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Journal of Microbiological Methods 49 (2002) 173–182

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Enumeration of small ciliates in culture by flow cytometry and nucleic acid staining

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Received 2 July 2001; received in revised form 30 October 2001; accepted 31 October 2001

Abstract

We developed a fast and simple protocol for accurate quantification of small freshwater ciliates by flow cytometry (FCM). The ciliates were stained with several nucleic acid stains such as TO-PRO-1, YO-YO-1 and PicoGreen, and analysed by a commercially available flow cytometer. The method was tested with cultures of the prostomatid species *Urotricha farcta* and *Balanion planctonicum*, including the small cryptophyte *Cryptomonas* sp. as food. Of the dyes tested, TO-PRO-1 gave the best results. Flow cytometric results agreed well with microscopic counts. Due to its greater speed and accuracy, FCM was superior to light microscopy. FCM was also superior to electronical particle counting and sizing (EPCS). Of particular importance, FCM in combination with TO-PRO-1 staining allowed unequivocal discrimination in cases of overlapping size distributions between the target population (i.e., the ciliate predators) and other particles (the cryptophyte prey, detritus).

Keywords: Ciliates; DNA stain; Flow cytometry; TO-PRO; *Urotricha*; *Balanion*

1. Introduction

Flow cytometry (FCM) is routinely being applied in aquatic microbial ecology, primarily to study abundance and activity of bacterioplankton (e.g., del Giorgio et al., 1996; Lebaron et al., 1998) and phytoplankton (summarised by Olson et al., 1993; Veldhuis and Kraay, 2000; Marie et al., 2000). Only a few studies, however, used FCM to investigate planktonic, non-pathogenic protozoa. This is probably because analysis of heterotrophic protozoa by FCM is more difficult, mainly due to their relatively low abundance in natural waters. The practical detection limit for FCM is in the order of 100–1000 cells ml^{-1} (Collier and Campbell, 1999), which is the upper limit of many natural protozoan populations. Furthermore, phagotrophic protozoa lack phototrophic pigments such as chlorophyll *a* or phycoerythrin which can be used to discriminate phytoplankton from detritus and other plankton. It is therefore extremely difficult to unequivocally detect and quantify protozoan species among the vast number of other particles found in natural samples. The highly specific flow cytometric detection and quantification in combination to in situ hybridisation with oligonucleotide probes (Lim et al., 1993; Rice et al., 1997) is a promising technique which is, however, currently still in its infancy.

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0167-7012/02/$ - see front matter
PII: S0167-7012(01)00366-9

Among freshwater protozoa, ciliates usually dominate in terms of biomass. Ciliates are also at times the most important algal predators and are significant as food for larger organisms (summarised by Weisse and Müller, 1998). Due to the central role ciliates play in lake ecosystems, knowledge of their ecophysiology is essential for an improved understanding of the functioning of planktonic food webs. Recent experimental studies revealed that large species-specific differences in ecophysiological key parameters such as growth and grazing rates exist, even among closely related species (Müller and Geller, 1993; Weisse and Montagnes, 1998; Müller and Schlegel, 1999). A common problem in such laboratory experiments, when a large number of samples needs to be processed in order to obtain statistically reliable results, is to accurately determine ciliate cell numbers. The most common method for determination of ciliate numbers, microscopic counting, is laborious and time consuming and is, therefore, of limited use in extensive laboratory experiments. Counting of a ciliate sample in a settling chamber or in a Sedgewick rafter cell takes approximately 15–20 min. Accordingly, there is a need for a fast and simple method to determine ciliate abundance in cultures. Since FCM allows cells to be counted at a speed of several hundreds per second, the use of FCM should substantially improve the possibilities to study ciliates in laboratory experiments. The abundance of ciliates in laboratory cultures ranges typically from several hundreds to several thousands per milliliter (e.g., Klaveness, 1984; Lavin et al., 1990; Montagnes and Weisse, 2000), thereby somewhat circumventing the problems originating from the low densities of natural populations. Flow cytometry has already been applied to measure grazing rates of cultured ciliates and dinoflagellates (e.g., Gerritsen et al., 1987; Lavin et al., 1990; Kenter et al., 1996; Weisse and Kirchoff, 1997).

