Chapter 4

On the Determination of the Size of Microbial Cells Using Flow Cytometry

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Introduction

In most flow cytometers the determination of cell size is based on the measurement of light scattered by cells as they pass through an illumination zone. In conventional instruments the source of this illumination is a laser (Shapiro 1988). Given that both the cell volume and the DNA content of bacteria is some 1000-fold less than that of higher eukaryotic cells, however, laser-based flow cytometers have generally proved unsuitable for the study of microorganisms (Steen et al. 1990). In the Skatron Argus 100 flow cytometer, a high-pressure mercury arc lamp is used as the excitation source, and the machine makes use of an open flow chamber in which a jet impinges at an angle onto the surface of a microscope cover slip (Fig. 4.1). The result is a flat, laminar flow of water across the glass surface. This flow has only two interfaces – the glass/water interface and the water/air interface – and of these only the former can collect particles that may cause background scattering of light (Steen et al. 1989). Furthermore the orientation of these surfaces perpendicular to the optical axis means that the surfaces themselves scatter only the minimum of light. Thus the system has a high signal-to-noise ratio and is therefore ideal for detecting light scattered by microorganisms (Boye et al. 1983).

When non-spherical cells such as erythrocytes or rod-shaped bacteria are carried in a fast-moving liquid (such as the sheath fluid in a flow cytometer) they will tend to align in the direction of their longest dimension, i.e. with their "flat" sides parallel to the flow (Kachel et al. 1990). When such oriented cells interact with a laser beam that is perpendicular to the flow, the scattering will be larger than if the cells were arranged randomly. However, the optical arrangement of the arc-lamp-based flow cytometer results in the cells being illuminated from a wider range of angles and therefore the scattering signal is less sensitive to cell alignment (Steen and Lindmo 1985).

When a particle (e.g. a cell) interacts with a beam of light, such as that

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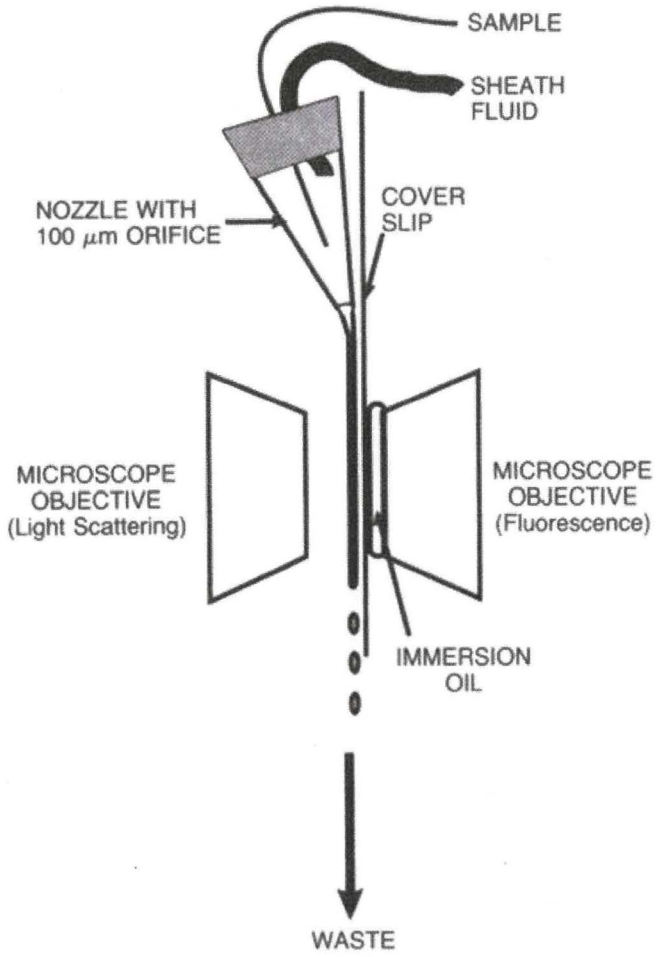

Fig. 4.1. The open flow chamber of the Skatron Argus flow cytometer. The design of this flow chamber causes very little background light scatter, thus giving a high signal-to-noise ratio, making it ideal for measurements on microorganisms.

produced by a mercury arc lamp, some of the light is scattered out of the beam (Mullaney and Dean 1970; McCoy and Lovett 1989; Salzman et al. 1990). The way in which cells scatter light is rather complex and is dependent on their size, shape and internal structure. The relative contributions that these cell characteristics make to the amount of light that is scattered varies with the range of angles over which scattered light is collected, and in particular are functions of the relationship between the cell size and the wavelength of light (Steen and Lindmo 1985; Steen et al. 1989). Generally, the amount of forward scattering (small-angle scattering) increases rapidly with cell size and is affected to a much lesser extent by cell shape and refractive index, while the cell structure and shape become more important at larger angles (Paau et al. 1977; Muirhead et al. 1985; Steen and Lindmo 1985; Wittrup et al. 1988; Boye and Løbner-Olesen 1990; Shapiro 1990). Whilst detection of light scattering at two separate angles has proved useful in the differentiation of blood cell types (Shapiro 1988; Steen et al. 1989), and occasionally for distinguishing different bacteria in mixed populations (e.g. Frelat et al. 1989; Steen 1990), it is usually assumed, after calibration with standards of known sizes, that the extent of low-angle forward light scatter provides an accurate representation of cell size.

It is important to note that flow cytometry yields information on individual cells rather than giving an average value for all of the cells measured, and the special power of the technique is that it allows one to quantify the *heterogeneity* of the sample of interest (Kell et al. 1991). A representation of the cell size distribution can be obtained by the detection of forward scattered light from cells passing in a hydrodynamically focussed stream past a measuring point (Shapiro 1983). Data are collected by appropriate detectors that may be gated electronically, and converted into pulses, the magnitude of which represent the amount of light that is scattered. The pulses are "binned" into channels that increase in number with increasing levels of scattered light (cell size). The data are usually plotted as a histogram in which the abscissa represents the channel numbers whilst the ordinate represents the number of cells measured in each channel (Dean 1990).

