 Cytometry 28:311–315 (1997)

Fluorescent Brighteners: Novel Stains for the Flow Cytometric Analysis of Microorganisms

Hazel M. Davey* and Douglas B. Kell

Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed, United Kingdom

Received 1 October 1996; Accepted 18 April 1997

Flow cytometry is a rapid method for measuring the optical properties of individual cells. The technique has found great utility in the study of mammalian cells, but microbiological applications have been more limited. We here show that UV-excited fluorescent whitening agents, in particular Tinopal CBS-X, are effective stains for both vegetative microbial cells and for spores of Gram-positive bacteria. Pretreatment of samples with ethanol speeds the staining process. Under favourable conditions, Tinopal CBS-X may be used to discriminate among organisms, a fact that may be useful when screening for a target microorganism against a high biological background. Cytometry 28:311–315, 1997.

Key terms: bacteria; discrimination; ethanol fixed; fluorescent brightener; microorganism

A wide variety of fluorescent stains have been used in flow cytometric studies (35) and include those which bind to molecules such as DNA (2,27,15), RNA (28,1,4), or protein (31,20,5), fluorogenic substrates for measuring the activity of a given enzyme (10,37,9,14), indicators of intracellular pH (12,34), fluorescent stains which are taken up or excluded by cells in a manner which reflects the intactness of their membranes (7,16), and, increasingly, antibodies (8) or oligonucleotides (36) 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 [the *Escherichia coli* chromosome is about 1,400 times smaller than that of a human diploid cell (33)] and other cellular constituents had proved to be below the resolution of most commercial flow cytometers. 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 (for recent reviews, see, e.g., 17,29,6,26).

Flow cytometers such as the Skatron Argus devised by Steen and colleagues (32) have been designed specifically for the analysis of microorganisms, and as such are fully capable of analysing bacteria without the requirement that they are stained. In other commercial flow cytometers, it is often desirable that bacteria are stained to aid separation of their signal from the background noise.

Fluorescent brighteners are stains which are widely used as additives in domestic washing powders to improve the apparent ''whiteness'' of clothes; they exert this effect by absorbing otherwise invisible UV light and giving off a blue fluorescence (11,38). They have occasionally been used in fluorescence microscopy (21) and are known to interact with a number of microbial cells (22–25). Some, such as calcofluor white, are well-known stains of the fungal cell wall, and have been exploited in flow cytometric analyses (13). The use of Tinopal LN was reported for the flow cytometric analysis of blood cells (30); however, we are not aware of the previous use of these types of molecules in the flow cytometric analysis of bacteria, except for a recent report (19) in which calcofluor white was used in a comparison with other stains to assess bacterial viability. As part of a study designed to explore the utility of cocktails of stains for the detection and discrimination of microbial cells, it therefore occurred to us that fluorescent brightening agents might be ideal components of a staining mixture, because 1) they are widely available at low cost, 2) they are nontoxic to higher organisms, 3) they have a high quantum yield, and 4) since they are excited by UV light, they should be ideal for use in staining cocktails in conjunction with other stains such as fluorescein isothiocyanate without significant interference.

In the work described herein, we show that fluorescent brightening agents can indeed serve as excellent stains for a variety of microorganisms and spores under flow cytomet-

Contract grant sponsor: United States Army, European Research Office; Contract grant sponsor: the Chemicals and Pharmaceuticals Directorate of the BBSRC.

*Correspondence to: Hazel M. Davey, Edward Llwyd Building, Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed, SY23 3DA, UK.

E-mail: HLR@aber.ac.uk

ric conditions, and can offer a level of discrimination among organisms.

MATERIALS AND METHODS

Fluorescent Brighteners

Four different fluorescent brighteners were studied in terms of their usefulness in staining *Bacillus subtilis* var *niger* (*B. globigii*) spores. Tinopal CBS-X, Tinopal DMS-X, and Tinopal SWN were obtained as a gift from Ciba Dyes and Chemicals (Macclesfield, UK) and Calcofluor White M2R was purchased from Sigma (Poole, UK).