The aim of this study was to develop a flow cytometric protocol that can be widely applied for quantifying ciliates in culture. It is critical for a successful enumeration to distinguish unequivocally the cells of interest from other particles in the sample. Ciliates and other protozoa can be discriminated from their prey based upon a combination of light scatter and fluorescence parameters, provided that prey and predator differ largely in size and the detritus content is low (Lavin et al., 1990; Weisse and Kirchoff, 1997). If the latter is higher, FCM cannot distinguish between ciliates and similar-sized detritus (Gerritsen et al., 1987). Owing to their macronucleus, ciliates have a higher relative DNA content compared to most other planktonic protists, and compared to debris. Accordingly, measurement of the relative DNA content has been used to distinguish ciliates from other particles (Kenter et al., 1996). These authors used the DNA stain DAPI (Porter and Feig, 1980) to label the ciliates. DAPI staining requires, however, a flow cytometer equipped with a UV light source, because DAPI has its absorption maximum at 358 nm (Haugland, 1996). Since flow cytometers equipped with mercury-arc lamps or UV lasers are less common than those equipped with an Argon laser, which emits light in the blue range, we wanted to develop a flow cytometric protocol that could be used in combination with an Argon laser. Therefore, instead of DAPI, DNA stains that can be excited at 488 nm and which became recently available were tested in this study. The performance of the FCM method developed was compared with that of electronical particle counting and sizing (EPCS) and light microscopy. While light microscopy is the standard technique which has been used in almost all previous studies to identify and count ciliates, EPCS has been applied only sporadically to measure the cell numbers and size of cultured protozoa (Lavin et al., 1990; Weisse and Kirchoff, 1997).

2. Materials and methods

2.1. Study organisms

Two species of prostomatid ciliates were used in this study: *Urotricha farcta* (in vivo cell size 20–30 × 15–20 μm, Foissner et al., 1999) and *Balanion planctonicum* (in vivo size approximately 15–20 μm, Foissner et al., 1999). *U. farcta* was isolated from Lake Schöhsee (Weisse and Montagnes, 1998). *B. planctonicum* was isolated from Lake Mondsee by Helga Müller in autumn, 1999. The ciliates were maintained at 15°C in modified Woods Hole medium (MWC, Guillard and Lorenzen, 1972; *U. farcta*) or in MWC medium plus an equal amount of sterile filtered lake water from Lake Mondsee (*B. planctonicum*). The small cryptophyte, *Cryptomonas* sp. strain 26.80 (approximately 11 × 7 μm, average cell volume 280 μm^3, Weisse and Kirchoff, 1997), obtained from the

Culture Collection of Algae in Göttingen (Germany), was used as prey. This alga has been used as a standard food organism by several laboratories in many previous experiments with prostome ciliates (Müller and Geller, 1993; Weisse and Montagnes, 1998; Müller and Schlegel, 1999; Montagnes and Weisse, 2000).

2.2. Preservation and DNA staining of the ciliates for flow cytometry

The ciliate cultures were preserved with formalin (final concentration of formaldehyde 3.7% v/v) prior to staining. To optimise distinguishing the ciliates from other particles in the cultures, three different nucleic acid binding dyes were tested using subsamples from one culture of *U. farcta*, and one culture of *B. planctonicum*. The dyes tested were: PicoGreen, TO-PRO-1 and YO-YO-1 (Molecular Probes Europe, Leiden, The Netherlands). The three different dyes used are all excitable by blue light (i.e., at 488 nm), and when they bind to nucleic acids they all emit green light (Haugland, 1996). The dyes were added directly to the preserved samples; PicoGreen was added to yield a concentration of 1/1000 of the stock solution, and TO-PRO-1 and YO-YO-1 were used at final concentrations of 30 nM each. The concentrations of the dyes added followed previously described protocols for staining phytoplankton for FCM (Marie et al., 2000). We also tested for the optimum dye concentration in additional experiments, in which *U. farcta* was exposed to different concentrations of PicoGreen (0.05/1000–10/1000 of the stock) and TO-PRO-1 (10–50 nM). Results showed that the concentrations given above gave the highest fluorescence intensities (PicoGreen), or that higher concentrations increased fluorescence intensity only slightly (TO-PRO). The cultures were stained in darkness at room temperature for 60 min. Fluorescence intensity of TO-PRO stained cells increased linearly with incubation time during the first 60 min (results not shown); thereafter, the rate of increase levelled off. Accordingly, a staining time of 60 min was chosen.

