Cytometry 44:126–132 (2001)

A Simple and Highly Efficient Fixation Method for *Chrysochromulina polylepis* (Prymnesiophytes) for Analytical Flow Cytometry

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Received 8 June 2000; Revision Received 15 February 2001; Accepted 27 February 2001

Background: To study the fragile Prymnesiophyte species *Chrysochromulina polylepis* by flow cytometry (FC), we needed an effective fixation method. This method must guarantee a high yield of fixed cells to achieve acceptable measurement times by FC and to allow quick processing of many samples. Moreover, we wanted a method that allows for storage of fixed samples when FC analysis cannot be done immediately.

Methods: Different aldehydes and methanol were tested at different final concentrations. Gravity sedimentation and centrifugation were applied to achieve higher cell concentrations. Storage of fixed samples was tested under different conditions.

Results: 0.25% glutaraldehyde (GA) fixation yielded a recovery rate of about 90%. The signals obtained by FC analysis were excellent. It is possible to centrifuge GA-fixed cells and to store them for several weeks.

Conclusions: GA is the fixative of choice for FC analysis of *C. polylepis* (and possibly other small delicate species) because it yielded highly significant recovery rates and high-quality FC signals. Cells can be centrifuged to increase the cell concentration, thereby achieving short measurement times with FC. The possibility of long-term storage of fixed cells presents an additional advantage if FC analysis cannot be done immediately. Cytometry 44: 126–132, 2001. © 2001 Wiley-Liss, Inc.

Key terms: aldehydes; *Chrysochromulina polylepis*; fixation; flow cytometry; glutaraldehyde

Flow Cytometry (FC) has become an increasingly important tool to answer urgent questions arising in marine biological sciences. One example from this research area is the analysis of physiological processes of phytoplanktonic algae in relation to their cell cycle. Similar studies have been performed using cultured (1,2) as well as wild species (3,4) with growth rate constants usually being determined by FC (3–5). Cell cycle analysis using FC visualizes the stages of the cell division cycle by quantifying the DNA in each phase (6). This is possible only after fixation of the cells and appropriate staining of the DNA.

For the investigation of the toxin production of the ichthyotoxic species *Chrysochromulina polylepis* (Prymnesiophyta) throughout its cell cycle, we needed to establish a suitable fixation method that allows efficient FC analysis. The main focus was on the high recovery yield of cells of this small flagellate (appoximately 8 μm in diameter) after fixation to ensure short measurement times by FC. This was not trivial because *Chrysochromulina* species are fragile and are easily destroyed by many chemicals often used for fixation and preservation of phytoplankton samples.

For our demands, the procedure, besides being as gentle as possible, must also be simple and quick. This is to guarantee that many samples (>100) can be managed within short time intervals throughout the experiment with samples that had to be taken in parallel for several other analytical purposes. We also explored possibilities to concentrate cells in samples that contain only low cell numbers. Because we could not perform FC analyses at our institute, it was also important to examine storage conditions for samples taken over a long period of time (which may also be a relevant point for experimentators who recover samples in the field, e.g., during a long ship cruise).

We began with a protocol developed for preservation of *Prorocentrum lima* (7). This method includes fixation with paraformaldehyde (PFA) dissolved in TE buffer and the addition of Nonidet P40 as well as methanol in TE buffer to increase membrane permeability. After sedimentation by gravity, 90% of the supernatant was removed. This procedure worked inefficiently for *C. polylepis*,

Grant sponsor: BMBF funded project; Grant number: TEPS 03F0161.

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especially because the addition of methanol caused precipitate formation in the seawater-based culture medium and resulted in a further dilution of the cell concentration. Sedimentation during the fixation process was used because centrifugation of living *C. polylepis* cells caused irreversible cell clumping. Recovery rates from sedimentation, however, were sometimes as low as 20%, resulting in either unacceptably long measurement times or even in statistically nonsignificant values due to low numbers of detectable events by FC.