Sample Preparation

B. globigii spores were obtained from the CBDE (Porton Down, Salisbury, UK) as a dry preparation. Prior to analysis, the spores were suspended in sheath fluid (see below) to give a concentration of approximately $1x10^6$ spores ml^{-1}. Unstained samples were run without further preparation. *E. coli* (Lab Strain C500) were grown on a medium containing 1% tryptone, 1% yeast extract and 70 $mg.l^{-1}$ $MgSO_4$. The medium was adjusted to pH 6.8 with HCl or KOH prior to autoclaving at 121°C for 15 min. Cells were grown in batch culture at a temperature of 37°C on a shaker for 3 days. *Micrococcus luteus* (NCIMB 13267) were grown on E-Broth (Lab M) on a shaker at 30°C for 3 days.

A strain of *Saccharomyces cerevisiae* was isolated from locally obtained baker's yeast and grown on YPG medium which contained 5% glucose, 0.5% yeast extract, and 0.5% bacteriological peptone. The medium was adjusted to pH 5 with phosphoric acid prior to autoclaving. Temperature was maintained at 30°C but the culture flask was not agitated during the 3-day incubation.

Fixed cells or spores were prepared by squirting a suspension of spores or cells from a syringe into ethanol to give a final ethanol concentration of 70%. Fixed samples could be stored at -20°C for several months without noticeable deterioration. All fixed samples were centrifuged and washed prior to resuspension in sheath fluid for flow cytometric analysis. Fixed samples were analysed within 2 hr of removal of the fixative.

Fluorimetry

Fluorimetry was performed using a Shimadzu RF-5301PC spectrofluorophotometer. Aqueous samples of the fluorescent brighteners were placed into 3 ml cuvettes (1 cm light path) for analysis. An excitation wavelength of 325 nm was chosen, as this was the UV wavelength available in the flow cytometer (see below). Emission scans were performed by measuring the intensity of the emitted light at 1 nm wavelength intervals between 300 and 800 nm.

Flow Cytometry

All flow cytometric analyses were performed using a Coulter Epics Elite flow cytometer (Coulter Electronics, Luton, UK) using a HeCd laser (325 nm) and an argon ion laser (488 nm) for excitation. The flow cytometer was set up as described in the manufacturer's 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 sheath fluid was adjusted to pH 6.8 with KOH and then filtered using a 0.1 µm Whatman WCN filter. Prepared sheath fluid was stored at 4°C but was allowed to reach room temperature before use. The lasers were aligned so that the sample intersected the HeCd laser 40 µsec after intersecting the argon ion laser. The signals were then recombined using the gated amplifier electronics. We have found that use of the gated amplifier improves resolution of bacterial scatter signals from the noise and in the present case the forward scatter signal from the argon laser was found to be a suitable trigger. On a channel number scale of 0–1,023 the scatter intensity from the background noise was all below channel 50, while light scatter for the microbial cultures was above channel 325. The wavelengths over which fluorescence was collected, together with the photomultiplier voltages used, are given in the legends to the appropriate figures.

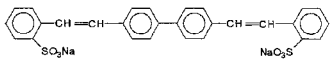
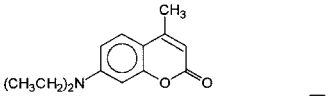
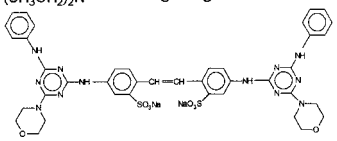
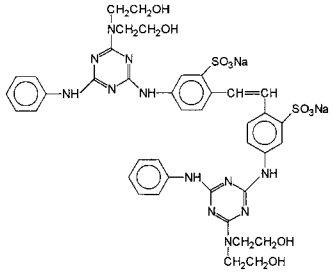
RESULTS

The majority of flow cytometric analyses are performed in aqueous media and so the ideal stain must be soluble in water at an appropriate concentration. The first test therefore, was to determine the solubilities of the fluorescent brighteners in water. Tinopal CBS-X and Calcofluor White M2R were fully soluble at the highest concentration used (10 $mg.ml^{-1}$). Tinopal SWN was less soluble and Tinopal DMS-X failed to dissolve even at 0.1 $mg.ml^{-1}$ (Table 1). Thus, based on these results we excluded Tinopal DMS-X from further study.

