R. A. Diamond
S. DeMaggio
(Eds.)

In Living Color

Protocols in Flow Cytometry and Cell Sorting

Springer
Lab Manual

ROCHELLE A. DIAMOND
Member of the Professional Staff
California Institute
of Technology
Division of Biology
1200 E. California Blvd.
Pasadena, CA 91125, USA
Tel: 626 395 - 4947
Fax: 626 449-0756
E-mail: diamond@its.caltech.edu

SUSAN DEMAGGIO
MS BSMT (ASCP)CQ
Optical Biology Core Facility
Developmental Biology Center
University of California
Irvine, CA 92687-2275, USA
Tel: (949) 824-4110
Fax: (949) 824-3571
E-mail: suedemag@uci.edu

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Flow Cytometry Analysis of Marine Picoplankton

D. MARIE, N. SIMON, L. GUILLOU, F. PARTENSKY, AND D. VAULOT

introduction

In the last decade, the use of flow cytometry (FCM) has become more and more popular among limnologists and marine biologists, both for laboratory studies and field research. FCM allows the analysis of phytoplanktonic cells that are too dim to be discriminated by epifluorescence microscopy. Its major advantages are to provide rapid and accurate measurements of individual particles and to allow the discrimination between auto- and heterotrophic populations as well as between cells and detritus or suspended sediments. FCM is particularly well suited for the study of the smallest size class of the plankton (below 2 μm), called picoplankton. Picoplankton is composed by 4 major groups: heterotrophic prokaryotes, prochlorophytes (*Prochlorococcus*),[1] cyanobacteria (*Synechococcus*)[2] and eukaryotes. These small organisms dominate the biomass in the open ocean, reaching respective concentration ranges of $10^6 - 10^5$, $10^5 - 10^3$, $10^5 - 10^3$ and $10^4 - 10^2$ cells per ml. The geographical distribution of these organisms, their biological characteristics (carbon and pigment content), and their dynamics in relation to the biotic factors are of major interest for the oceanographers. Initially used to discriminate and enumerate the different populations of phytoplankton, the application of flow cytometry has been extended to physiological analyses (e.g. DNA analysis) and more recently to phylogenetic analyses with the help of fluorescent molecular probes.

We present here four methods that are useful for the analysis of marine picoplankton both for natural samples and cultures.

1. Photosynthetic picoplankton. Photosynthetic picoplankton possess naturally fluorescing pigments (chlorophyll, phycoerythrin). Therefore the straight analysis of marine samples

allows one to obtain information on the abundance, cell size and pigment content of the major photosynthetic picoplankton groups (prochlorophytes, cyanobacteria and eukaryotes).[3] This type of analysis can be performed either on unfixed samples on board ships or on preserved samples that are brought back to shore.

2. Heterotrophic bacteria and cell cycle analysis of photosynthetic prokaryotes. Since bacteria do not contain naturally fluorescing pigments, they need to be stained prior to analysis. Nucleic acid stains are very useful in this respect. Initially the UV-excited dyes DAPI or Hoechst 33342 have been used for this purpose.[4,5] However, recently, 488 nm-excited dyes such as YOYO-1, PicoGreen or SYBR™ Green-I have been introduced [6,7,8] that make this type of analysis possible on small low-cost flow cytometers. Moreover, nucleic acid stains provide information on the cell cycle distribution of photosynthetic prokaryotes, which allows to estimate directly growth rates in the ocean. [9,10]

3. Taxonomy of eukaryotic picoplankton. The taxonomy of small eukaryotic picoplankton is still poorly known nowadays. Most species have very few morphological features and can hardly be discriminated, even at the class level, by classical methods such as optical microscopy. Fluorescent oligonucleotide probes targeted to 18S rRNA appear as very promising tools for this purpose[11,12] allowing the identification of specific groups within complex communities.

4. DNA content and G-C% of isolated eukaryotic nuclei. For many eukaryotic marine strains, taxonomy is also very uncertain at finer levels, typically the species level. Species differences can be resolved by assessing their DNA content and G-C%. This has proved very useful for example to resolve the taxonomy of the ubiquitous genus *Phaeocystis*.[13] Another application of DNA content determination in microalgae is to assess ploidy levels in order to resolve the sexual cycle.[14] Such determination can be achieved on cultures using the range of DNA stains which are currently available and which present different G-C% sensitivity.