To improve the fixation of *C. polylepis* for FC analysis, we tested fixatives at different concentrations, explored alternatives of concentrating cell numbers in samples with low cell densities, and looked for suitable storage conditions in the event that FC analysis cannot be done immediately.

MATERIALS AND METHODS

Culture Conditions

C. polylepis was grown in IMR-enriched seawater medium (8,9) in batch cultures at 15°C under white fluorescent light with a photon flux of 45 μmol s^{-1} m^{-2}.

Fixation

Fixation of *C. polylepis* cells was performed with (1) PFA (Sigma-Aldrich, Steinheim, Germany; powder dissolved in seawater to 10%), (2) formaldehyde (FA; Polysciences, Warrington, PA; 10% methanol-free ready-to-use solution), (3) glutaraldehyde (GA; Plano, Wetzlar, Germany; 70% double-distilled solution diluted to 10% with seawater), and (4) methanol (Merck, Darmstadt, Germany; analytical grade). Aldehyde stock solutions were freshly prepared (except FA) and were used at 0.25, 0.5, 1.0, 2.0, and 4.0% final concentrations (without further addition of detergents or methanol); methanol was used at 15, 20, 25, 30, and 35% final concentrations. Cells were incubated at 4°C throughout different time periods (at least 1 week). Cell numbers were counted with a Coulter Multisizer II (Coulter Electronics, Krefeld, Germany) equipped with a 100-μm aperture. Cell size profiles were calculated with Coulter-Multisizer software.

Cell Concentrating Procedures

Cells were incubated at 4°C in 10-ml volumes in 15-ml Sarstedt tubes (Sarstedt, Nümbrecht, Germany) containing the culture sample plus fixative. Concentration was achieved by decanting 8 ml of supernatant after 1 week of sedimentation. Alternatively, fixed cells were centrifuged. This was done with the same type of sample as described above. After 1 week of fixation, samples were centrifuged with a swing-out rotor applying 3,200 g for 15 min at 4°C. After centrifugation and decantation of 8 ml of supernatant, the cell pellets were resuspended by vigorous pipetting and vortexing.

Storage Experiments

Culture samples containing fixative were stored at 4°C from 1 to 3 weeks. Alternatively, cells that have been fixed for 1 week at 4°C after centrifugation and decantation of the supernatant were stored as pellets at -20°C for another 2 weeks.

FC–Analysis

Fixed algal cells were stained with 5 μM SytoxGreen (Molecular Probes, Leiden, The Netherlands) by either adding the dye directly to the cells (i.e., fixed cells in seawater containing the fixatives) or by adding the dye to fixed cells that have been pelleted by centrifugation and resuspended in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). Incubation with SytoxGreen was performed in the dark and usually overnight, which was typically 16 h. Stained and fixed cells were analyzed for DNA fluorescence using a FACS Vantage flow cytometer (Becton and Dickinson, San Jose, CA) equipped with an Innova Enterprise II 621 laser light regulated at 20 mW UV, emitting at 488 nm. Green fluorescence of the Sytox stain was detected at 530 nm (FL1). Chlorophyll autofluorescence was detected at 675 nm (FL3, 450 V, linear gain 1). SSC-H was set as threshold parameter at 20 to account for both the unstained and stained samples (360 V, linear gain 1). The pulse-processing parameters used were FL1-H (375 V, gain 1), FL1-A (508 V, gain 1), FL1-W (544 V, gain 1), which were all set to linear scale. Twenty thousand cells were analyzed per sample at a pressure of 1 psi, resulting in a count rate of about 200 events × s^{-1}. Dot plots and histograms were created by WinMDI 2.8 (Joseph Trotter, The Scripps Research Institute, La Jolla, CA).

RESULTS

Fixation of *C. polylepis* Cells With Different Aldehydes and Methanol

Highest recovery rates of fixed cells after 1 week of incubation at 4°C were obtained with GA and methanol, reaching up to 90% cell recovery or higher (Fig. 1). Whereas GA was already effective at final concentrations as low as 0.25%, methanol worked better at higher concentrations (≥30%). PFA and FA yielded maximum 75% recovery, with PFA being more or less equally effective throughout all the concentrations tested and FA being most effective when used at ≥2% final concentration.

