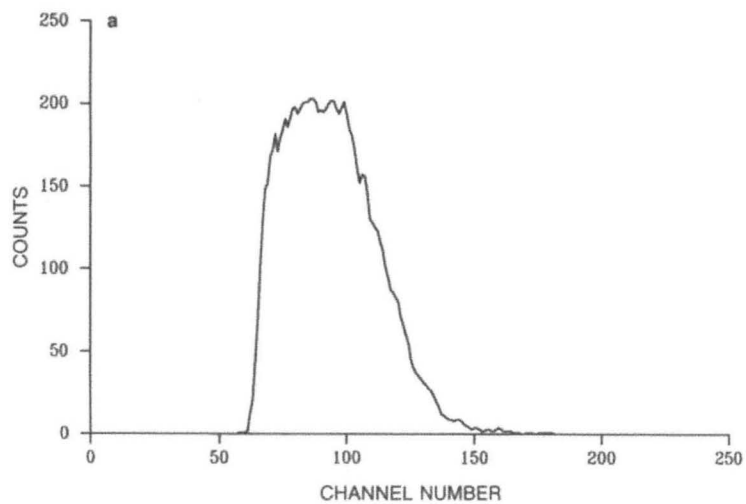
Total numbers of cells were determined by manually counting unstained cells loaded into an improved Neubauer counting chamber. Because of the small size (less than  $1\mu\text{m}$ ) of the cells the counts were performed under oil-immersion at a total magnification of  $\times 1000$ .

### Chemicals

All chemicals were obtained from Sigma or BDH. Water was singly-distilled in all glass apparatus; water used in the sheath fluid of the flow cytometer was treated with a Millipore MilliQ apparatus.

## Results

Figure 6.1 shows the light scattering and fluorescence properties of cells of *M. luteus* grown in C-limited continuous culture and prepared and stained with rhodamine 123 as described above. Previous work had shown that under these conditions the uptake of the dye is essentially completely reversed by the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (Kaprelyants and Kell 1992). It can be seen here (Fig. 6.1b) that the uptake of rhodamine 123 varies substantially between the individual cells in the population as, to a lesser extent, does the forward light scatter or cell size (Fig. 6.1a). For a given internal concentration, large cells will obviously take up more rhodamine 123 than will small cells, but it is apparent from the dual-parameter histogram (Fig. 6.1c) that even cells of the same size exhibit a broad range of fluorescences. It can also be seen (Fig. 6.1b,c) that under these conditions there is a continuous variation of fluorescence between individuals, from those with very low fluorescences (dead cells) to those with high fluorescence (viable, culturable cells). The cells fluorescing between these extremes will include damaged cells which, given suitable conditions, could be resuscitated and returned to a culturable state (Kaprelyants and Kell 1992). The viability of this particular culture, as judged by a comparison between plate counts and total cell counts, was some 14%. This is somewhat lower than that (c. 40%) observed previously for a different run



(Kaprelyants and Kell 1992), and may be ascribed to the greater length of time for which this low growth rate had been enforced in the present work.

Figure 6.2 shows the effect of varying the concentration of rhodamine 123 added to the cells prior to flow cytometry. From the lowest concentration of rhodamine used ( $0.03 \mu\text{mol/l}$  Fig. 6.2a) up to  $0.5 \mu\text{mol/l}$  (Fig. 6.2d) the peak channel number of the fluorescence histogram increases. Increasing the rhodamine 123 concentration above this level does not further increase the peak channel number of the fluorescence (Fig. 6.2e). With concentrations of rhodamine of  $0.5 \mu\text{mol/l}$  and below there is a good fit by linear regression (correlation coefficient = 0.99) to a plot of the peak channel number of fluorescence versus the rhodamine concentration (Fig. 6.3). In other words, within this linear region the extent to which a cell will take up the dye is, as expected, proportional to the dye concentration. This provides a novel and convenient method for effecting a calibration of the flow cytometer.

From Fig. 6.3 it can be seen that if an average (modal) cell from the population is stained with  $0.4 \mu\text{mol/l}$  rhodamine one would expect it to fluoresce at channel number 131. If the same cell were stained with  $0.2 \mu\text{mol/l}$  rhodamine then it would fluoresce at channel number 102. For any population histogram of rhodamine fluorescence such as those shown in Fig. 6.1b or 6.2a–e, one can therefore say that a cell fluorescing at channel 131 has taken up twice as much rhodamine as a cell fluorescing at channel 102. By repeating this argument for other rhodamine concentrations within the linear region (Fig. 6.3), one can conclude that a difference in peak channel number of 65 units corresponds to a factor of 10 in cell fluorescence. Thus it is possible to obtain a *quantitative* idea of the extent of the heterogeneity of the sample (Fig. 6.4).