While channel numbers allow one to express the data in terms of the *relative* light scattering of each cell, it is also useful for many purposes to be able to express cell sizes in *absolute* terms. This is necessary, for example, if one wishes to determine the mode of growth of individual cells (Davey et al. 1990a). The extent of light scattering can of course be affected by the way in which the flow cytometer is set up (e.g. the flow rate of the sample and sheath fluids, and especially the deterioration of the arc lamp over time (Horan et al. 1990)), and this makes comparisons between work done on different days impossible. By using a cocktail of monodisperse latex beads of known diameters, however, it is possible to obtain a calibration curve of channel number versus cell size, and thus to convert channel numbers to a measure of cell size that is independent of the performance of the flow cytometer. This method is convenient as monodisperse latex beads of high uniformity (coefficient of variation typically <2%) are available in a variety of sizes. However, we have noticed that calibration by this method results in an underestimation of the true cell size, as measured microscopically (Davey et al. 1990a,b) or via the Coulter counter (unpublished observations).

The heterogeneity of cell populations, even in stationary phase, can be quite large (Kell et al. 1991) and therefore it is often desirable to use logarithmic amplification when acquiring flow cytometric data. The latex bead calibration described above also serves to linearize the relationship between the channel number and the diameter of the latex beads.

Often in flow cytometric studies it is a requirement that cells are fixed, either for storage or as a preliminary step in a staining procedure. When cells are stained, for example with a fluorescent DNA stain, measurements are often gated on cell size and the data are plotted as a dual-parameter histogram of DNA versus size versus counts. It is therefore important to determine what effects fixing has on cell size and light scattering behaviour. The purpose of fixing cells prior to staining is to permeabilize the cell membrane, allowing entry of the probe. However, this will also facilitate leakage of cell contents and allow the suspension medium to enter the cell. This may be expected to affect both the "true" cell size and the light scattering properties of the cell as compared both with an unfixed cell and with a latex bead of equivalent size. The effect of a variety of fixatives (ethanol, formalin, etc.) on samples for flow cytometry was investigated by Alanen et al. (1989), who concluded that ethanol fixation produced data for

the distribution of DNA content most similar to those obtained with unfixed cells. Many workers (e.g. Kogoma et al. 1985; Skarstad et al. 1985, 1986; Scheper et al. 1987) use 70% ethanol for fixing their cells, although we are not aware of any report describing the effect of this on the apparent cell size.

In view of the above, it seemed important to perform a detailed and systematic study of the effect of fixation and sample preparation on the relationship between the channel numbers observed and the "true" cell size. We describe here the results of just such a study, using strains of *Saccharomyces cerevisiae* and *Micrococcus luteus*. These organisms were chosen because they are almost spherical, since calibration in flow cytometry is usually done with latex spheres. It was anticipated that this choice would help to reduce artifacts due to effects of shape on the (calibrated) data. Since the cells used were all in stationary phase the percentage of (yeast) cells with buds was very low – in all cases 13% or less as judged by scoring 100 cells; thus cell asymmetry due to buds was kept to a minimum.

Materials and Methods

Organisms Used

Three industrial strains of the yeast *Saccharomyces cerevisiae* (BB1, DCL1 and DCL2) were used. BB1 was grown up to high biomass in batch culture in a medium consisting of 1.3% E-broth and 5% malt extract (both from Lab M), pH 6. The working volume of the fermentor was 5 litres, and the temperature was controlled at 30 °C. After 22 h of growth, by which time the culture had reached stationary phase, the contents of the fermentor were harvested by centrifuging the medium from the fermentor to give a pellet. The two other strains of yeast, DCL1 and DCL2, were obtained as a paste; from the low bud counts (less than 13%) these were deemed to have been grown to stationary phase.

Micrococcus luteus (Fleming strain 2665) was grown in batch culture in 5 litres of E-broth (pH 7.4) at 30 °C at pH 7.4. After 34 h the contents of the fermentor were harvested as described for the BB1 yeast above.

Buffers

Phosphate-buffered saline (pH 7.4) was obtained from the Sigma Chemical Company, Poole, Dorset (cat no. P-1000-3). When reconstituted in distilled water it contained (in mmol/l) NaCl 120, KCl 2.7 and phosphate buffer salts 10. This was stored at 4 °C between experiments.

Suspension buffer contained (in mmol/l) KH_2PO_4 50, $MgSO_4$ 5. It was adjusted to pH 7.0 with 5 mol/l KOH. SB was made up freshly for each experiment.

Fluorescein isothiocyanate (FITC) for protein staining was from Sigma. All other reagents were from BDH, Poole, Dorset. Water was singly-distilled in all-glass apparatus.

Preparation of Cells

The pellet of cells, either harvested from a batch culture as described above or cut from a block of paste, was suspended in at least three times its own volume of suspension buffer. The resulting suspension was centrifuged at 1000 *g* (yeast) or 2750 *g* (*M. luteus*) for 10 min at room temperature. The supernatant and any precipitated medium from the top of the pellet were discarded and the cells were resuspended in fresh suspension buffer. These were left at room temperature for 45 min, and were occasionally mixed to resuspend them. The cells were recentrifuged as described above and the supernatant discarded. A final wash was performed in suspension buffer followed by another centrifugation to obtain a pellet of cells.

A small amount of the pellet was resuspended in fresh suspension buffer to give approximately 10^7 cells/ml. Two 1 ml aliquots of this suspension were placed in Eppendorf tubes and centrifuged in a bench-top centrifuge at 13 000 rpm. One aliquot was resuspended in suspension buffer, the other was suspended in phosphate-buffered saline. Both were washed twice in their respective buffers before being finally resuspended in suspension buffer or phosphate buffer.

Fixed Cells

The cells in buffer were added to absolute ethanol to give a final ethanol concentration of 70% (v/v). These cells were then allowed to fix at room temperature for 20 min prior to being stored at 4 °C until required. All cells stored in this way were examined within 48 h. Prior to flow cytometry and/or photography of these fixed samples, the cells were centrifuged and resuspended in their original buffers at room temperature.

Cell Treatments Used in the Experiments

Four treatments of cells from each organism were used for flow cytometry and photography. These were:

1. Cells in Phosphate-Buffered saline which were Unfixed (PBU).
2. Cells in Phosphate-Buffered saline which were Fixed (PBF).
3. Cells in Suspension Buffer which were Unfixed (SBU).
4. Cells in Suspension Buffer which were Fixed (SBF).