Besides determining the cell numbers, we measured size profiles before and after fixation with the Coulter-Multisizer software (Fig. 2). All fixatives led to a reduction in cell size, which was biggest with the use of methanol (26.1%), followed by FA (11.6%) and PFA (8,7%). Fixation with GA, however, led only to a minor reduction of the cell size (4.4%).

Concentration of Fixed Cells

Sedimentation analysis was performed with the lowest concentration for all fixatives for at least 1 week, until a cell pellet was visible in the tip of the tubes (Table 1). After decanting 4/5 vol of supernatant, the PFA sample showed a loss of more than 50% of the cells. The situation was even worse with FA and methanol-fixed cells; only

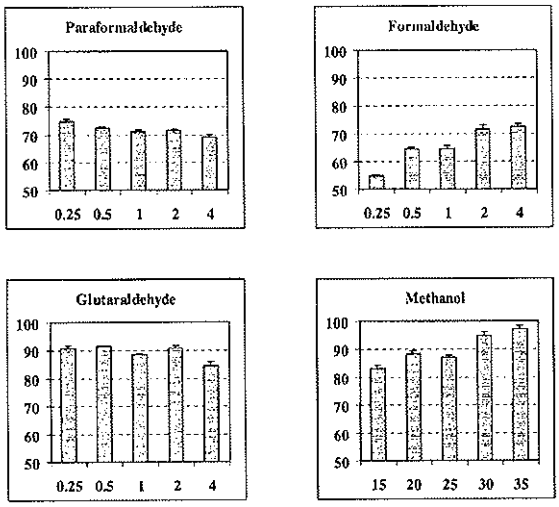

FIG. 1. Concentration-dependent fixation efficiency of different aldehydes and methanol. *C. polylepis* cells were fixed with different concentrations of fixatives (x-axis) for 1 week at 4°C. Fixation efficiency is expressed as percent recovery of fixed cells from cells counted prior to fixation (y-axis). All values are calculated as the mean of three measurements. Error bars indicate SDs.

23.1% and 30.6% were recovered, respectively. GA, 60.7%, showed the best total cell number recovery.

We also checked the possibility of increasing the cell concentration by centrifugation, which is not possible prior to fixation. Our results show that centrifugation after fixation significantly increases the yield of recovered cells when applying 3,200 g for 15 min. It is possible to harvest

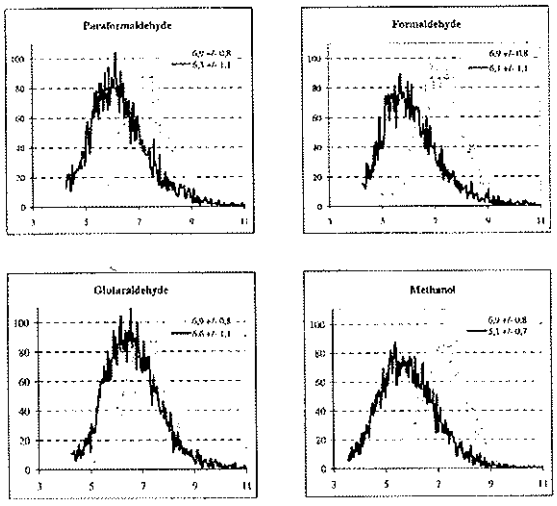

FIG. 2. Coulter Counter cell size profiles (equivalent spherical diameter [μm] versus cell number) of *C. polylepis* cells before (grey plots) and after fixation (black plots) with aldehydes (0.25%) and methanol (15%). Legends indicate mean cell sizes ± SD.