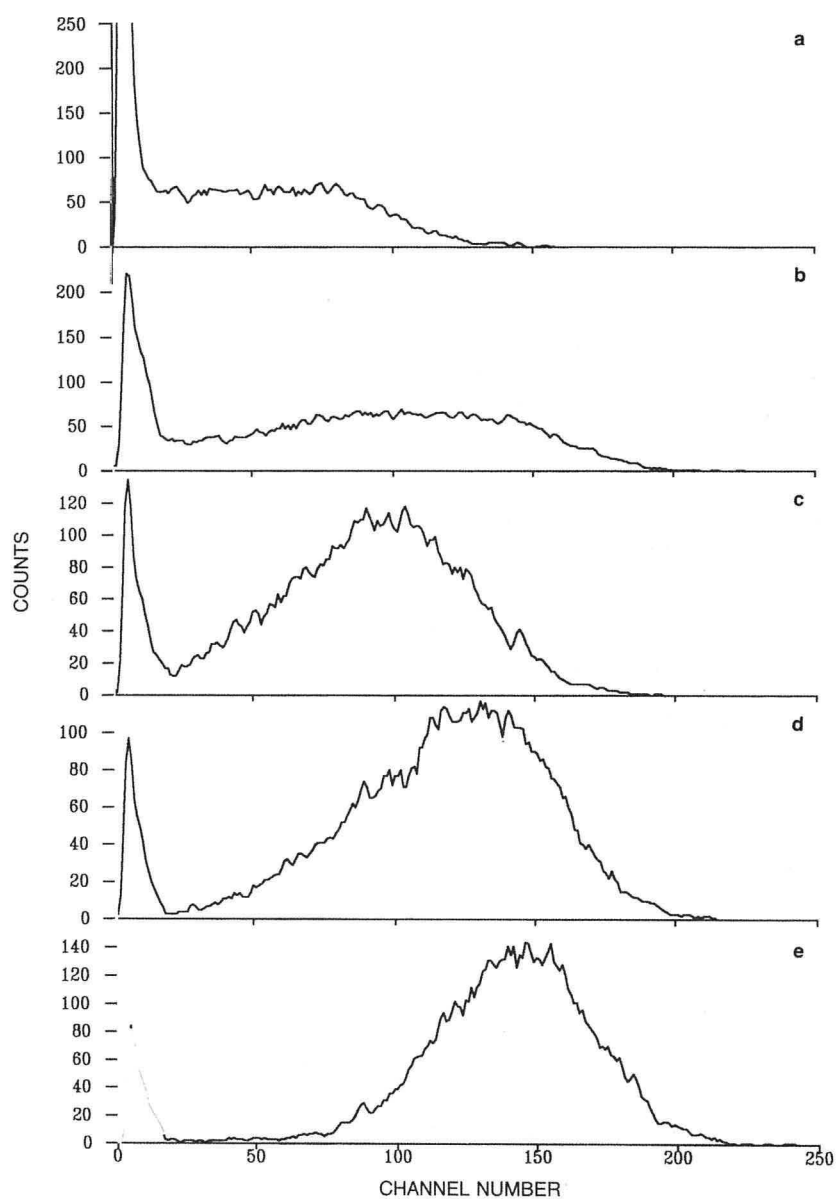
Finally, the addition of  $1 \mu\text{mol/l}$  rhodamine 123 to cells prior to plating them out did not affect their viability, since there were, within experimental error, the same number of colonies produced by the cell samples irrespective of whether rhodamine had been added (data not shown).

## Discussion

In many microbiological studies it is essential, or at least desirable, to be able to determine the number of viable cells in a sample. Plate counts are the usual way of obtaining viable counts, but the method is slow and does not necessarily give a measure of the number of *viable* cells; more accurately it measures the number of *culturable* cells. Viability staining followed by microscopic examination should give a more accurate measurement but several hundred cells have to be examined for the results to be statistically