These cell treatments will be referred to using the three-letter abbreviations shown in parentheses after the descriptions above. The fixed cells were stored as described; the unfixed cells were examined as soon as they were prepared.

Protein Staining

A stock solution of FITC was prepared consisting of 1 mg/ml FITC in acetone; this was stored at 4 °C. On the day of the experiment 50 μl of stock

solution was diluted to 2 ml with phosphate buffer (final FITC concentration 25 μg/ml).

Two samples of *Micrococcus luteus* cells in phosphate buffer, one of which had been fixed and the other unfixed, were used for protein staining. The fixed cells were washed twice in phosphate buffer to remove traces of the fixative (ethanol) prior to the addition of 1 ml of staining buffer to the pelleted cells.

Flow Cytometry

Flow cytometry was carried out using a Skatron Argus 100 flow cytometer (Skatron, Ltd, PO Box 34, Newmarket, Suffolk), as described by Steen, Boye and colleagues (Steen et al. 1990; Boye and Løbner-Olesen 1991). The flow cytometer was set up as described in the manufacturer's manual, save that an additional 0.1 μm filter was placed in the sheath fluid line. The sheath fluid was prepared from water that had been filtered using Millipore Milli-Q apparatus. Sodium azide (1 mmol/l) was added to inhibit microbial growth in the sheath fluid tank.

The B1 filter block (excitation, 395–440 nm; band stop, 460 nm; emission, >470 nm) was used for all cell size measurements unless otherwise stated. For experiments in which cells were stained with FITC the FITC filter block was used (excitation, 470–495 nm; band stop, 510 nm; emission, 520–550 nm). The photomultiplier (PMT) setting chosen necessarily differed between the cell types used so that the cell size distribution was on scale. In all cases the gain was logarithmic. A sheath fluid pressure of 1.5–2 kPa/cm^2 was used and the sample flow speed was set at 1 or 2 μl/min. In the experiments described here data were collected from the low-angle light scattering (LS1) detector and, if appropriate, from the FITC fluorescence detector. The flow cytometer was controlled by a Viglen IIHDE microcomputer (IBM-PC-AT-compatible, 80286 processor, EGA screen) with software supplied by the manufacturer. Using this software, data were acquired and saved according to the flow cytometry data file standard outlined by Murphy and Chused (1984).

Initial calibration and linearization of the cell size measurements were achieved by using a cocktail of monodisperse (cv <2%) latex particles (Dyno Particles AS, PO Box 160, Lillestrøm, Norway). Beads of diameter 2, 5, 7 and 10 μm were run through the flow cytometer using the same settings as for the cells. A three-term polynomial ($y = a + bx + cx^2$) was used to fit a curve of channel number versus cell size to the bead data. The cell data were imported into the spreadsheet program FLOWTOVP.WKS (Davey et al. 1990b) via a filter program written in-house (in Microsoft Quick BASIC v4.5). FLOWTOVP.WKS was used to convert channel numbers to apparent cell diameters using the values fitted to the polynomial.

Photography

Light microscopy was used for all photomicrographs because, although this gives lower resolution than does electron microscopy, it avoids the need

for special cell preparation methods that may affect cell size. A Polyvar microscope incorporating a 35 mm camera was used for all photography. Photographs were taken under oil immersion at a total magnification of ×1250. Photographs of the 10 μm beads used in the calibration of the flow cytometer were also used in the calibration of the photomicrographs.

The photographs were developed and printed at a magnification of ×3.5 except for the photographs of *Micrococcus luteus* (and the corresponding set of latex beads), which were magnified to ×10. The resulting photographs for each organism were mixed into random order, identified only by a code number, so that size measurements were carried out blind. The photographs were placed in turn under a ×1.5 bench magnifying glass and the long and short axes of the cells were measured (to the nearest 0.1 mm) using a pair of vernier callipers; once the magnification had been taken into account this corresponded to approximately 0.1 μm. Every cell (apart from unseparated buds) of *Saccharomyces cerevisiae* that was in focus was measured, but in the case of the photographs of *M. luteus*, only the in-focus cells from one randomly selected quarter of the photograph were measured. In most cases 100–200 cells were measured per treatment for each organism. However in some cases, where excessive clumping occurred (see later discussion of this problem), fewer cells were measured. The lowest number of cells measured was 47 and the highest number was 222. All measurements were made by the same person, with the same pair of callipers, to keep systematic errors constant throughout.

The dimensions of the cells (recorded in millimetres from the photographs) were entered into a spreadsheet and converted to their true sizes by using a calibration based on the mode size of a sample of 10 μm beads that had been measured as described above. The length (in micrometres) of the long and short axes of the cells were then used to calculate the diameter of the sphere of equivalent volume.

The volume of an ellipsoid of revolution (Grant et al. 1978) is given by:

$$V = \frac{4}{3} \cdot \pi \cdot a \cdot b^2 \tag{4.1}$$

where V is the volume, a is the long semi-axis and b is the short semi-axis of the ellipsoid. From this one can derive Eq. (4.2) that gives the equivalent diameter for a sphere with the same volume as the ellipsoid:

$$D = 2 \cdot [(L/2) \cdot (S/2)^2]^{\frac{1}{3}} \tag{4.2}$$

where D is the diameter of the equivalent sphere, L is the length of the long axis of the cell and S is the length of the short axis of the cell.

Histograms of the resulting equivalent diameters were produced using in-house software written in Microsoft Quick BASIC v4.5. The program (BIN.BAS/EXE) read in an ASCII list of numbers separated by carriage returns and reported the smallest and largest numbers in the file. The user then entered the required lower and upper bounds (in all cases these were set to 0 μm and 15 μm respectively, which easily encompassed all of the data) and also a suitable "bin" size. Here a bin size interval of 0.2 μm was chosen as, in preliminary tests with a range of bin sizes, it gave good sensitivity without producing an excessively noisy plot. Cells were placed in

a given bin if they were equal to or greater than the lower bound and were less than (but not equal to) the upper bound of that particular bin. The output from the BASIC program was in the form of two columns; column one contained the centre value of each bin interval (i.e. 0.1, 0.3, 0.5, . . . , 14.9) and the second column contained the number of cells that had been placed into each of the bins. These were then used to construct a histogram of counts versus cell size.