Subprotocol 1
Abundance and cell characteristics of photosynthetic picoplankton

Flow cytometry is now routinely used for the analysis of marine oceanic samples. Ideally, samples should be first analyzed fresh on board ships. Alternatively, if fresh analysis is not possible, a simple method for the preservation of marine samples, that interfere minimally with the cellular properties of phytoplanktonic cells, was developed by Vaulot et al.[15] The combined analysis of the light-scattering parameters and of the fluorescence of natural photosynthetic pigments (chlorophyll, phycoerythrin) allows the identification of different groups that differ in terms of size and pigment contents. Several aspects are critical to successful analysis of picoplankton samples:

- careful sample preservation (if necessary)
- good discrimination of populations from noise
- accurate identification of populations
- careful determination of flow sample rate.

Figure 1-3: Example cytograms were obtained for samples collected at 2 different depths, 60 m (Fig. 1, 2) and 5 m (Fig. 3), in oligotrophic waters (Pacific Ocean) containing *Prochlorococcus (Proc), Synechococcus (Syn)* and picoeukaryotes (Euk). Data acquisition is triggered by red fluorescence to reduce interferences from non fluorescent particles. Subpopulations are interactively defined with gates and identified by the combination of all recorded parameters. The Synechococcus population is discriminated from other phytoplankters by its orange fluorescence (due to the presence of phycoerythrin) on the orange versus red fluorescences cytogram (Fig. 1). *Prochlorococcus* cells that are smaller and less fluorescent are distinguished from picoeukaryotes on the bivariate distribution of the SSC (a function of size) versus red fluorescence (Fig. 2). The low red chlorophyll fluorescence of *Prochlorococcus* in surface waters does not allow complete separation of them from noise (Fig. 3). Fluorescent microspheres (0.95 μm beads) are added as internal reference. All other particles are non photosynthetic detrital particles.

Materials

Equipment

- FACSort flow cytometer
- Pipetmen and tips for 1 to 1000 µl
- Waterbath
- 1.8 ml Cryovials
- Vortex mixer
- Disposable 0.2 pm-filter units and plastic syringes

Reagents

- Paraformaldehyde powder (SIGMA P-6148)
- Glutaraldehyde: 25% aqueous solution (SIGMA G-6257)
- RNase A : SIGMA (R-4875)
- RNase B: SIGMA (R-7884)
- SYBR™ Green-I: Molecular Probes (Ref S-7563)
- 0.95 pm Yellow-Green fluorescent beads: Polysciences (Ref 71825)

Solutions

Fixatives solution
- 10 ml of para-formaldehyde 10%
- 200 µl of glutaraldehyde 25%

SYBR™ Green-I
- 1% of the commercial solution in distilled water (store frozen in aliquot of 1 ml)

RNase mixture 0.1 g/l
- 50% RNase A
- 50% RNase B

0.95 µm Beads solution
- 10^6 beads per ml in 0.2 µm filtered seawater

Sheath fluid
- 0.2 µm filtered seawater

Citrate stock solution
- 1 M potassium citrate in distilled water

Preparation

paraformaldehyde (see previous section)
RNase. The mixture of RNase A and B is boiled for 10 min at 90°C to degrade any contaminating DNase

Procedure

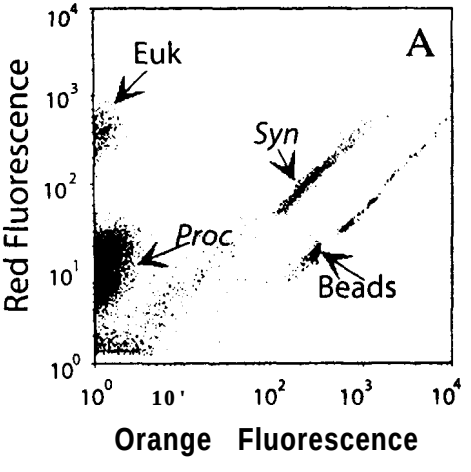
Sample collection and preservation

1. Put 1 ml of sample in a pre-labeled cryovial.
2. Add 100 µl of a mixture of paraformaldehyde 10% and glutaraldehyde 0.5%.

Note: The preservation is not necessary if samples are run immediately. The mixture of fixatives may be aliquoted and preserved at -20°C.

3. Wait for 15 min at room temperature then freeze the samples in liquid nitrogen and transfer at -80°C until further analysis.