For analysis by excitation with the HeCd laser, it was essential that the stains fluoresced when illuminated with light at 325 nm. This was tested using a fluorimeter with the excitation wavelength set to 325 nm and emission collected at 1 nm intervals between 300 and 800 nm (Fig. 1). The peak at 325 nm seen with all samples is due to light scattering. Peak fluorescence emission wavelengths for the three brighteners were all between 430 and 436 nm. It can clearly be seen that Tinopal CBS-X gave the brightest fluorescence. The results obtained from flow cytometric analysis of stained *B. globigii* spores (not shown) also showed that Tinopal CBS-X gave the brightest fluorescence; fluorescence from Calcofluor White M2R-stained spores could also be detected but spores stained with Tinopal SWN were not appreciably more fluorescent than unstained spores. The structures of the fluorescent brighteners are shown in Table 1 and it can be seen that the extent of staining is correlated with the ionic character of the stain, which may indicate the target of the molecules within the spore.

Thus, from these preliminary results we decided to use Tinopal CBS-X as our dye of choice in subsequent studies. The effect of concentration on the extent of staining of the spores with Tinopal CBS-X was investigated by using a range of concentrations (0–100 $\mu g.ml^{-1}$) of the stain with a suspension of unfixed spores. Figure 2 shows that between 0 and 40 $\mu g.ml^{-1}$ the extent of staining increases with concentration of the stain, but that on more than

Table 1
Characteristics of the Four Fluorescent Whitening Agents Studied

Fluorescent brightener	Ionic character	Chemical structure	Solubility in water (mg · ml^{-1})
Tinopal CBS-X	Strongly anionic		>10
Tinopal SWN	Weakly cationic		1
Tinopal DMS-X	Anionic		<0.1
Calcofluor White (Tinopal LPW)	Anionic		>10

While low concentrations (μg.ml^{-1}) are typically used in flow cytometric analyses, it is usually desirable to make stock suspensions of dyes at 1 mg.ml^{-1} or higher. Tinopal DMS-X was excluded from further analysis since it had a low solubility in water <100 μg.ml^{-1}. The remaining three were all soluble at 1 mg.ml^{-1} or higher, although Calcofluor White was slow to dissolve in cold water at this concentration.

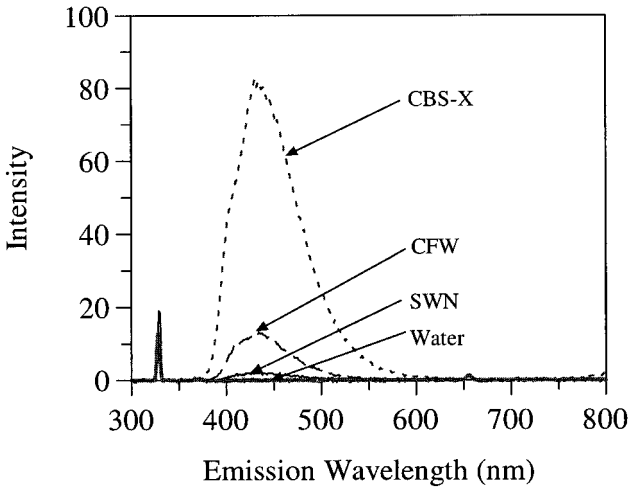


FIG. 1. Fluorimetric analysis of the three soluble fluorescent brighteners (CFW = Calcofluor White). The excitation wavelength was set to 325 nm and fluorescence emission was collected at 1 nm intervals from 300 to 800 nm. The concentration of the fluorescent brighteners was 0.33 μg.ml^{-1} in each case. Intensity is measured in arbitrary units.