It has been previously suggested that formaldehyde-fixed ciliate cells degrade faster than cells fixed with Lugol's iodine solution (Sherr and Sherr, 1993). We therefore tested if samples which were fixed with 2% (v/v) acid Lugol's iodine and then destained with a few drops of sodium thiosulphate (Sherr and Sherr, 1993) could thereafter be stained with TO-PRO and measured by FCM. The staining worked well, yielding a cellular fluorescence intensity comparable to the formaldehyde fixed material. In contrast to working with the formalin-fixed cells, we could, however, not obtain reproducible FCM cell counts with the Lugol's fixed samples. We assumed this was caused by faster settling of the Lugol's preserved cells compared to the formalin-fixed cells. Therefore, fixation by Lugol's iodine was abandoned. Glutaraldehyde, which is commonly used for preservation of protists (Sherr and Sherr, 1993), is known to give an undesired background of green fluorescence (e.g., Veldhuis et al., 1997; Vives-Rego et al., 2000) and was, therefore, not tested.

2.3. Flow cytometry

The samples were analysed using a FACSCalibur™ flow cytometer (Becton Dickinson Austria, Schwechat, Austria). The flow cytometer was equipped with an Argon-ion laser emitting light at 488 nm. The forward scatter (FSC) signal was collected by a forward scatter diode, while side scatter (SSC) as well as green, orange and red fluorescence were collected by photomultiplier tubes (PMT). A filter passing light of 530 ± 15 nm was placed in front of the PMT collecting green light. The corresponding filters for the orange and red fluorescence passed light of 585 ± 21 and > 650 nm, respectively. For details of the optical system of the FACSCalibur see Collier (2000). FSC and SSC parameters were collected using either linear or logarithmic amplification, while the fluorescence parameters were always collected with logarithmic amplification. Green fluorescence was used as the triggering parameter, i.e., only signals from particles with a green fluorescence intensity exceeding a given threshold value were measured. We used the following PMT voltages and settings: E^{-1} and amplification gain 7 in linear mode (FSC) 276 and amplification gain 1.7 in linear or 240 in log mode, respectively (SSC), 270 in log mode (green fluorescence) and 300 in log mode (red fluorescence). The number of log decades was four. The data were analysed with CELLQuest™ version 3.0 and Attractors™ version 3.0 software packages (Becton Dickinson). We used FACSFlow™ (Becton Dickinson) as sheath fluid for all analyses.

2.4. Quantification of ciliates by flow cytometry

The preserved and TO-PRO-1 stained ciliate samples were gently mixed and run for 5 min at the highest flow rate possible, giving a count rate of less than 300 particles per second. The abundance of ciliates (c; cells ml^{-1}) in the samples were calculated according to:

$$c = (n/V) \times 1000 \times 1.13 \quad (1)$$

where n = the number of ciliates counted by the flow cytometer, 1.13 is a factor to compensate for the dilution of the sample by formaldehyde and TO-PRO-1 and V is the volume of sample analysed (μl) according to:

$$V = vt \quad (2)$$

with t = time of sample run (5 min) and v = flow rate of the sample in the cytometer (μl min^{-1}).

The flow rate of the cytometer was determined by running a sample of a known concentration of latex beads (TruCount™, Becton Dickinson). Initially, we only determined flow rate at the end of each day of analysis, and found it to be between 58 and 62 μl min^{-1}. However, later on we found the flow rate to vary slightly during the day. Therefore, we instead determined the flow rate at regular intervals between the samples.

We also tested an alternative approach to determine the volume of the sample analysed by adding a known number (2000–9000 ml^{-1}) of latex beads (Nile Red, Becton Dickinson) directly to each sample analysed. This approach resulted, however, in a usually larger variation of ciliate numbers between subsamples. This was probably mainly due to pipetting errors upon the addition of the beads. We used a relatively low beads concentration of a few thousands ml^{-1} to avoid unfavorable high ratios between nontarget populations (all other particles including beads) and target populations (ciliates). Additions of such small amounts of beads require well-mixed and well-calibrated beads stock solutions to be accurate. It seems possible that the pipetting errors could have been reduced if we had put more effort into sonicating and mixing of the beads prior to analysis. Such efforts would, however, have increased the handling time of the samples. Therefore, we used the volumetric method described above for the determination of ciliate numbers.

Since the samples were run for 5 min, approximately 300 μl of sample was analysed. Every sample was run in duplicate, and the mean value of the two measurements was used.

2.5. Comparison of the cell numbers estimated by flow cytometry and light microscopy

Four different subcultures of *U. farcta* were diluted by MWC medium to obtain 18 samples with ciliate abundances ranging from 1000 to 18,000 cells ml^{-1}. Similarly, four different cultures of *B. planctonicum* yielded 19 samples with ciliate numbers varying between 200 and 30,000 cells ml^{-1}. The cultures used differed in their prey concentrations. Some ciliate samples had received ample food supply, others were deplete of food upon fixation. From each of these samples, two subsamples were taken. One subsample was preserved with formalin (final formaldehyde concentration 3.7% v/v) and stained with TO-PRO-1, and the ciliates were then enumerated by FCM, as described above. The other subsample was preserved with 2% acid Lugol's iodine, and the ciliates were enumerated by light microscopy in a Sedgewick Rafter cell of 1-ml volume.