Fig. 1



Note: The samples can be stored at -80°C for a period ranging from a few days to more than one year. They degrade very quickly if kept at -20°C.

Flow cytometric analysis

4. Preserved samples are quickly thawed in a water bath at 37°C.

Note: Dense culture samples can be diluted in 0.2 pm-filtered seawater in order to avoid coincidence on the flow cytometer.

5. Add 10 µl of the bead solution and 1 ml of the sample in a pre-labeled flow cytometric tube.

Note: The beads solution should be prepared daily. Beads are electrostatically charged and can stick to the wall of the tube. Moreover a progressive degradation of their fluorescence occurs when kept at room temperature.

Note: 0.2 urn-filtered seawater is preferred to distilled water as sheath fluid because the latter induces changes on both forward and side scatters as well as cell counts.

Instrument settings

6. Run a characteristic sample in order to adapt the configuration and the settings (see Comments).

Note: On the FACSort flow cytometer, for a natural sample collected at low depth, typical settings are FSC=E02, SSC=450, Green=650,Orange=650, Red=600. All parameters are collected *on* logarithmic scale. The discriminator is set on the red fluorescence and the threshold at 0.

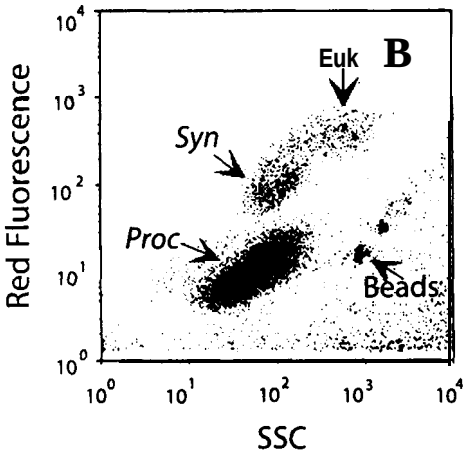


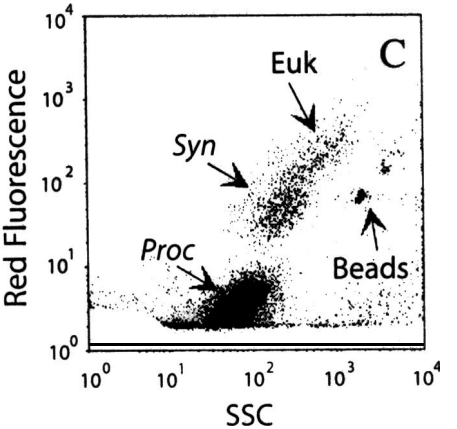
Fig. 2

Flow rate calibration

7. Weigh a tube containing a sample. Start the acquisition. Start the chronometer as the injection of the sample begins.
8. Simultaneously remove the sample tube and stop the chronometer. Weight the sampling tube.

Note: The chronometer indicates the time of injection allowing the evaluation of the flow rate. The difference between weights before and after the injection gives the delivered volume (see Comments).
A Pipetman-1000 or 100 can be used instead of a balance. (see Notes and Comments for more details)

Fig. 3



Comments

Instrument choice

Sensitivity is of critical importance when analyzing oceanic picoplankton. *Prochlorococcus* are very dim in surface oligotrophic waters and may be easily missed if the instrument sensitivity is poor or if optimal settings are not used. In our hand, the best commercially available instrument for picoplankton analysis is the FACSort from Becton Dickinson because of its excellent sensitivity and its compact design allowing easy use on board ships. Still it does not allow to completely resolve *Prochlorococcus* in most oceanic surface waters (Fig. 3). A custom modifica-

tion of the laser focalization and of the forward scatter detection has been described[16] to allow *Prochlorococcus* detection even in extremely oligotrophic waters. Another critical aspect of picoplankton analysis is the necessity to obtain absolute (and not relative) abundance of the different cell populations. Unfortunately, most available instruments are not set to deliver well-defined sample volumes. Therefore in all cases it is necessary to precisely estimate the volume of sample analyzed. On the FACSort, the most accurate method consist in determining the flow rate very precisely and then recording the time of analysis for each sample.