Results

Determination of a Constant Calibration Factor

Ideally, one would expect that a plot of cell size measured by flow cytometry versus cell size measured from photomicrographs would have a gradient of 1. However, as can be seen from Fig. 4.2 this is not the case. Figure 4.2 shows the data for the four types of unfixed cells measured in phosphate buffer (PBU cells). When the peak cell diameter determined by flow cytometry is plotted against the modal cell diameter measured from the photomicrographs, the gradient of the line $y = 0 + bx$, which is well fitted by linear least squares, is only 0.76. This suggests that calibration of the flow cytometric data with the latex calibration beads leads to a constant underestimation of the "true" size of these cells of 24%, presumably because cells

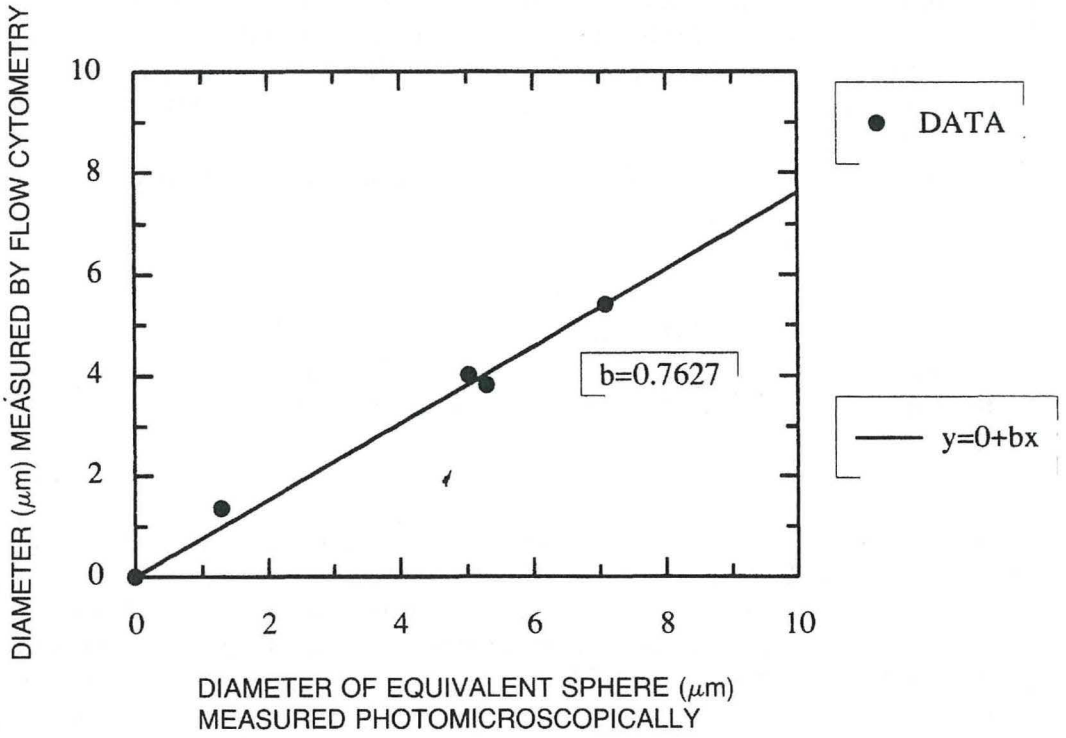

Fig. 4.2. Data for the unfixed cells in phosphate buffer (PBU) showing (in order of increasing size) *M. luteus* and yeast strains DCL2, DCL1 and BB1. The graph shows that calibration by latex beads gives an underestimation of true cell size. However, the gradient (b) of the line $y = 0 + bx$ gives an additional calibration factor that can be used to convert the apparent cell size measured by flow cytometry into a "true" cell size.

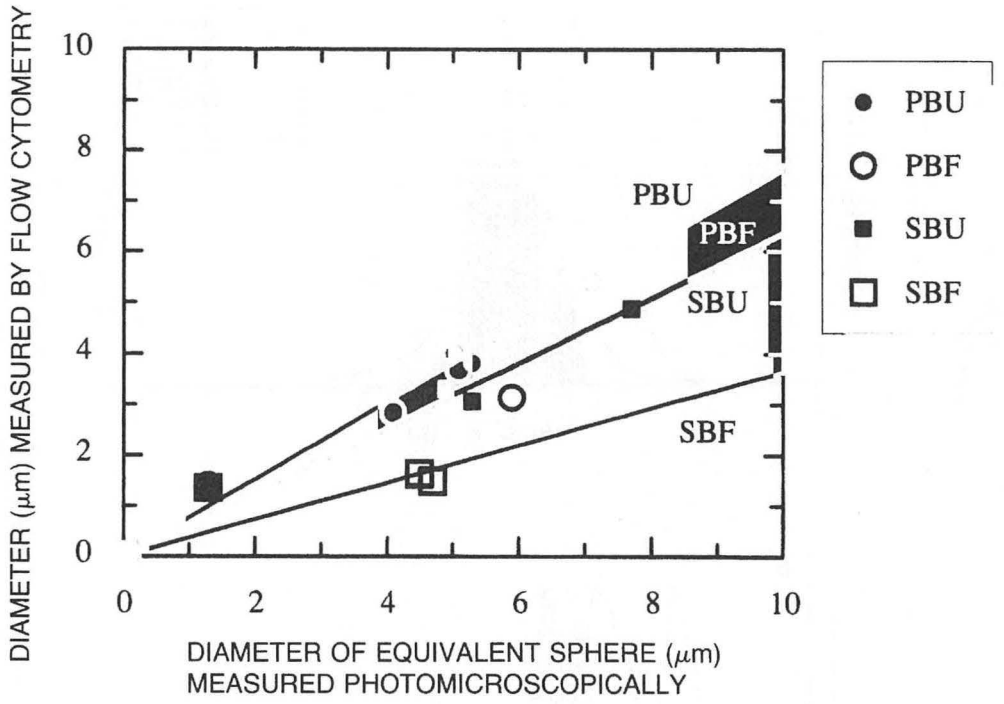

Fig. 4.3. Data from all four cell treatments for *M. luteus* and the three yeast strains. The data point for the yeast strain (BB1) is missing from the SBF plot because excessive clumping made interpretation of the flow cytometric data impossible. The gradients of the line ($y = 0 + bx$) for each treatment are as follows: PBU, 0.763; PBF, 0.637; SBU, 0.634; SBF, 0.365. It is obvious that a different calibration factor is required for each buffer and for fixed and unfixed cells in the same buffer.

do not scatter light as much as do latex beads of the same diameter. Since much of what is measured by flow cytometry is directly related to volume rather than to diameter (e.g. protein content), it should be noted that a 24% underestimation of diameter corresponds to a 56% underestimation of volume. Once the extent of the underestimation of cell size has been determined, however, it can be used as a constant factor for calibrating flow cytometric light scattering data obtained from microorganisms, apparently independently (within reason) of their size and nature.