To check the potential effect of storage time on ciliate counts in formaldehyde fixed samples, six of the *U. farcta* samples mentioned above were remeasured by FCM after 3, 6, 24 and 48 h of storage at 4°C.

2.6. Statistics

The correspondence between ciliate numbers determined by microscopic counts and those determined by flow cytometry was analysed by least-squares linear regression. The effect of storage time on ciliate numbers determined by flow cytometry was analysed by a two-way ANOVA, with time and initial abundance of *U. farcta* as factors and the actual ciliate abundance as dependent variable. All statistical analyses were performed in Microsoft Excel 98 for Macintosh.

2.7. Electronical particle counting and sizing (EPCS)

EPCS measures particle concentrations, equivalent spherical diameter (ESD) and volume according to a resistance measuring principle (e.g., Kachel, 1990; Boyd and Johnson, 1995). This method has previously

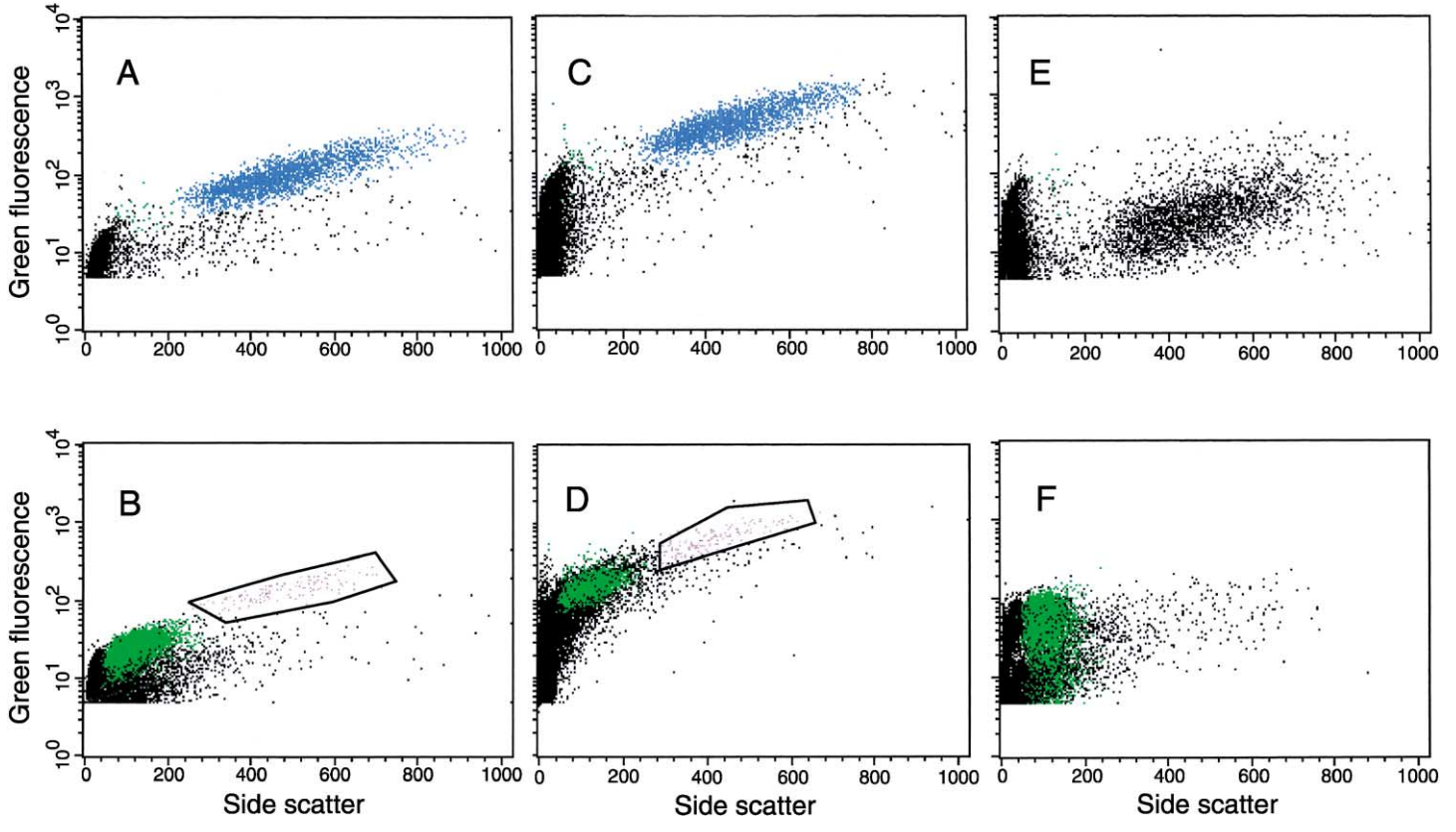

Fig. 1. Flow cytometric dot plots showing green fluorescence vs. side scatter (SSC). Cultures of *U. farcta* (A, C and E) and *B. planctonicum* (B, D and F) stained with TO-PRO-1 (A and B), PicoGreen (C and D) or YO-YO-1 (E and F). Blue: *U. farcta*; pink and marked by gate: *B. planctonicum*; green: the prey *Cryptomonas* sp.; black: unidentified particles. The *Cryptomonas* population was identified according to its SSC vs. red fluorescence signature (not shown).