Preservation

Best results are obtained on fresh samples run immediately after collection. Fresh samples can be stored at 4°C for up to 12 hours with minimal effect. Fixation will always result in cell loss (about l0%), in change of scatters signals and in a sharp increase of orange fluorescence (up to two fold), The choice of the preservative may depend of the nature of the cells. Glutaraldehyde (0.1 to 1%) and paraformaldehyde (0.5 to 3%) are the most common chemicals used for preserving seawater samples. Glutaraldehyde at high concentrations (> 0.5%) generates crystallization, inducing noise that emits in the green region of the spectrum. The use of paraformaldehyde is an acceptable alternative to glutaraldehyde since it does not induce autofluorescence of the preserved cells. We found that a mixture of paraformaldehyde 1% and glutaraldehyde 0.05% (final concentrations) was an acceptable compromise.

Preparation of the fixative solution requires special care. Paraformaldehyde is a polymerized formaldehyde having poor crosslinking properties and cannot be used in that state as a fixative. By heating paraformaldehyde in water, the polymer dissociates into formaldehyde which is more water soluble and an efficient fixative. It is very difficult to completely dissolve the paraformaldehyde powder, it must be vigorously mixed in distilled water for 2 hours or more at 70°C. The solution obtained can be clarified, after cooling at room temperature, by addition of small amounts of sodium hydroxide 1N. The pH is then adjusted, but the solution must be always filtered on Whatman pa-

per filters (Grade 113) and then through 0.2 m-filter before aliquoting and storage. We did not observe any significant difference between analysis of samples fixed with paraformaldehyde freshly prepared or preserved at -20°C for up to one year. However, after thawing, paraformaldehyde aliquots must be kept at +4°C and should not be used beyond one week.

Calibration of the flow rate

Most flow cytometers do not record the volume of the sample that has been analyzed; they only record the duration of the analysis. Therefore it is necessary, in general, to determine the sample flow rate very precisely. Some instruments can be equipped with automatic sampling devices that deliver known volumes with high reproducibility. Even in this case, the nominal volume must often be calibrated and corrected for a dead volume that must be determined. To calibrate such systems, a suspension of fluorescent beads can be used. After enumeration of the beads suspension by epifluorescence microscopy, 5 to 10 replicates are analyzed by FCM under fixed delivery conditions. The actual volume (V) delivered is given by:

$$V = A/S$$

where:

A = number of beads analyzed

S = number of beads per ml determined by epifluorescence microscopy in the initial suspension.

The use of fluorescent microspheres as internal standards at known concentration has sometimes been reported to calculate the analyzed volume. We do not recommend this method, however, because the electrostatic properties of beads are generally modified by seawater and they tend to stick to the plastic tubes or into the sample line, modifying their initial concentration.

A precise method for the calibration of the flow rate is described hereafter for a FACSort flow cytometer. After setting the rate (HI, MED, LO) to be calibrated, the outer sleeve of the injection needle is removed to inactivate the vacuum and to avoid aspiration of liquid. A tube containing two ml of 0.2 urn-filtered seawater is set and the lower arm is immediately moved to the central position. Simultaneously a chronometer is started. After 10 min or more, the injection of the sample is stopped by moving the arm left or right, the sample tube is quickly removed and the chronometer stopped. The remaining volume is measured and the rate is deduced as follows:

$R = (Vi - Vf) / T$
where:
R = Rate µl. min-1)
Vi = Initial volume (µl)
Vf = Final volume (µ1)
T = Time (min)

Instead of using volume measurements, a balance can be used for weighing the tube containing filtered seawater before and after running and the following formula can be used:

$R = (Wi - Wf) / (T * d)$
where:
R = rate (µ1. min-1)
Wi = initial weight (mg)
Wf = final weight (mg)
T = Time (min)
d = density of the solution (seawater; typically 1.036).

The method described here is designed for a FACSort flow cytometer but can be adapted to a majority of instruments. On the FACSort, the flow rate remains relatively constant over a large period. Nevertheless, it can be affected by environmental parameters such as room temperature and must be calculated daily at the beginning and at the end of the enumeration experiments. If it is suspected that the rate varies or drifts, it must be determined every 5 or 10 samples, since its determination is critical for abundance estimates. Instability may occur in the flow rate when aggregates or big cells are present in the sample

which may clog the flow cell. A pre-filtration through a 10 μm nylon mesh is necessary in such cases.