When similar data for the other cell treatments are plotted (Fig. 4.3), it may be observed that the magnitude of the "constant factor" (ratio) is dependent on the buffer in which the cells are suspended and also on whether or not the cells have been fixed. The goodness of fit to a straight line is also clearly better for the unfixed cells. For each cell *treatment* used it will therefore be necessary to use a different calibration factor.

The effect of using the constant calibration factor for a *particular* treatment is a much improved measure of cell size. This can be seen in Fig. 4.4, which shows the photomicrographic measurements along with the flow cytometric data calibrated either with calibration beads alone or using both calibration beads and the relevant calibration factor. For the unfixed cells, the agreement of the latter flow cytometric measurements with the photomicrographic measurements is practically perfect (Fig. 4.4a,c). However, this is not the case for fixed cells (Fig. 4.4b,d).

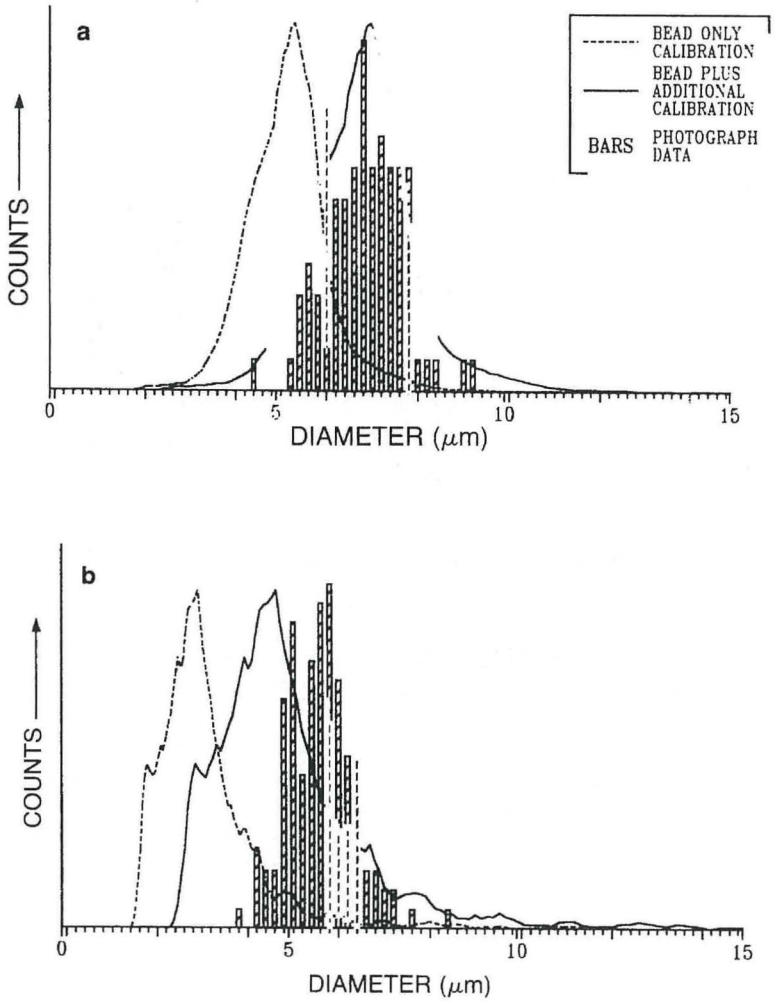

Fig. 4.4a–d. Effect of using the additional calibration factor obtained from the gradient for the relevant treatment shown in Fig. 4.3. By using the additional calibration factor a more accurate measure of cell size is obtained. **a** PBU cells; **b** PBF cells; **c** SBU cells; **d** SBF cells. It should be noted that even when the additional calibration factor is used the overlap of the data for the fixed cells is not perfect. The data shown in **a–c** are for yeast strain BB1, whilst **d** shows data for the DCL2 yeast. In each graph the flow cytometric data represent size measurements made on approximately 10000 cells while the data from the photomicrographs represent only 100–200 cells. In order to plot these together the absolute counts have been deleted from the ordinate axes.

Effect of Fixing the Cells

When the cells were fixed with 70% ethanol some clumping occurred. This hindered cell size measurement from the photomicrographs to some extent as it was difficult to see the edges of cells in the clumps. More importantly though, it also affected the cell size distributions from the flow cytometer and, in the case of the largest strain of yeast considered (BB1), the clumping resulted in the peak channel number being outside the range encompassed by the calibration beads and so the data for the SBF cells of this yeast were lost. More, and larger, clumps were formed by the SBF cells (up to 28 cells per clump; average 30% of cells unclumped) than by the PBF cells (up to 9