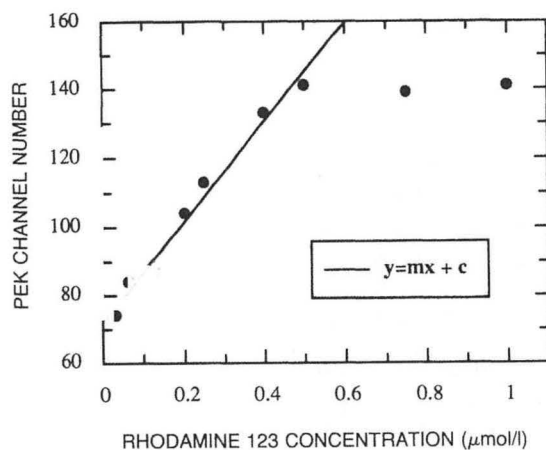
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**Fig. 6.1.a–c.** Flow cytometry of *Micrococcus luteus* stained with  $0.06 \mu\text{mol/l}$  rhodamine 123. There is heterogeneity in both the forward light scattering (**a**) and the rhodamine 123 fluorescence (**b**) of the sample. However the dual-parameter histogram of forward light scatter and fluorescence (**c**) shows that even cells of comparable size exhibit a broad range of fluorescences (rhodamine uptake).

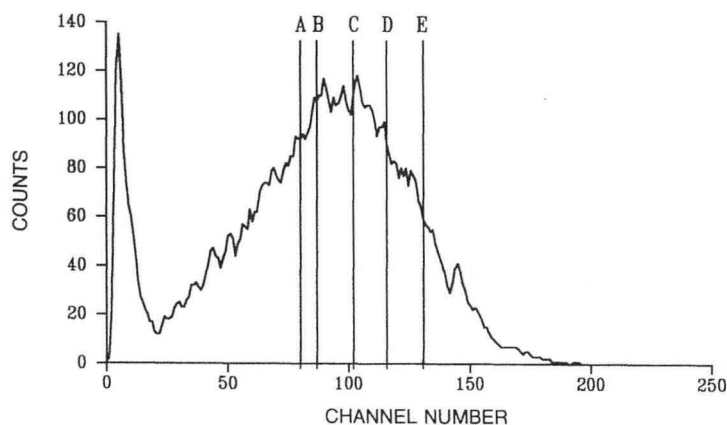


**Fig. 6.2.a-e.** Increasing the concentration of rhodamine 123 up to  $0.5 \mu\text{mol/l}$  increases the peak channel number of fluorescence (a-d). Increasing the rhodamine concentration above  $0.5 \mu\text{mol/l}$  does not increase the fluorescence further (e). Peak channel numbers and rhodamine 123 concentration ( $\mu\text{mol/l}$ ) for each sample were as follows: **a** 74, 0.03; **b** 87, 0.125; **c** 104, 0.2; **d** 141, 0.5; **e** 141, 1.





**Fig. 6.3.** The relationship between rhodamine 123 concentration and the peak channel number of fluorescence is well fitted by linear regression ( $r = 0.99$ ) at concentrations of  $0.5 \mu\text{mol/l}$  and below. Using higher concentrations of rhodamine 123 does not further increase the fluorescence.



**Fig. 6.4.** The extent of rhodamine 123 uptake between individual cells is very heterogeneous. Cells were actually stained with  $0.2 \mu\text{mol/l}$  rhodamine 123. Lines A–E represent the expected fluorescence of an average (modal) cell stained with different concentrations of rhodamine 123. These concentrations (in  $\mu\text{mol/l}$ ) are as follows: A 0.05, B 0.1, C 0.2, D 0.3, E 0.4. It may be seen that the most fluorescent cells have taken up almost  $\times 1000$  the amount of dye taken up by the most weakly fluorescent, but non-dead, cells (at approx. channel 20).

meaningful. As a consequence these tests are tedious to perform, and as such are prone to error.

Cells stained with a suitable viability stain can be studied by flow cytometry, a technique that allows rapid acquisition of data. Furthermore, flow cytometry enables judgements to be made on the “degree of viability” (in terms of dye uptake) of *each* cell in the sample, allowing quantification of

the heterogeneity of "viability" within the sample. Such a technique has potential for studies of the mechanisms of cell death (Kell et al. 1991; Kaprelyants and Kell 1992). Since, in suitably equipped flow cytometers, multiparameter fluorescence measurements can be made it may be possible to study cell viability in conjunction with other cellular parameters, in order to determine whether they have any role in, or correlation with, cell death.

Since rhodamine 123 does not affect the viability (culturability) of the cells as judged by plate counts, staining of cells does not rule out their use in subsequent physiological studies. In flow cytometers equipped with sorting facilities a fraction of the viable cells could be selected for further study.

We conclude that provided the dye concentration is kept below  $0.5 \mu\text{mol/l}$ , flow cytometry in conjunction with rhodamine 123 staining provides a rapid method of assessing cell viability. Furthermore because of the nature of flow cytometric data there are many possibilities for further studies of viability that could not be easily undertaken using any other existing technique.