Detection of phytoplanktonic cells

The detection and identification of phytoplanktonic groups is the main difficulty encountered by the operator. The intensity of the cellular parameters varies throughout the whole water column and the PMT voltages have to be adjusted frequently, depending on the size of organisms of interest, and for a given organism or community (e.g. picoplankton) depending on the depth sampled. The forward and the side scatters as well as the intensity of fluorescence from natural pigments usually increase with depth, due to photoacclimatation processes.[18]

The analysis of natural samples presents difficulties even for an experienced operator and one should be careful to collect all events of interest. The acquisition of small photosynthetic cells such as *Prochlorococcus* may be difficult particularly in surface samples of oligotrophic ocean waters (Fig. 3). The chlorophyll content is too low to be detected by the majority of the existing instruments.

If different depths have to be analyzed at a given site, it will be better to start with the deeper sample since the chlorophyll content per cell is then maximum. The threshold is set on the red detector and the red PMT voltage is increased until obtaining a clear separation between noise and the lowest population.

If only surface samples are available, the best approach is to start with a flow cytometric tube containing 0.2 pm-filtered seawater. The acquisition is started, the discriminator set on the red fluorescence (chlorophyll) and the threshold set at the minimum value, The red PMT voltage is increased until noise can be detected. The total number of events per second must be maintained below 100. Then a surface sample is run and the red PMT value is decreased if necessary in order to obtain a total number of events below 1000 per second.

In both cases, it is critical to adjust the PMT values in order to use all the dynamic range of the logarithmic scales (see Fig. 2).

Sheath fluid

Filtered seawater is preferred to distilled water, because this latter can induce modifications of the refractive indexes of the cells resulting in changes of the measured forward (FSC) and side (SSC) scatters.

Data acquisition

Samples are collected as listmode files and routinely 20,000 to 40,000 events are recorded typically during 1 to 4 min on a FACSort flow cytometer using the high (HI) sample flow rate. List mode storage gives more flexibility in data analysis, while requiring large storage space (typically a cruise would require 1 Go of disk space). We usually transfer all data to another computer to keep the instrument available for more analyses. In marine samples, rare cells (e.g. nano- or microplankton) are difficult to study and require the analysis of large sample volumes.

Data processing

Absolute cell concentrations for each population in a given sample are computed as follows:

$$C_{pop} = N_{pop} / (R*T) *(Vtotal / Vsample)$$

where:

C_{pop} = Concentration of population in cell µl-1

N_{pop} = Number of cells acquired

T = Acquisition time (min)

R = Sample flow rate (µ1. min-1) as determined for the sample series.

V_{total} = Volume of sample plus additions (fixatives, beads, etc...) (µl) V_{sample} = Volume of sample (µ1)

Data were processed by the custom-designed software CYTOWIN (Vaulot unpublished) running under Windows, available freely at http://www.sb-roscoff/Phyto/cyto.html. All parameters are reported relative to the beads added to the samples:

$$X_{rel} = X_{pop} / X_{beads}$$

where: X_{pop} is the average value of a cell parameter (scatter or fluorescence) for given population and X_{beads} the same parameter for the beads. Before ratioing, both X_{pop} and X_{beads} must be expressed as linear values (not channels) after conversion from the logarithmic recording scale. Since beads and cells have very different refractive index, such ratioing does not constitute a size characterization. For example in Fig. 2, although *Synechococcus* and beads have similar size (about 1 pm), the latter have a 10 times larger side scatter.

Su bprotocol 2
Abundance of heterotrophic bacteria. Cell cycle analysis of photosynthetic prokaryotes

Materials

The following methods are used both for the enumeration of prokaryotes in marine assemblages and for the cell cycle analysis of photosynthetic prokaryotes. Flow cytometric analysis of bacteria, that have generally a very low DNA content, requires the combination of highly fluorescent stains and sensitive instruments. Nucleic acid stains are used for this purpose. However since they stain both DNA and RNA and since bacteria may have a relatively high RNA content when grown under optimal conditions, RNA must be removed enzymatically. For years, only the UV-excited dyes Hoechst 33258 (H0258), Hoechst 33342 (H0342) and DAPI could be used for analysis of phytoplanktonic cells. Recently, new dyes from Molecular Probes Inc, (Eugene, Oreg.) TOTO-1, TO-PRO-l, YOYO-1, YO-PRO-1 and PicoGreen have been introduced for the detection of small amounts of nucleic acids on electrophoretic gels. TOTO- and YOYO-1 are cyanine dyes that are chemically different but possess similar optical properties. They can be excited by blue light, emit in the green region of the spectra, are cell impermeant and can be used on fixed cells. TOTO-1 seems to exhibit strong affinity for CTAG sequences, while YOYO- 1 was found to have two bind-