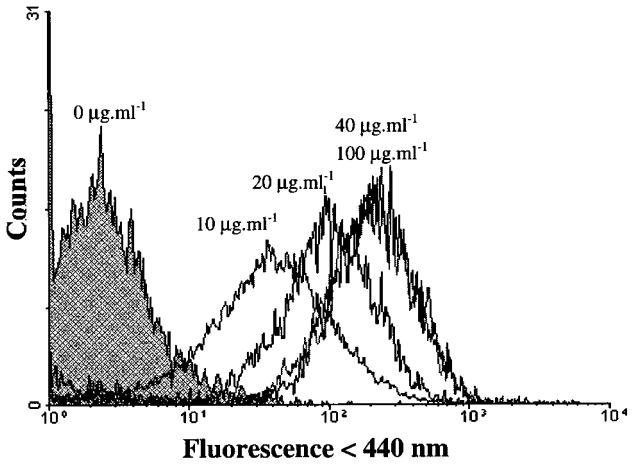


FIG. 2. *B. globigii* spores stained with Tinopal CBS-X. The flow cytometric analysis was carried out as described in Materials and Methods. Fluorescence resulting from excitation with the HeCd laser (325 nm) was collected below 440 nm (to exclude the strong 488 nm side scatter signal from the Argon ion laser) on PMT1. The voltage was 630 and the amplification was logarithmic.

doubling the concentration to 100 μg.ml^{-1}, there is no further increase in fluorescence.

Figure 3 illustrates the time dependency of staining of the *B. globigii* spores with Tinopal CBS-X. While some 20 min are required for maximum staining to be achieved with unfixed spores (Fig. 3A), following fixation of the spores in 70% ethanol (Fig. 3B) maximum staining is reached immediately.

The general applicability of Tinopal CBS-X for staining of microbial samples is demonstrated in Figure 4, where the stain has been used with other representative microorganisms including Gram-positive and -negative strains and