been used in combination with flow cytometry to study feeding rates of cultured freshwater ciliates and dinoflagellates (Lavin et al., 1990; Weisse and Kirchoff, 1997). Since this technique measures cell numbers and size with high speed similar to FCM, we wanted to compare the performance of EPCS with that of the FCM method developed. Cell concentrations and dimensions of ciliates (*U. farcta* and *B. planktonicum*) and their cryptophyte prey were determined using a CASY 1-Model TTC (Schärfe System) electronic particle counter. Results are presented in 1024 channels. The orifice of the measuring capillary was 150 μm. Between 0.2 and 1.0 ml of the sample volume was diluted to 10 ml with isotonic solution (CASYton, Schärfe System), and 0.2 or 0.4 ml of the total volume was then measured in four replicates. Both live and fixed samples can be used for EPCS measurements. Live samples were measured immediately after sampling, while samples fixed with Lugol's solution were measured within two weeks after fixation. The typical time of analysis was 12–15 s per measurement. To analyse one sample in four replicates, therefore, took about 1 min.

3. Results and discussion

The *Cryptomonas* sp. prey populations could be easily detected and gated from the ciliate cultures based on their side scatter (SSC) vs. red fluorescence (chlorophyll autofluorescence) signals (data not shown). In the dot plots showing green fluorescence (i.e., DNA-induced fluorescence) vs. SSC, the ciliates could be separated from other particles. Staining by both TO-PRO-1 and PicoGreen yielded relatively easily distinguishable populations of *U. farcta* and *B. planctonicum* (Fig. 1A–D), while staining with YO-YO-1 did not allow distinguishing ciliates unequivocally from other