### Acknowledgements

We thank the Science and Engineering Research Council, UK, and the Royal Society, under the terms of the Royal Society/USSR Academy of Sciences exchange agreement, for financial support of this work.

## References

- Back JP, Kroll RG (1991) The differential fluorescence of bacteria stained with acridine orange, and the effects of heat. *J Appl Bacteriol* 71:51–58
- Boye E, Løbner-Olesen A (1991) Bacterial growth control studied by flow cytometry. *Res Microbiol* 142:131–135
- Boye E, Steen HB, Skarstad K (1983) Flow cytometry of bacteria: a promising tool in experimental and clinical microbiology. *J Gen Microbiol* 129:973–980
- Chen LB (1988) Mitochondrial membrane potential in living cells. *Annu Rev Cell Biol* 4: 155–181
- Chen LB, Summerhayes IC, Johnson LV, Walsh ML, Bernal SD, Lampidis TJ (1982) Probing mitochondria in living cells with rhodamine 123. *Cold Spring Harbor Symp Quant Biol* 46:141–155
- Darzynkiewicz Z, Staiano-Coico L, Melamed MR (1981) Increased mitochondrial uptake of Rhodamine 123 during lymphocyte stimulation. *Proc Natl Acad Sci USA* 78:2383–2387
- Davey CL, Dixon NM, Kell DB (1990) FLOWTOVP: A spreadsheet method for linearizing flow cytometric light-scattering data used in cell sizing. *Binary* 2:119–125
- Dean PN (1990) Data processing. In: Melamed MR, Lindmo T, Mendelsohn ML (eds) *Flow cytometry and sorting*, 2nd edn. Wiley-Liss, New York
- Grogan WM, Collins JM (1990) *Guide to flow cytometry methods*. Marcel Dekker, New York
- Harris CM, Kell DB (1985) The estimation of microbial biomass. *Biosensors* 1:17–84
- Hattori T (1988) *The viable count: quantitative and environmental aspects*. Springer, Berlin
- Iwagaki H, Fuchimoto S, Miyake M, Oirta K (1990) Increased mitochondrial uptake of rhodamine 123 during interferon-gamma stimulation in Molt 16 cells. *Lymphokine Res* 9: 365–369
- Johnson LV, Walsh ML, Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci USA* 77:990–994
- Johnson LV, Walsh ML, Bockus BJ, Chen LB (1981) Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J Cell Biol* 88:526–535
- Jones RP (1987) Measures of yeast death and deactivation and their meaning. Part 1. *Process Biochem* 22:118–128

- Kaprelyants AS, Kell DB (1992) Rapid assessment of bacterial viability and vitality using rhodamine 123 and flow cytometry. *J Appl Bacteriol* 72:410–422
- Kell DB, Ryder HM, Kaprelyants AS, Westerhoff HV (1991) Quantifying heterogeneity: flow cytometry of bacterial cultures. *Ant Van Leeuw* 60:145–158
- Lizard G, Chardonnet Y, Chignol MC, Thivolet J (1990) Evaluation of mitochondrial content and activity with nonyl-acridine orange and rhodamine 123: flow cytometric analysis and comparison with quantitative morphometry. *Cytotechnology* 3:179–188
- McFeters GA, Singh A, Byun S, Callis PR, Williams S (1991) Acridine orange staining as an index of physiological activity in *Escherichia coli*. *J Microbiol Meth* 13:87–97
- Murphy RF, Chused TM (1984) A proposal for a flow cytometric data file standard. *Cytometry* 5:553–555
- Pollack A, Ciancio G (1990) Cell cycle phase-specific analysis of cell viability using Hoechst 33342 and propidium iodide after ethanol preservation. In: Darzynkiewicz Z, Crissman HA (eds) *Flow cytometry*. Academic Press, San Diego
- Postgate JR (1969) Viable counts and viability. *Meth Microbiol* 1:611–628
- Postgate JR (1976) Death in microbes and macrobes. In: Gray TRG, Postgate JR (eds) *The survival of vegetative microbes*. Cambridge University Press, Cambridge, pp 1–19
- Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. *Microbiol Rev* 51:365–379
- Shapiro HM (1988) *Practical flow cytometry*, 2nd edn. Alan R Liss, New York
- Steen HB, Skarstad K, Boye E (1990) DNA measurements of bacteria. In: Darzynkiewicz Z, Crissman HA (eds) *Flow cytometry*. Academic Press, London
- Stoicheva NG, Davey CL, Markx GH, Kell DB (1989) Dielectric spectroscopy: a rapid method for the determination of solvent biocompatibility during biotransformations. *Biocatalysis* 2:245–255