Table 1
*Comparison of the Two Methods Used to Concentrate Fixed Cells**

Fixative	Percent cell recovery after sedimentation	Percent cell recovery after centrifugation (3,200 g)
0.25% PFA	47.2 ± 2.3 (62.6)	73.4 ± 3.2 (97.3)
0.25% FA	23.1 ± 2.8 (39.8)	50.9 ± 0.5 (87.8)
0.25% GA	60.7 ± 2.1 (70.5)	87.7 ± 3.0 (100.0)
15% Methanol	30.6 ± 1.4 (36.7)	79.3 ± 6.4 (95.2)

*Cells used for sedimentation and centrifugation were fixed for 1 week at 4°C with 0.25% aldehydes and 15% methanol, respectively. All values (mean of three measurements ± SD) present the recovery in percent from total cell number measured live prior to any treatment. Numbers in parentheses additionally indicate recovery in percent from total number of fixed cells measured prior to sedimentation or centrifugation.

up to 100% of the cells that have been successfully preserved by the respective fixative (indicated by the numbers in parentheses in Table 1). These data are in good accordance with the data in Figure 1, which show fixation efficiencies for 0.25% aldehydes and 15% methanol.

Storage of Fixed Cells

Cells stored at 4°C (culture sample plus fixative) were counted after 1, 2, and 3 weeks (Fig. 3). Additionally, we examined the possibility of storing cells as pellets at -20°C for 2 weeks after they have been fixed for 1 week at 4°C. We recovered more than 80% of GA and methanol-fixed cells after 1 week and still counted ≥70% after 3 weeks. PFA, with >70% recovery after 1 week and about 50% after 3 weeks, and FA, with <60% recovery after 1 week and about 30% after 3 weeks, worked significantly less efficiently. Storage of fixed cells as pellets at -20°C led to huge cell losses in all cases. However, recovery of GA and methanol-fixed cells with about 40% was significantly

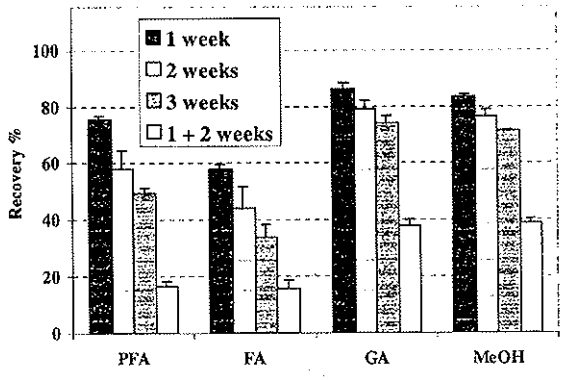

FIG. 3. Influence of storage of fixed cells on cell recovery. Cells were fixed with 0.25% aldehydes and 15% methanol and stored in culture medium containing the respective fixative either up to 3 weeks at 4°C or for 2 weeks at -20°C as cell pellets after 1 week of fixation at 4°C (nonshaded bars). All values (mean of three measurements plus SD) present the recovery in percent from total cell number measured prior to fixation.

higher than for PFA and FA-fixed samples, which show a reduction to <20%.

FC Analysis of Fixed *C. polylepis* Cells

FC analysis was performed using the same settings and number of events for all samples to allow for comparison of the influence of the different fixatives on cellular parameters (Figs. 4–7). The scatter characteristics (Figs. 4A, 5A, 6A, 7A) already show important differences between the different aldehydes and methanol. Both PFA and FA-fixed samples do not show a homogenous population of cells. Besides the main population, there was a subpopulation in the lower part of the scatter plots that consists of destroyed cells, which becomes evident by the chlorophyll fluorescence shown in Figures 4A and 5A. In contrast, the cell population in the GA-fixed sample (Fig. 6A) appeared more homogenous and there were only a few destroyed cells detectable by their red fluorescence (Fig. 6B). The scatter characteristics of the methanol-fixed sample is completely different from the aldehyde-fixed samples. Forward scatter (FSC) and side scatter (SSC) signals appear to be reversed (Fig. 7A), indicating major changes of the cell morphology induced by methanol. The red chlorophyll fluorescence (Fig. 7B), although not completely reduced, is significantly quenched by methanol compared with aldehydes (methanol concentrations higher than 30% led to complete loss of red chlorophyll fluorescence). GA-fixed cells retained the highest chlorophyll fluorescence (Fig. 6B).