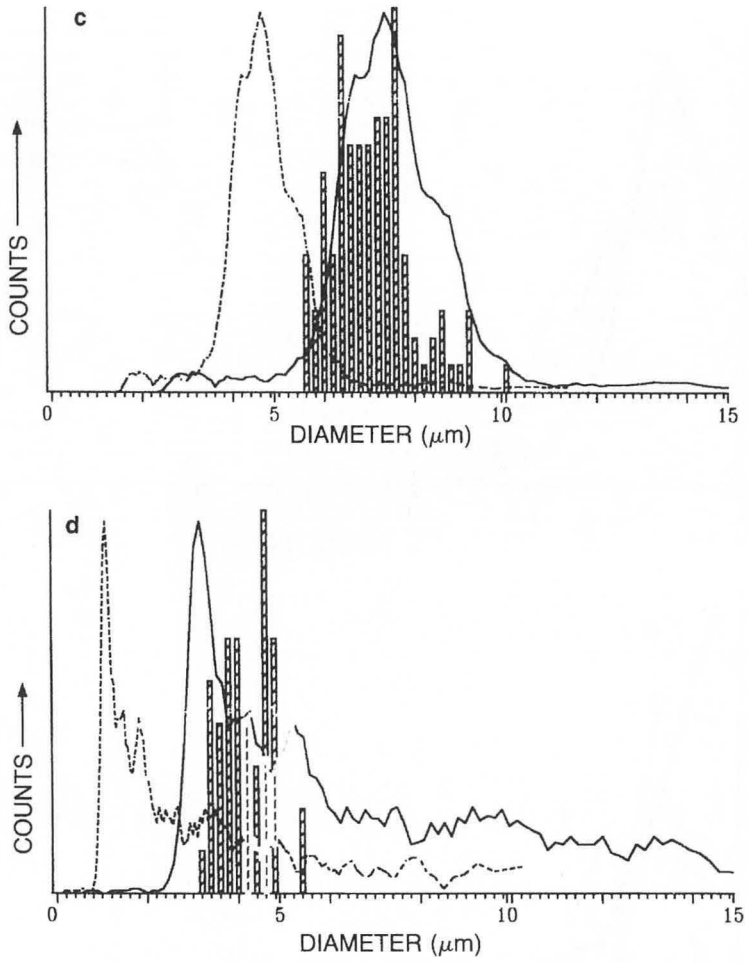

Fig. 4.4. *Continued*

cells per clump; average 61% of cells unclumped). The figures quoted here are for the three yeast strains only; with *M. luteus* no obvious clumping occurred with the PBF cells but the clumps formed by the SBF cells were so large that the number of cells per clump could not be determined. In Fig. 4.4d the effect of cell clumping on the flow cytometric data is clearly visible: a "tail" to the cell distribution is produced to the right of the unclumped cells in the main peak.

Another effect of fixing the yeast cells was to reduce their diameters. This was apparent both from the flow cytometry data and also from the photomicrographs, although the effect on the flow cytometric data was greater. No such reduction in cell size was observed in the case of *M. luteus*. The average reduction in the apparent cell diameter on fixing for the PBU cells of the three yeast strains was 13% when measured from the photomicrographs but was 25% when measured by flow cytometry. For the SBU cells the reduction was 16% when measured from the photomicrographs but 54% when measured by flow cytometry.

Related to the reduction in cell size caused by fixing with ethanol was a third effect on the yeast cells, namely the effect on the axial ratios of the cells. As can be seen in Fig. 4.5 the axial ratio of the cells increased after

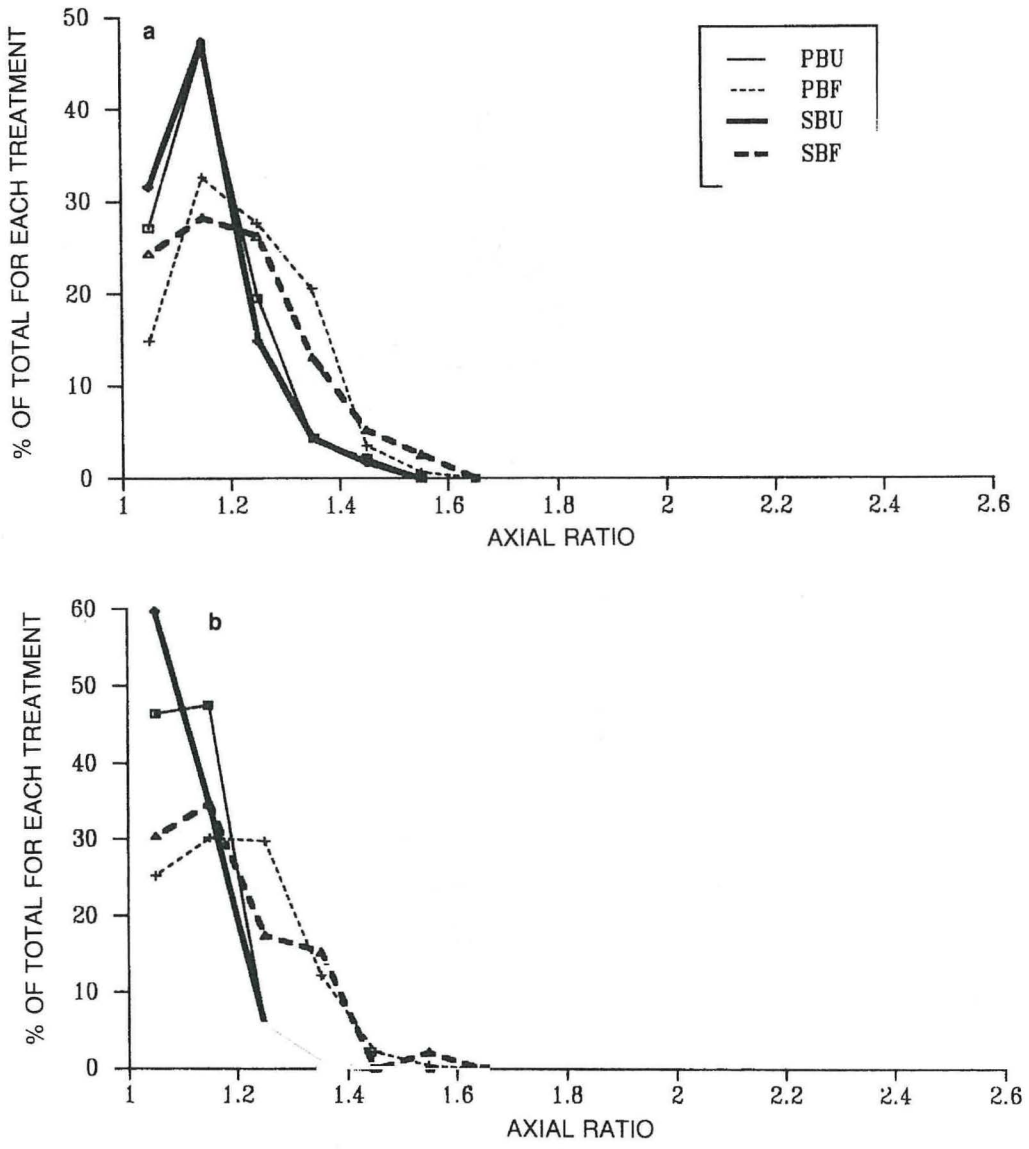

Fig. 4.5a–d. The effect of fixing the cells with 70% ethanol on their axial ratios. **a** BB1 yeast; **b** DCL2 yeast; **c** DCL1 yeast; **d** *M. luteus*.

fixing. This indicates that although the cells do shrink in both length and width (data not shown), they do not do so in equal proportions.