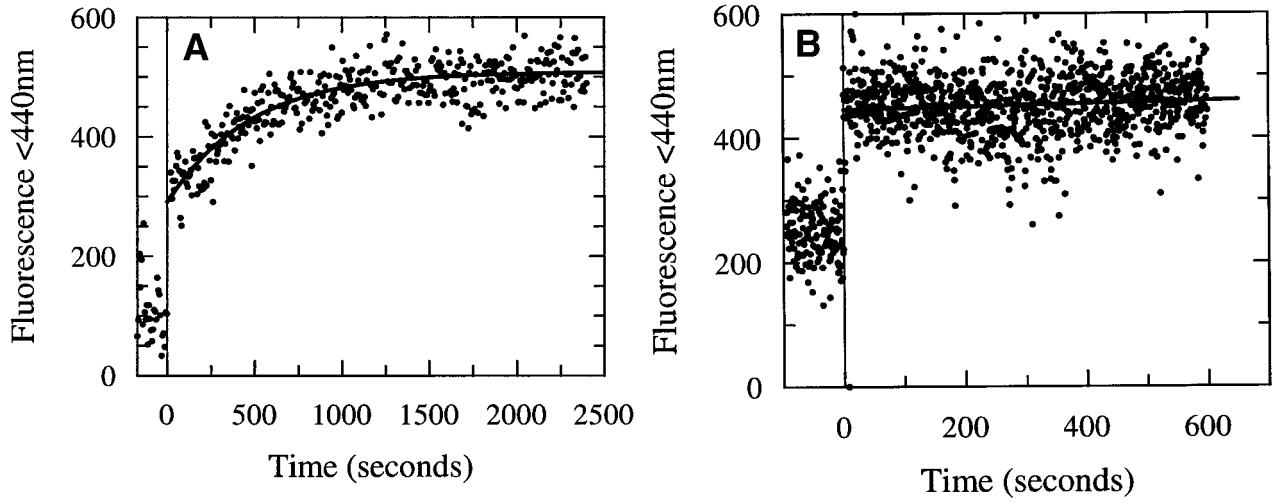


FIG. 3. Kinetics of staining of *B. globigii* spores with 10 μg.ml^{-1} Tinopal CBS-X. (**A**) Unfixed cells, (**B**) fixed cells. Samples were prepared as described in Materials and Methods and the flow cytometer was set up as described in the legend to Figure 2.

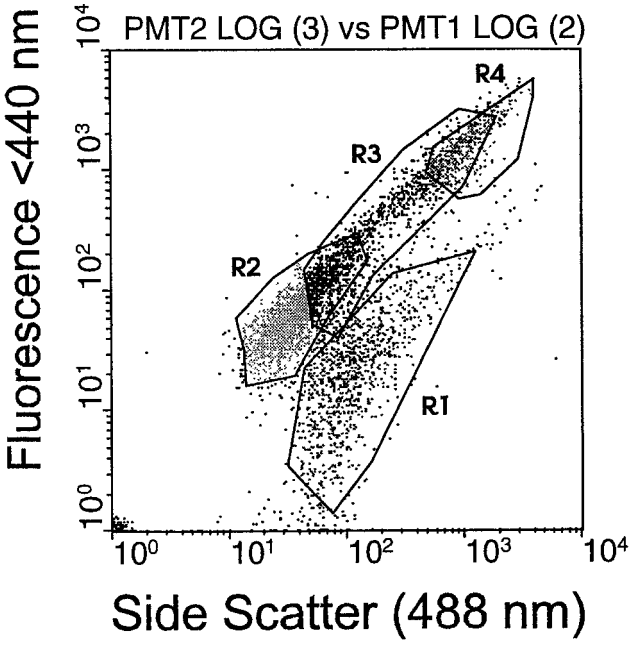


FIG. 4. Tinopal CBS-X is an effective stain for a variety of microorganisms. Each of the organisms was analysed separately and regions were drawn around the data on the resulting two-parameter dotplots using the WinMDI package (available freely via http://www.bio.umass.edu/mcbfacs/flowhome.html). These regions were then overlaid onto the corresponding dotplot for the analysis of a mixture of the four organisms. R1 = *B. globigii*, R2 = *E. coli*, R3 = *M. luteus*, R4 = baker's yeast. In all cases, the samples were fixed prior to analysis as described in Materials and Methods. The flow cytometer settings were as described in the legend to Figure 2. Side scatter resulted from excitation with the argon ion laser (488 nm) collected on PMT 2 with the voltage set at 400 and a logarithmic gain.

yeast. Although all organisms are stained, the extent of staining differs among the organisms and thus Tinopal CBS-X offers the ability to assist discrimination between these organisms.

DISCUSSION

Flow cytometry is a technique that has great advantages in the field of microbiology, but its application is limited by problems of instrument sensitivity where bacteria are concerned. Development of brightly fluorescent dyes is of great importance if bacteria are to be analysed successfully by flow cytometry and the work described herein shows that fluorescent brightening agents are useful stains for this purpose.

It is often necessary to discriminate among microbes, not least when the presence of a pathogen is suspected. This may, for example, be the case when one is analysing clinical samples (18) or when the release of biological warfare agents is suspected. In this latter role, suitable stains for spores are very important, as *B. anthracis* spores are often considered to be the most credible biowarfare threat (3). The difference in the extent of staining of microorganisms with fluorescent brightening agents, as shown here, can be exploited for cell discrimination. A multiparameter flow cytometric analysis using fluorescent brighteners together with other stains could also be used for this purpose (6).