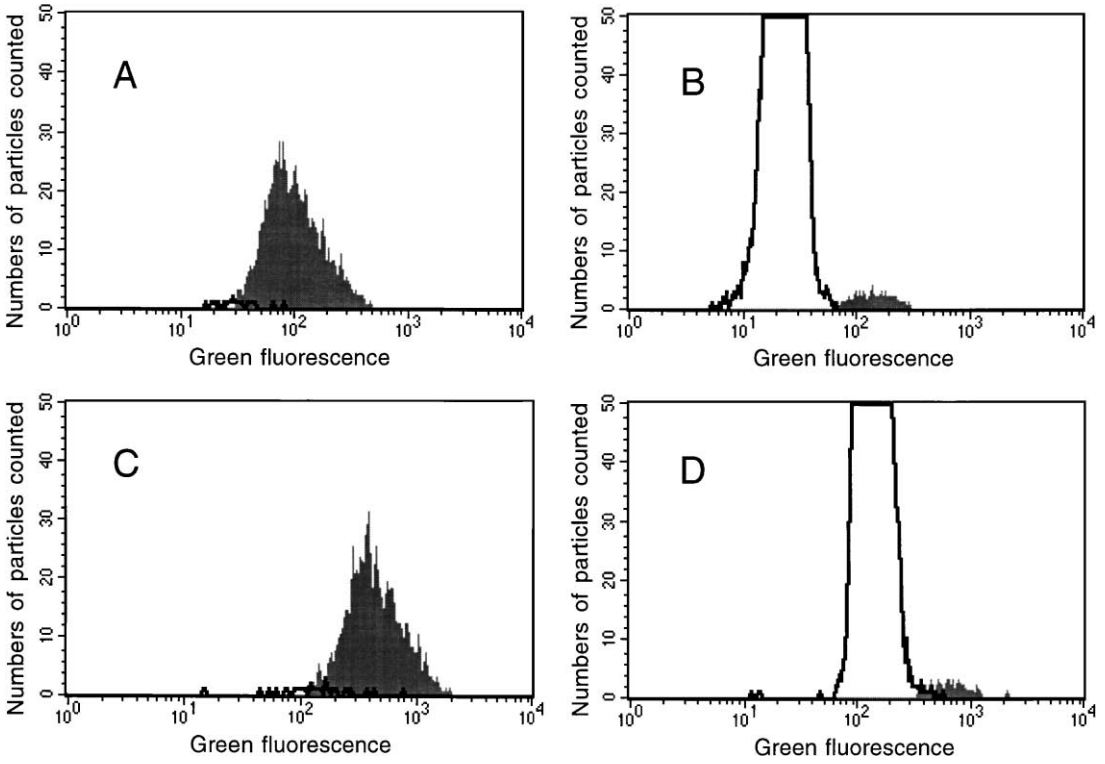

Fig. 2. Flow cytometry histograms of green fluorescence intensity of the ciliates and their prey. Subsamples of cultures of *U. farcta* (A and C) and *B. planctonicum* (B and D) stained with TO-PRO-1 (A and B) or PicoGreen (C and D). Filled grey: ciliates; black unfilled: the prey, *Cryptomonas* sp.

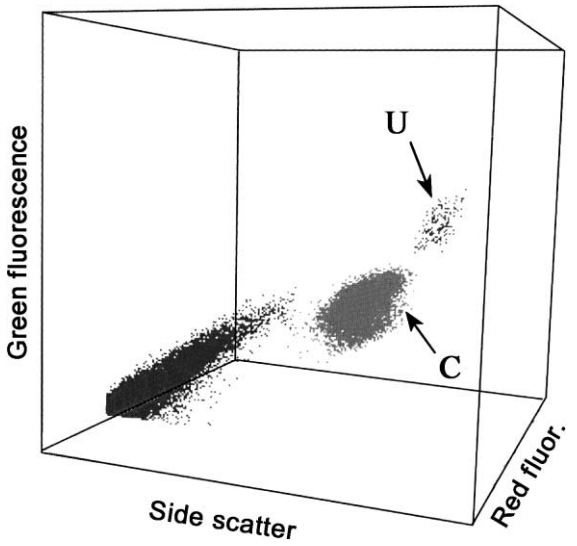

Fig. 3. Three-dimensional dot plot of *U. farcta* (U) and *Cryptomonas* sp. (C). The unlabeled particles are debris.

particles (Fig. 1E and F). The results, therefore, showed that both PicoGreen and TO-PRO-1 are potentially useful dyes for our application. However, for both ciliate species, the addition of TO-PRO-1 resulted in larger differences in green fluorescence between the ciliates and the *Cryptomonas* cells than did PicoGreen (Fig. 2). TO-PRO-1 was, therefore, considered the most adequate of the dyes tested, and was chosen for the further experiments reported below. For enumeration of ciliates, we used a gate in the SSC vs. green fluorescence dot plots (CELLQuest software) or a combination of SSC or FSC with green fluorescence and red fluorescence (Attractors software) to distinguish them from other particles. Discrimination between ciliates, cryptophytes, and detritus particles was most obvious in 3-D plots using the Attractors software (Fig. 3).

Least-squares linear regression analysis (Fig. 4) revealed a highly significant correlation between the ciliate cell numbers obtained by light microscopy and FCM of TO-PRO-1 stained, formaldehyde fixed ciliates ($r^2 = 0.956$, $p < 0.001$). The dotted line indicating ideal agreement between the two methods (Fig. 4) fell into the 99% confidence interval of the regression line. We conclude that FCM accurately measured cell numbers of both ciliate species, for cultures of different physiological status, and at ciliate abundances ranging from 200 to almost 30,000 ciliates ml^{-1}. This range covers the span which is commonly used in ecophysiological investigations with cultured ciliates (e.g. Lavin et al., 1990; Müller and Geller, 1993; Weisse and Frahm, in press).