Because GA is reported to possess intrinsic fluorescence properties, we compared fixed cells with and without the green fluorescent DNA stain SytoxGreen (C and D, respectively, in Figs. 4–7). The results clearly show that there is only slightly more intrinsic fluorescence in the GA samples (Fig. 6C) compared with the other aldehydes (Figs. 4 and 5). This autofluorescence of the fixatives, however, is clearly distinguishable from the green fluorescence conferred to the cells by SytoxGreen (D in Figs. 4–7). The methanol sample proved once more to be exceptional because it does not show green fluorescence either in the unstained (Fig. 7C) or in the SytoxGreen-stained sample (Fig. 7D).

For this reason, the peak characteristics (peak width and area in E and F in Figs. 4–7) for DNA signals could only be appraised for the aldehyde-fixed samples. The cell populations (gated in details E) gave similar shaped peaks (shown as histograms in details F) for PFA and FA-fixed cells (Figs. 4 and 5). However, compared with the sharp peak obtained for GA-fixed cells (Fig. 6F), they appear sturdier. This is also reflected by the coefficient of variation (CV) values gained for 20,000 events per sample, which was lowest for the GA sample (12–13% compared with 16–17% for the PFA and FA samples, respectively).

Neither centrifugation as a means to increase cell concentration nor storage either at 4°C or at -20°C for 2 weeks negatively influenced the quality of the signals obtained. However, a big difference was observed between fixed cells being resuspended in TE buffer (a buffer generally recommended [10] for DNA storage and manipulation) compared with cells taken directly from culture medium containing the fixative. The former showed a significant reduction of almost 50% in fluorescence intensity relative to the seawater samples as well as increased CV values (18–20% compared with 11–12% for a GA-fixed sample).

DISCUSSION

The use of fixatives is often necessary in the analysis of phytoplankton by FC. In many cases, the fresh samples cannot be measured directly at the sampling site or the staining or labeling procedure requires a fixation step. The aim of the present study was to develop a protocol that could be used for effective fixation of *C. polylepis* (Prymnesiophyta), which is known to be fragile and very sensitive to many chemicals used for the preservation of phytoplankton.

GA, FA, PFA have been used almost exclusively. They preserve the autofluorescence of chlorophyll best, which is a prerequisite for ecological studies in natural communities (11). However, there have been contradictory reports on the applicability of any of the three fixatives. There is also some confusion about the proper definition of FA and PFA (see info at http://www.cyto.purdue.edu/hmarchiv/99/1226.html). To preserve wild phytoplankton mixtures, Vaulot et al. (12) proposed 1% GA, followed by immediate storage in liquid nitrogen. This basic method (fixation and shock freezing) has been adopted by many authors (13). However, PFA was soon regarded as superior to GA, due to a better long-term preservation (14,15), lower intrinsic fluorescence (16), and a lower cell loss of the picoplankton (17). Some authors have used mixtures of both GA and PFA (18,19), but PFA has been increasingly preferred over GA in field studies (20–22).

In studies involving DNA analyses, GA (6), FA (3), and PFA (23, 24) have been used satisfactorily. PFA is mostly preferred over FA because the latter breaks down after some time, which reduces fixation efficiency (25). GA is known as the fixative of choice for electron microscopy sample preparation. Although its penetration rate is slower compared with FA, it causes more effective cross-linking of proteins because of the presence of two functional groups (dialdehyde). This leads to extraordinarily good preservation of ultracellular structures (26,27). Additionally, we tested the usefulness of methanol for cell preservation and subsequent FC analysis.