One final effect that was noticed following fixing the cells in suspension buffer was that in some cases the contents of the cells were released. This can clearly be seen (along with the other effects of fixing) in Fig. 4.6.

Finally, Fig. 4.7 shows the relationship between forward angle light scattering (size) and FITC staining (for protein content) for fixed and unfixed samples of *M. luteus*. Steen (1990) states that light scattering is proportional to the total protein content of the cells, and although there is some scatter on the plots, this seems generally to be true for both unfixed and fixed cells of *M. luteus*.

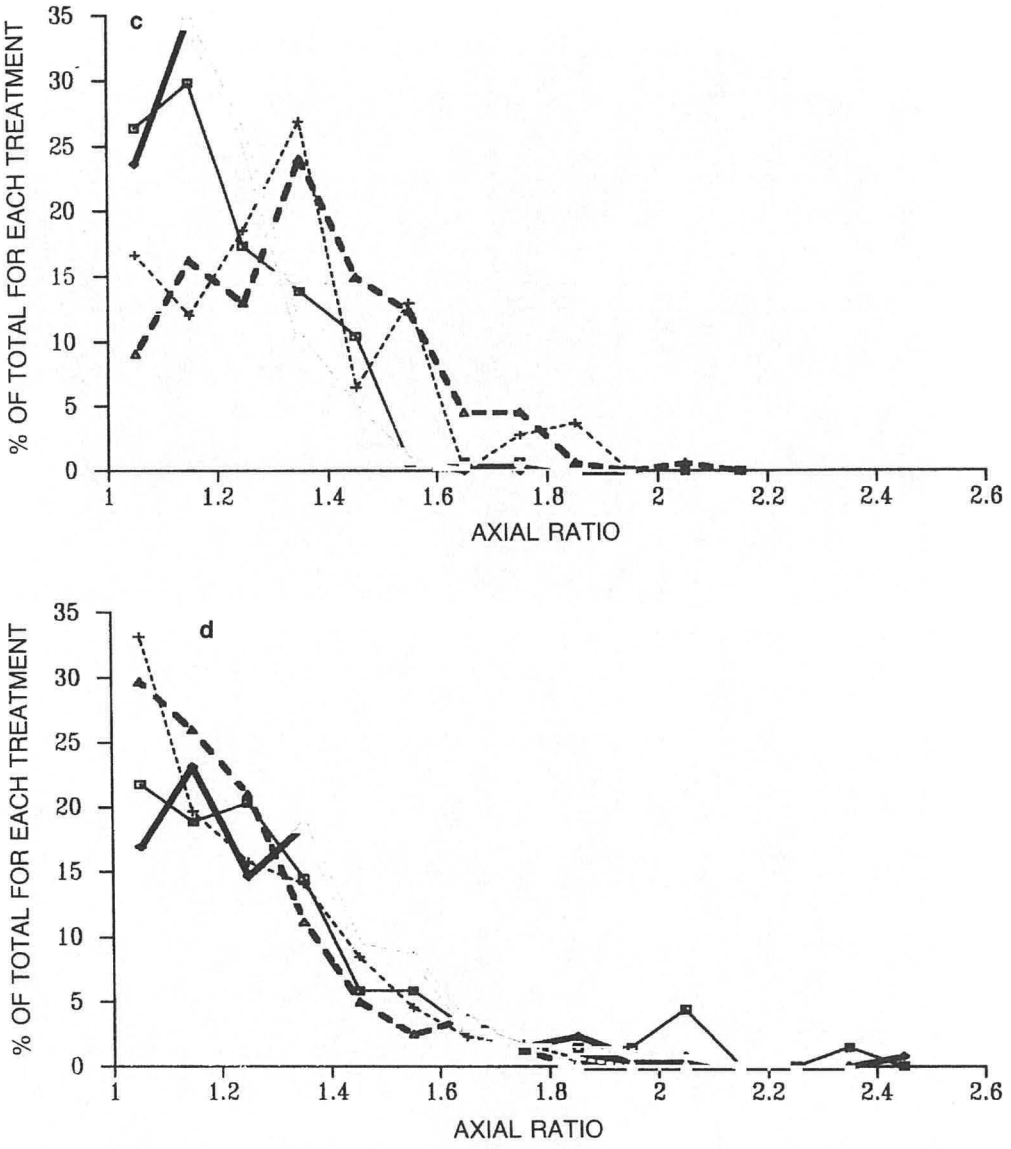

Fig. 4.5. *Continued*

Discussion

For a variety of flow cytometric applications it is essential to express cell sizes in absolute rather than relative terms. Figures 4.2–4.4 show that the amount of light scattered by a cell is always less than that of a latex bead of the same diameter, but by a constant fraction. The linearity of the data in Figs. 4.2 and 4.3 shows that the error is a constant *fraction* and not a constant *amount*. By comparing cell size distributions measured by flow cytometry with those obtained from photomicrographs we have obtained a set of additional calibration factors that enable one to convert bead-calibrated flow cytometric data to "true" cell sizes. Thus we have the convenience of a simple bead calibration coupled with an improved determination of cell size.