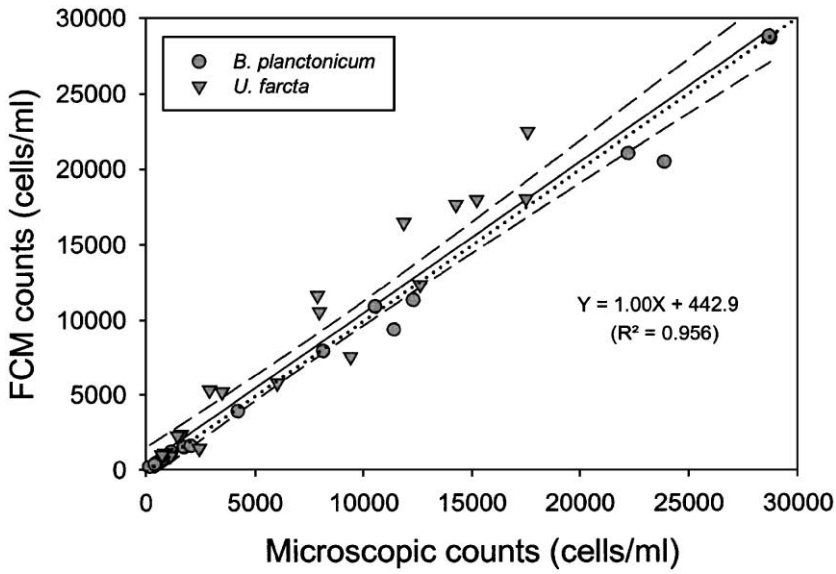

Fig. 4. Abundance of *U. farcta* and *B. planctonicum* measured by flow cytometry vs. light microscopy. The solid line denotes least-squares linear regression, dashed line denotes 99% confidence intervals, dotted line indicates the theoretical perfect agreement between the two methods.

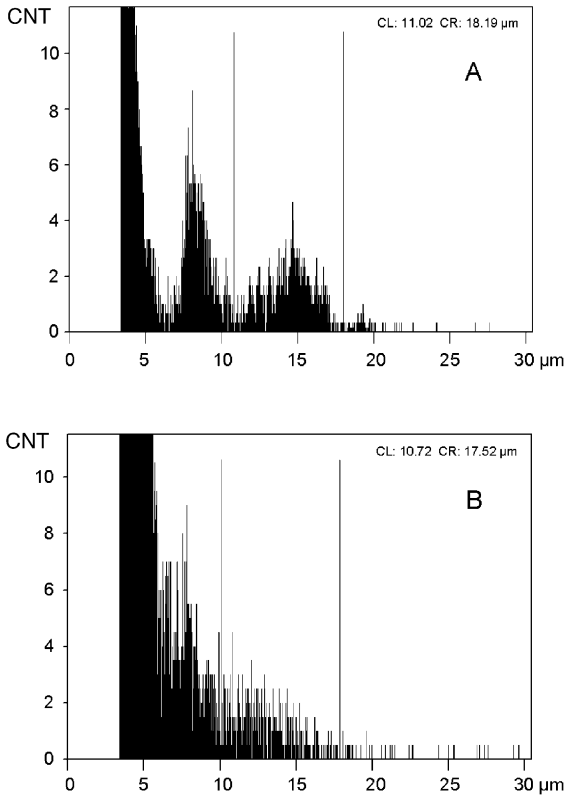

Fig. 5. Typical examples of EPCS plots of cultures of *U. farcta* (A) and *B. planctonicum* (B) and their cryptophyte prey. The *x*-axis shows equivalent spherical diameter (ESD), the *y*-axis shows counts per ESD channel. Vertical cursors (CL = cursor left, CR = cursor right) mark the presumed size range of the ciliates.

Repeated FCM analysis of six formalin-fixed *U. farcta* samples stored for up to 48 h showed a trend of decreasing numbers with time (results not shown). This decrease was, however, statistically insignificant after 48 h ($p = 0.064$). Not surprisingly, the effect of time was even less pronounced 24 h after fixation ($p = 0.126$). These results are in agreement with results previously obtained by microscopic counting, when storage for 24 h did not have a significant effect on ciliate numbers ($p = 0.881$, D.J.S. Montagnes, unpublished data). Thus, storage of formalin-fixed ciliate samples before analysis by FCM should be possible for at least 24 h.

Compared to light microscopy, the flow cytometric method outlined here is less time-consuming and more accurate, since a larger number of cells can be analysed by FCM. In addition, sample statistics are available for each parameter measured cytometrically. Even minor changes in the target parameters, such as cellular DNA content or autofluorescence induced by ingestion of autotrophic prey (e.g., Cucci et al., 1989; Weisse and Kirchoff, 1997), can be assessed in the course of an experiment.