Our data show that PFA, especially if used at lower concentrations, yielded higher recoveries compared with FA, which was applied as a ready-to-use solution. The latter may contain less reactive FA because of the presence of degraded products than freshly prepared PFA solution. In contrast to other reports (17), GA at its lowest concentration was more effective than PFA and FA. Because of the small cell size of *C. polylepis*, it is not likely that these results were due to the different penetration capabilities of the aldehydes. Fixed samples were counted after 1–3 weeks of incubation. Therefore, better preservation and storage capacity seem to be the more likely explanations, resulting from the more effective fixation of GA compared with FA (according to Reimer [28], GA-

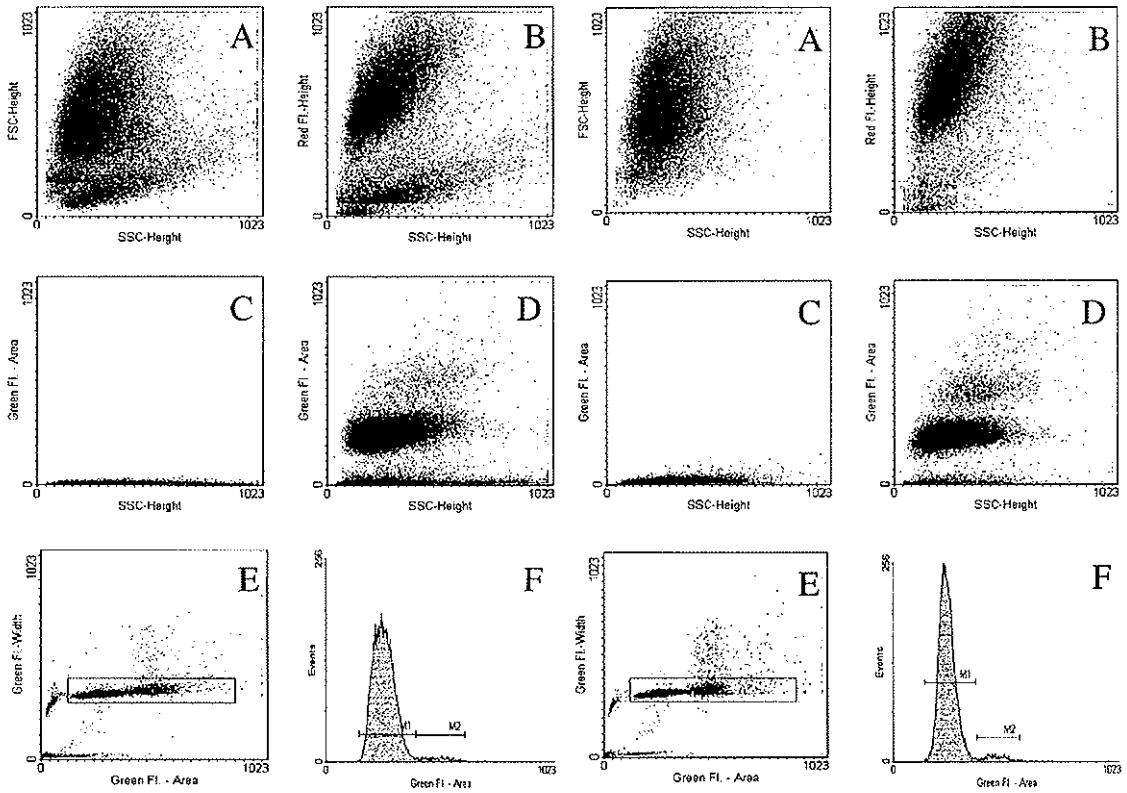

Fig. 4

Fig. 6

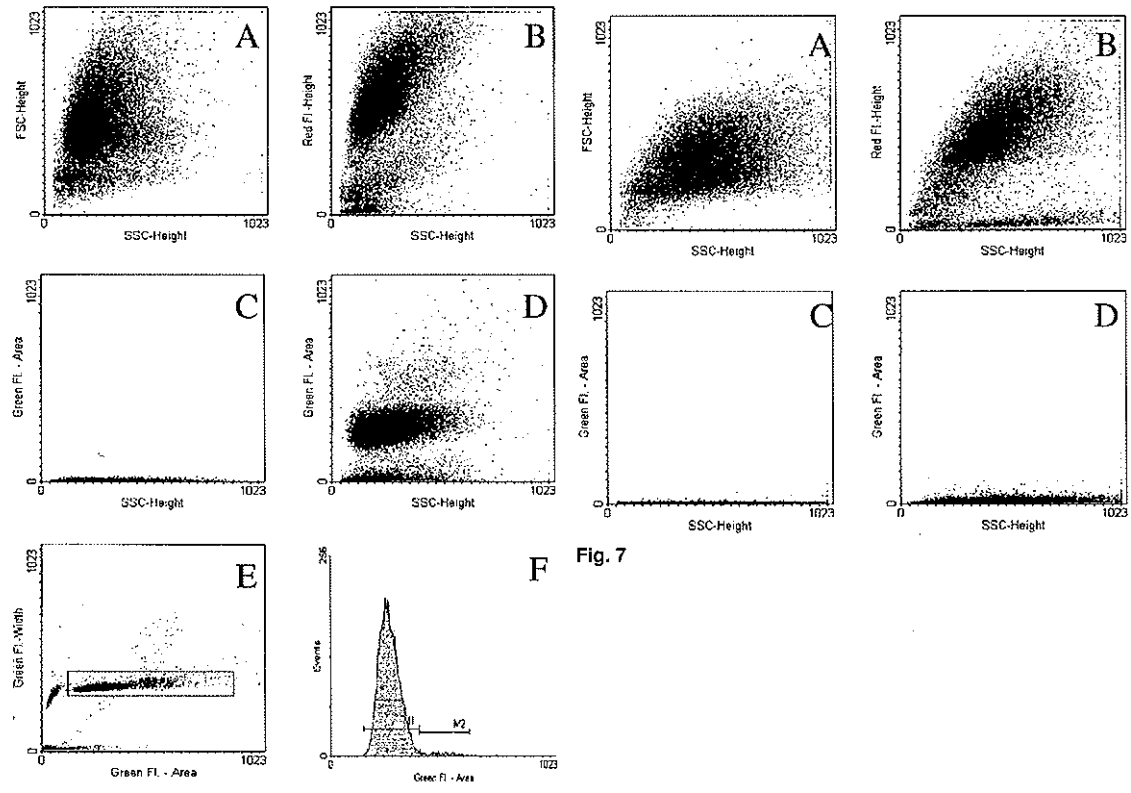

Fig. 5

Fig. 7

fixed material can be stored for months in aqueous buffer).

The superior preservation capacity of cellular structures by GA is also indicated by the fact that the cell size of *C. polylepis* is much less affected by GA than by the other fixatives (Fig. 2). Further support for the careful preservation of the cells by this agent was obtained with FC analysis. The scatter and chlorophyll fluorescence measurements revealed a homogenous cell population for GA-fixed cells in contrast to PFA and FA-fixed cells, which showed a significant portion of destroyed cells. Methanol-fixed cells, although appearing homogenous in the scatter analysis, apparently underwent major morphological changes as indicated by the reversal of the scatter characteristics and the extreme shrinkage of the cells (Fig. 2).

The most convincing argument for the use of GA as the fixative of choice for *C. polylepis*, however, is given by the DNA fluorescent signals obtained after staining with SytoxGreen. The signals from GA-fixed cells are superior to the signals from the two other aldehydes with respect to the sharpness of the peaks and CV values. In contrast to other reports (16), the intrinsic fluorescence of GA (measured with unstained cells) is only slightly higher than for PFA and FA and does not at all interfere with the fluorescence of the DNA stain. Methanol, despite its excellent preservation properties, is definitely not suitable for FC because cells fixed with this agent did not stain with SytoxGreen. One reason for this effect could be the strong dehydrating capacity of methanol, which results not only in considerable shrinkage of the cells but also in extraction of hydration water from macromolecules. This may prevent an effective intercalation of SytoxGreen into DNA. In this context, it should be mentioned that in contrast to the original protocol, no methanol was added to the aldehyde-fixed samples. This not only prevents the risk of precipitate formation and a further dilution of the samples, but also avoids bleaching of the chlorophyll. The red autofluorescence of chlorophyll is maintained as an additional parameter that can be used for FC analysis. Again, it is best preserved in GA-fixed cells, which show the highest chlorophyll fluorescence.