ing modes: at low concentrations it appears to be intercalating and at high concentrations, external binding occurs. PicoGreen has a strong affinity for double-stranded DNA and was designed to quantify this molecule in solution. The fluorescence of these dyes is proportional to DNA concentration and does not depend on the G-C content. They are, however, very sensitive to the ionic strength of seawater and cannot be used directly on natural samples. Nevertheless the quality of DNA distributions obtained with YOYO-1 or PicoGreen on cultured samples after dilution in a low hypotonic buffer such as Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.2), make them useful for culture studies.' Even more recently, Molecular Probes has released a new family of nucleic acid dyes (SYBR family) for gel staining purposes. SYBR™ Green-I (SYBR-I) has a strong affinity for double-stranded DNA, but also binds with single-stranded nucleic acids with lower affinity. It can be excited by UV-light but is optimally excited at 495 nm. It has the advantage of being less mutagenic than ethidium bromide or propidium iodide. Since it has a very high fluorescence yield and is not sensitive to ionic strength, it appears ideally suited for analysis of marine samples.' The method involves initial fixation by aldehyde fixatives (see above), and samples can be either analyzed immediately or after preservation in liquid nitrogen for delayed analysis.

We only present here the method with the SYBR-I stain. However, for cultures, SYBR-I can be replaced either by PicoGreen or YOYO-1.[7]

Fig. 4,5: Flow cytometric analysis of a natural seawater sample collected in the Pacific Ocean after staining with SYBR-I. Fig. 4 represents the DNA fluorescence versus chlorophyll content and shows the distribution of the cell cycle of *Prochlorococcus* and of the bacteria. Fig. 5 represents the distribution of the SSC (as a function of the size) versus DNA-fluorescence of the prokaryotic fraction. Two bacterial populations referred as BI-like and B-II-like groups can be discriminated. *Synechococcus* and picoeukaryotes were present at low concentrations but are not visible on these graphs due to the level selected and because *Prochlorococcus* and heterotrophic bacteria are largely outnumbering them.

Equipment

- FACSort flow cytometer
- Pipetmen and tips for 1 to 1000 µl
- Waterbath
- 1.8 ml Cryovials
- Vortex mixer
- Disposable 0.2 pm-filter units and plastic syringes

Reagents

- Paraformaldehyde powder (SIGMA P-6148)
- Glutaraldehyde: 25% aqueous solution (SIGMA G-6257)
- RNase A : SIGMA (R-4875)
- RNase B: SIGMA (R-7884)
- SYBR™ Green-I: Molecular Probes (Ref S-7563)
- 0.95 µm Yellow-Green fluorescent beads: Polysciences (Ref 71825)

Solutions

Fixatives solution
- 10 ml of para-formaldehyde 10%
- 200 µl of glutaraldehyde 25%

SYBR™ Green-I
- 1% of the commercial solution in distilled water (store frozen in aliquot of 1 ml)

RNase mixture 0.1 g/l
- 50% RNase A
- 50% RNase B

0.95 µm Beads solution
- 10^6 beads per ml in 0.2 µm filtered seawater

Sheath fluid
- 0.2 µm filtered seawater

Citrate stock solution
- 1 M potassium citrate in distilled water

Preparation

- paraformaldehyde (see previous section)
- RNase. The mixture of RNase A and B is boiled for 10 min at 90°C to degrade any contaminating DNase

Procedure

Sample collection and preservation

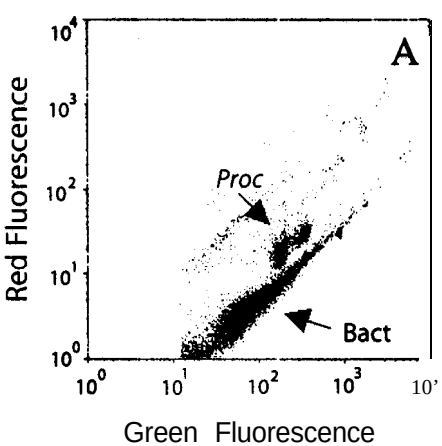
1. Put 1 ml of sample in a pre-labeled cryovial.
2. Add 100 µl of a mixture of paraformaldehyde 10% and glutaraldehyde 0.5%.

Note: The mixture of fixatives may be aliquoted and preserved at -20°C.