In conclusion, Tinopal CBS-X is a potentially useful stain for microbiological analyses, especially when congeners are used to bring about more rapid staining. The discriminatory powers of this stain may be used with mixed samples in order to distinguish broadly among the types of organisms present.

ACKNOWLEDGMENTS

The work described herein has been sponsored in part by the United States Army through its European Research Office. We thank the Chemicals and Pharmaceuticals Directorate of the BBSRC for financial support, and Ciba Dyes and Chemicals Ltd. for the gift of Tinopal CBS-X, Tinopal DMS-X and Tinopal SWN.

LITERATURE CITED

1. Bertin B, Van Broux O, Hoegaerden M: Flow cytometric detection of yeast by *in situ* hybridization with a fluorescent ribosomal RNA probe. J Microbiol Methods 12:1–12, 1990.
2. Crissman HA, Tobey RA: Specific staining of DNA with the fluorescent antibiotics, mithramycin, chromomycin, and olivomycin. In: Flow Cytometry. Darzynkiewicz Z, Crissman HA (eds). Academic Press, London, 1990, pp 97–103.
3. Dando M: Biological Warfare in the 21st Century. Brassey's, London, 1994.
4. Darzynkiewicz Z: Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine orange. In: Flow Cytometry, Vol. 33. Darzynkiewicz Z, Crissman HA (eds). Academic Press, San Diego, 1990, pp 285–298.
5. Davey HM, Davey CL, Kell DB: On the determination of the size of microbial cells using flow cytometry. In: Flow Cytometry in Microbiology. Lloyd D (ed). Springer-Verlag, London, 1993, pp 49–65.
6. Davey HM, Kell DB: Flow cytometry and cell sorting of heterogeneous microbial populations: The importance of single-cell analyses. Microbiol Rev 60:641–696, 1996.
7. Diaper JP, Tither K, Edwards C: Rapid assessment of bacterial viability by flow cytometry. Appl Microbiol Biotechnol 38:268–272, 1992.
8. Dietrich MA, Truax RE, French DD, Lea DF, Stear MJ, Newman MJ: Measurement of antibody binding to intact bacteria using flow cytometric techniques. J Microbiol Methods 13:281–291, 1991.
9. Dolbeare F: Fluorescent staining of enzymes for flow cytometry. In: Flow Cytometry. Darzynkiewicz Z, Crissman HA (eds). Academic Press, London, 1990, pp 81–88.
10. Dolbeare FA, Smith RE: Flow cytoenzymology: Rapid enzyme analysis of single cells. In: Flow Cytometry and Sorting. Melamed MR, Mullaney PF, Mendelsohn ML (eds). Wiley, New York, 1979, pp 317–333.
11. Gold H: The chemistry of fluorescent whitening agents. Major structural types. In: Fluorescent Whitening Agents. Anliker R, Müller G, Raab R, Zinkernagel R (eds). Academic Press, New York, 1975, pp 25–46.
12. Grogan WM, Collins JM: Guide to Flow Cytometry Methods. Marcel Decker, New York, 1990.
13. Hejtmánek M, Dolezel J, Holubová I: Staining of fungal cell walls with fluorescent brighteners: Flow cytometric anlaysis. Folia Microbiol 35:437–442, 1990.
14. Jasin M, Zalamea P: Analysis of *Escherichia coli* β-galactosidase expression in transgenic mice by flow cytometry of sperm. Proc Natl Acad Sci USA 89:10681–10685, 1992.
15. Jernaes MW, Steen HB: Staining of *Escherichia coli* for flow cytometry: Influx and efflux of ethidium bromide. Cytometry 17:302–309, 1994.
16. Kaprelyants AS, Kell DB: Rapid assessment of bacterial viability and vitality using rhodamine 123 and flow cytometry. Journal of Applied Bacteriology 72:410–422, 1992.