For experiments with many samples, or for samples containing only few cells, it is desirable to have a suitable tool at hand to concentrate the cells in order to achieve acceptable analysis times with FC. However, it is not possible to centrifuge living cells of *C. polylepis* because they will clump and they cannot be resuspended without destroying a large percentage of the cells. On the other hand, it is possible to pellet cells after fixation with high-speed centrifugation and subsequent resuspension in a suitable volume. This relatively rough treatment had no negative influence on the quality of FC results and led with all fixatives to a highly significant recovery of up to 100% of fixed cells. Concentrating cells by means of sedimentation, however, did not always result in satisfying recoveries of cells, but led to an acceptable recovery of 60% in the case of GA-fixed cells.

Because of the small cell size of *C. polylepis*, sedimentation had to be extended to at least 1 week before a clearly visible pellet had formed in the tube. This relatively long sedimentation time is not objectionable, if GA or methanol is chosen for preservation. No great cell loss (less than 10%) was observed even after 3 weeks of incubation in seawater plus the respective fixatives. On the other hand, PFA and FA-fixed samples showed a decrease in cell numbers by approximately 30% after 3 weeks. This again is in contrast to what is reported by others (14,15) because preservation of *C. polylepis* cells is definitely better with GA than with FA or PFA. The possibility to store fixed samples in a refrigerator for several weeks is a big advantage, especially when samples have to be collected throughout an extended period of time and cannot be analyzed by FC immediately. Our data indicate that long-term storage of fixed cells is also possible at -20°C as pellets. However, significant cell losses must be taken into account.

Finally, it is worth mentioning that fluorescence staining of GA-fixed cells with SytoxGreen works most efficiently in culture medium (seawater). Staining of fixed samples after resuspension in TE buffer, which is recommended for many DNA applications (10) and by product information for SytoxGreen stain provided by Molecular Probes, was less sensitive (decreased fluorescence intensity) and less reproducible (increased CV values) in subsequent analyses performed by FC.

In conclusion, we now have a very simple and highly efficient fixation method for *C. polylepis* based on the use of GA. Although our findings contrast with the extensive use of PFA in field studies, we have shown that our method works well for the delicate flagellate *C. polylepis* and may be the method of choice when these or closely related organisms are of particular interest. Dependent on the respective requirements, it is possible to prepare high-quality samples for FC either with gentle sedimentation requiring a minimum of instrumentation or very rapidly and with quantitative recovery of fixed cells by centrifugation.

FIGS. 4–7. FC plots of *C. polylepis* cells from batch cultures. Samples were fixed for 1 week at 4°C with 0.25% PFA (Fig. 4) FA (Fig. 5), GA (Fig. 6), and 15% methanol (Fig. 7). To concentrate cells, samples were centrifuged prior to FC analysis. Twenty thousand events were measured per sample. **A:** Scatter characteristics (FSC versus SSC). **B:** Chlorophyll autofluorescence (red fluorescence versus SSC). **C:** Green fluorescence of fixatives measured with cells not stained with SytoxGreen (green fluorescence versus SSC). **D:** Green fluorescence of cells stained with SytoxGreen (green fluorescence versus SSC). **E:** Peak characteristics of green fluorescent signals (green fluorescence width versus green fluorescence area) with gate for histogram in F. **F:** Histograms of DNA peaks gated in (E) (green fluorescence versus the number of events). E and F not shown for methanol-fixed samples because of the lack of green fluorescence in these samples.

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