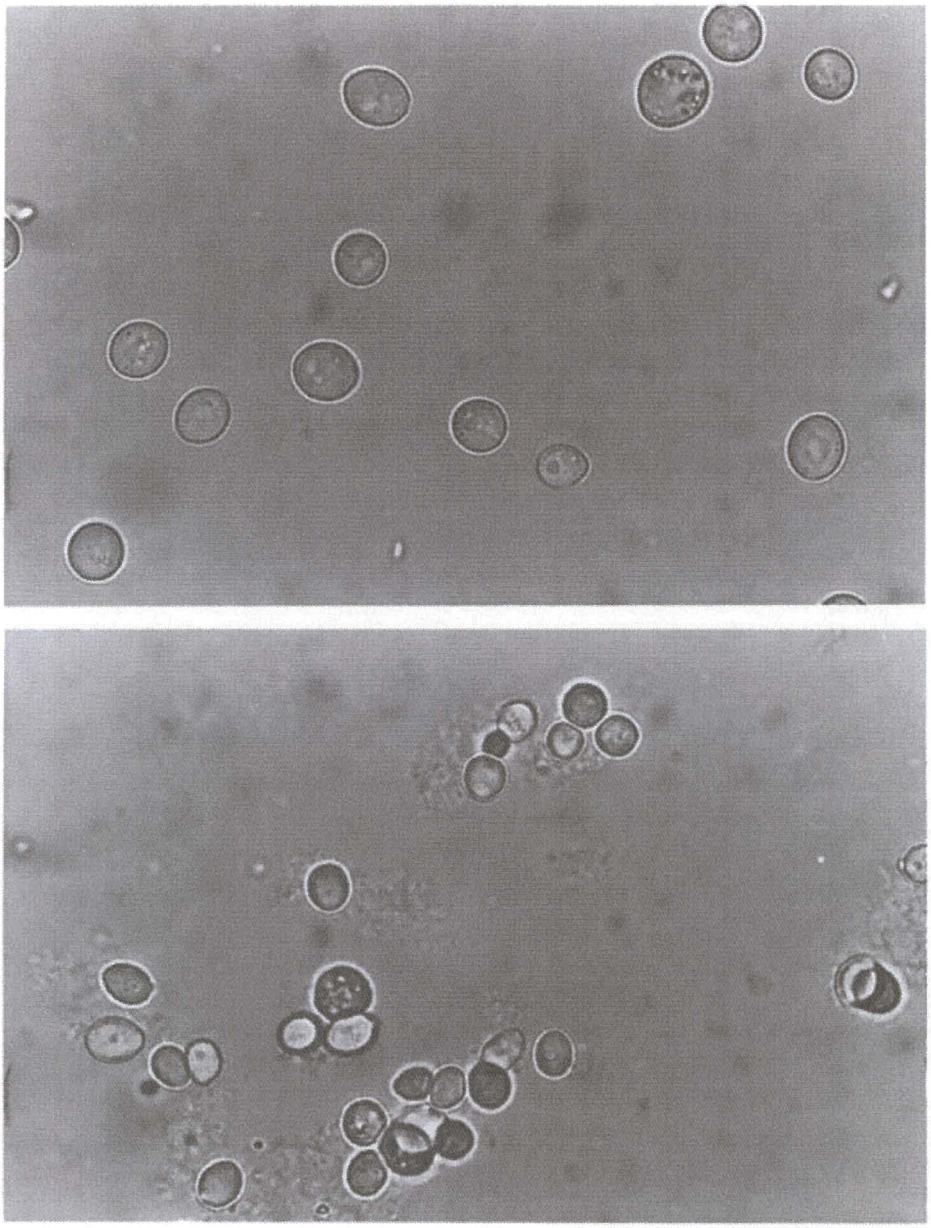

Fig. 4.6a,b. Photomicrographs of the BB1 yeast. **a** Unfixed yeast in suspension buffer (SBU yeast); **b** fixed yeast in suspension buffer (SBF yeast). Following the fixation step of the cell preparation several differences can be seen: the cells are smaller, large clumps of cells are formed and some cell contents have escaped into the medium.

It is apparent, however (Fig. 4.3), that a different calibration factor is required for each cell treatment (sample preparation) used.

The fact that fixing cells with 70% ethanol decreases their size as determined both by flow cytometry and by photomicrograph measurement means that one cannot determine "true" cell size distributions once cells have been fixed. This means, for example, that one cannot directly relate measurements of protein or DNA content obtained from flow cytometry of fixed cells to the cellular volume.

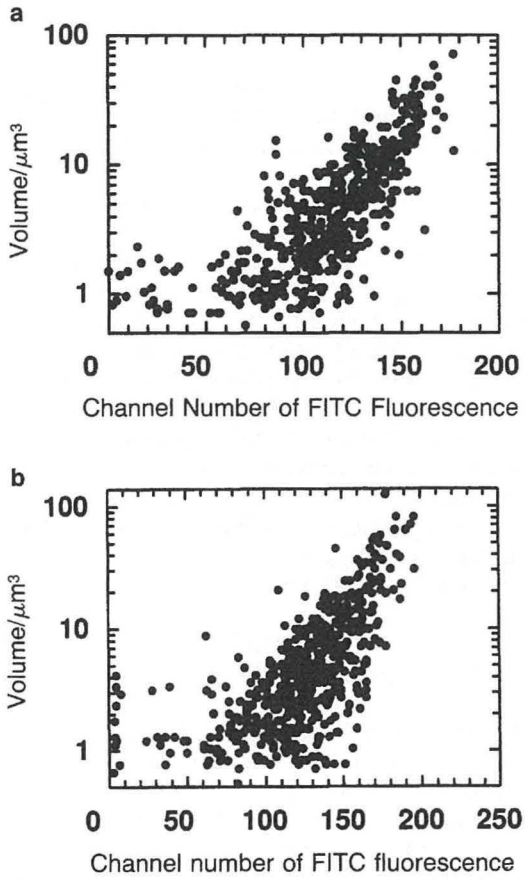

Fig. 4.7a,b. Relationship between volume and protein content of *M. luteus*. **a** Unfixed cells; **b** ethanol-fixed cells.

As shown in Fig. 4.4 the calibration factor improves the accuracy of cell size determination for all samples. However, while the cell size distributions for the unfixed (PBU and SBU) cells measured from the photomicrographs coincided almost exactly with the relevant flow cytometric data obtained by the improved calibration method, there was still a substantial discrepancy between the cell size distributions obtained by the two different methods for the PBF and SBF cells.

Flow cytometry has the prerequisite that the cell suspension of interest should consist of single cells, and, as shown here, the extent to which this is true can be strongly affected by fixing. The degree to which the cells are affected by fixing depends to some extent on which pre-fixation buffer is used. The correct interpretation of flow cytometric analyses therefore requires the determination of the effects of all stages of sample preparation on the cells.

We conclude that only in the case of unfixed cells is it possible to obtain a reliable value for their diameter by measuring the extent to which they scatter light at low angles in a flow cytometer.

Acknowledgement

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