17. Lloyd D: Flow Cytometry in Microbiology. Springer-Verlag, London, 1993.
18. Mansour JD, Robson JA, Arndt CW, Schulte TE: Detection of *Escherichia coli* in blood using flow cytometry. Cytometry 6:186–190, 1985.
19. Mason DJ, López-Amorós R, Allman R, Stark JM, Lloyd D: The ability of membrane potential dyes and calcofluor white to distinguish between viable and non-viable bacteria. J Appl Bacteriol 78:309–315, 1995.
20. Miller JS, Quarles JM: Flow cytometric identification of microorganisms by dual staining with FITC and PI. Cytometry 11:667–675, 1990.
21. Paton AM, Jones SM: Techniques involving optical brightening agents. In: Methods in Microbiology, Vol. 5A. Norris JR, Ribbons DW (eds). Academic Press, London, 1971, pp 135–144.
22. Phillips MK, Kell DB: A benzoxazole inhibitor of NADH dehydrogenase in *Paracoccus denitrificans.* FEMS Microbiol Lett 11:111–113, 1981.
23. Phillips MK, Kell DB: A novel inhibitor of NADH dehydrogenase in *Paracoccus denitrificans.* FEBS Lett 140:248–250, 1982.
24. Phillips MK, Rhodes-Roberts ME, Kell DB: The anti-bacterial action of tinopal AN. J Gen Microbiol 130:1999–2005, 1984.
25. Phillips MK, Rhodes-Roberts ME, Kell DB: Tinopal AN as a selective agent for the differentiation of phytopathogenic and saprophytic pseudomonas species. J Appl Bacteriol 58:283–292, 1985.
26. Porter J, Deere D, Pickup R, Edwards C: Fluorescent probes and flow cytometry: New insights into environmental bacteriology. Cytometry 23:91–96, 1996.
27. Sanders CA, Yajko DM, Hyun W, Langlois RG, Nassos PS, Fulwyler MJ, Hadley WK: Determination of guanine-plus-cytosine content of bacterial DNA by dual-laser flow cytometry. J Gen Microbiol 136:359–365, 1990.
28. Scheper T, Gebauer A, Sauerbrei A, Niehoff A, Schugerl K: Measurement of biological parameters during fermentation processes. Analytica Chimica Acta 163:111–118, 1984.
29. Shapiro HM: Practical Flow Cytometry (3rd edition). Liss, New York, 1995.
30. Shapiro HM, Schildkraut ER, Curbelo R, Laird CW, Turner RB, Hirschfeld T: Combined blood cell counting and classification with fluorochrome stains and flow instrumentation. J Histochem Cytochem 24:396–411, 1976.
31. Steen HB, Boye E: *Escherichia coli* growth studied by dual-parameter flow cytophotometry. J Bacteriol 145:1091–1094, 1981.
32. Steen HB, Lindmo T: Flow cytometry: A high-resolution instrument for everyone. Science 204:403–404, 1979.
33. Steen HB, Skarstad K, Boye E: DNA measurements of bacteria. In: Flow Cytometry. Darzynkiewicz Z, Crissman HA (eds). Academic Press, London, 1990, pp 519–526.
34. van Erp PEJ, Jansen MJJM, de Jongh GJ, Boezeman JBM, Schalkwijk J: Radiometric measurement of intracellular pH in cultured human keratinocytes using carboxy-SNARF-1 and flow cytometry. Cytometry 12:127–132, 1991.
35. Waggoner AS: Fluorescent probes for cytometry. In: Flow Cytometry and Sorting (2nd edition). Melamed MR, Lindmo T, Mendelsohn ML (eds). Wiley-Liss, New York, 1990, pp 209–225.
36. Wallner H, Amann R, Beisker W: Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14:136–143, 1993.
37. Wittrup KD, Bailey JE: A single-cell assay of β-galactosidase activity in *Saccharomyces cerevisiae.* Cytometry 9:394–404, 1988.
38. Zweidler R, Hefti H: Brighteners, fluorescent. Encyclopaedia of Chemical Technology. 4:213–226, 1978.