With respect to the time effort needed, EPCS is similar to FCM, since results can be obtained within approximately 1 min. In comparison to FCM, EPCS has the advantage that ciliate samples can be measured immediately, i.e., without fixing and staining. Similar to FCM, the population of *U. farcta* was relatively easy to define by use of EPCS (Fig. 5A), based on the differing equivalent spherical diameters (ESD) of the ciliates and their prey. In contrast, the population of the smaller ciliate *B. planctonicum* was not always unequivocally distinguishable from the cryptophytes.

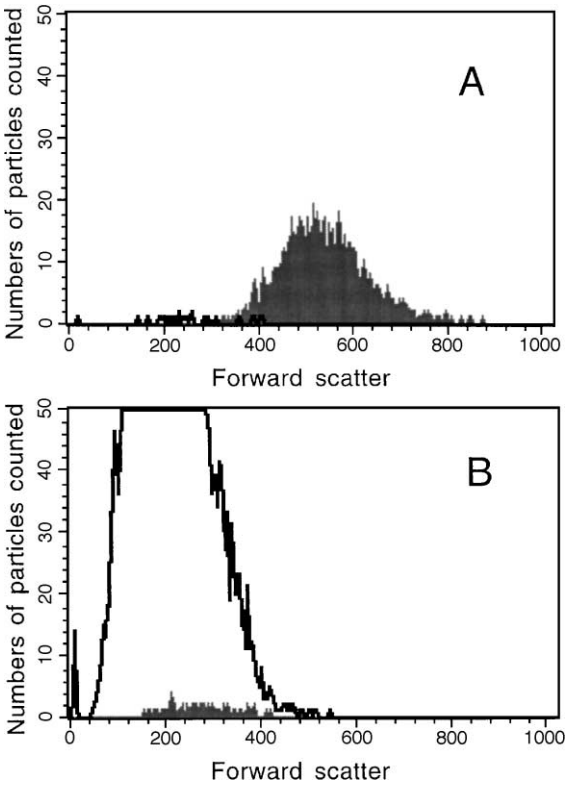

Fig. 6. Forward scatter (FSC) of the TO-PRO-1-stained samples analysed in Figs. 1 and 2, determined by flow cytometry. (A) *U. farcta* and the prey *Cryptomonas* sp.; (B) *B. planctonicum* and the prey *Cryptomonas* sp. Filled grey: ciliates; black unfilled: the prey, *Cryptomonas* sp.

This was a major problem in aged cultures where *Balanion* were relatively small, had depleted their food, and in which detritus particles had accumulated (Fig. 5B). In such cases, it was impossible to separate the two protist populations from each other and from the debris. Accordingly, EPCS is an alternative to FCM only in those cases in which the target population is clearly separated in size from all other particles present in the sample.

Similar to the ESD measured electronically by EPCS, forward scatter (FSC) measured optically by FCM is proportional to particle volume although the relationship is complex and depends on the particular cell type (e.g., Salzman et al., 1990; Watson, 1991 and references therein). Our flow cytometric measurements of the TO-PRO-1 stained samples confirmed the results obtained by EPCS. The overlapping in FSC between the *Cryptomonas* and the larger ciliate, *U. farcta*, was small (Fig. 6A). The FSC signal of *Cryptomonas* sp. overlapped, however, completely with that of the smaller ciliate, *B. planctonicum* (Fig. 6B). The starved *Balanion* population shown in Fig. 6B was approximately 10 μm in length (own unpublished research), i.e., it was on average only slightly larger than their prey. These results suggest that the assumption of a linear relation between FSC and cell size is correct in small protist populations.

To summarise, we have developed a simple and fast flow cytometric protocol for the quantification of small ciliates in cultures. In contrast to previous studies with ciliates (Kenter et al., 1996), our protocol does not require excitation in the UV range, but can be applied using commercially available benchtop cytometers equipped with Argon lasers. Several DNA stains that can be excited at 488 nm have recently become available for the analysis of aquatic microbes (e.g., Li et al., 1995; del Giorgio et al., 1996; Marie et al., 1996, 1997, 2000; Veldhuis et al., 1997; Lebaron et al., 1998). Among the three dyes tested here, TO-PRO-1 gave the best results for our application. We assume that several other dyes that are commonly used in FCM in the study of bacterioplankton, especially SYBRGreen-1 and SYTO-13 (del Giorgio et al., 1996; Lebaron et al., 1998), can be applied to protozoans as well. Based on the results obtained here, we expect that FCM will be of great use in future studies with cultured ciliates when processing of a large number of samples is necessary.

Acknowledgements

We thank Nicholas Crosbie and David J.S. Montagnes for fruitful discussions about FCM and the experimental design of this study.

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