3. Samples are fixed for 15 min at room temperature. If samples cannot be analyzed immediately, after fixation, freeze them in liquid nitrogen and transfer them at -80°C until further analysis.

Note: The samples can be stored at -80°C for a period ranging from a few days to more than one year. Otherwise they may be preserved at -20°C for a few weeks.

Fig. 4



Sample preparation

4. If samples have been preserved, they are thawed for 5 min at 37°C.

Note: In order to avoid coincidence on the flow cytometer, culture samples can be diluted in 0.2 pm-filtered seawater or in a less hypotonic buffer for the cell cycle analysis.

5. In a pre-labeled FACSort tube, mix:
   - 250 µl of sample
   - 2.5 µl of RNase mixture
   - 7.5 µl of citrate
   - 5 µl of beads solution

Note: Incubation must be performed in the dark and analysis in a darkened room because most of the fluorescent dyes are light-sensitive

6. Incubate for 30 min at 37°C.

Staining

7. Add 2.5 µl of SYBR-I solution.

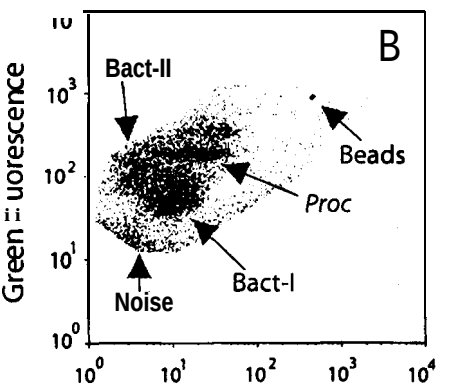
8. Incubate l5min at room temperature in the dark.

Flow Cytometric analysis

9. Analyze the samples on the flow cytometer.

Note: Sample rate is set to medium speed (MED) in order to avoid coincidence. Typical settings are FSC=E0l, SSC=450, Green=650,Orange=700, Red=630. All parameters are collected on logarithmic scale. The discriminator is set on the green fluorescence and the threshold at 200.

Fig. 5



Comments

(see also Comments of the previous section)

Staining

Common problems are excessive noise and broad Gl-like peaks. Noise can result from inadequate fixative solutions (see previous section). Make sure that all your solutions, including buffers, dyes or detergents are free of contaminating microorganisms. However, dye stock solutions must not be sterilized by filtration through 0.2 urn because some types of membranes can adsorb dyes. Broad Gl-like peaks generally result from too high dye concentration, inducing nonspecific binding. Signal compensation may be necessary to better discriminate photosynthetic cells from heterotrophic bacteria.

In the first seconds of analysis, instability may occur or acquisition may look noisy (high numbers of events per second). A sample of stained sheath fluid can be used to initially equilibrate or to clean the sample line between two samples.

Data acquisition

Logarithmic amplification gives the multidecade dynamic range necessary for the analysis of natural populations of planktonic cells, that present a wide and complex distribution. Nevertheless for cell cycle analysis, acquisition needs to be performed both on logarithmic and linear scales. For the cell cycle analysis of photosynthetic cells, a T-connector is set on the output of the green photomultiplier to record simultaneously both signals for the green fluorescence of the SYBR-I. Then we disconnect the output from the orange photomultiplier and use the corresponding analog-to-digital converter (used previously for the orange fluorescence) to collect the linear signal for the green fluorescence.

Data processing

Data analysis is performed with cytowin as described in previous protocol. Care must be taken to include correction factors to account for sample dilution with the different solutions added.

Su bprotocol 3
Taxonomy of eukaryotic picoplankton (In situ hybridization)

From knowledge of the ribosomal DNA sequences of microorganisms, nucleic acid probes specific for taxa can be designed.[19] They are complementary to a region of the ribosomal RNA molecule which is unique to the target group and can be used as "phylogenetic stains" once they have been labeled with a fluorochrome.[20] Oligonucleotide probes will hybridize to their homologous strand on the rRNA molecule within preserved cells. Labeled cells are detected by the probe-conferred fluorescence. This method is now commonly used for the identification of bacteria (see review from ref.[19]), but can also be used for the detection and identification of phytoplankton. Such probes are especially useful for the study of the smallest algae (picoeukaryotes) for which identification requires much time and expertise using traditional techniques because morphological characters are not readily available. Routinely used on cultured species,[11,12] whole-cell hybridization has yet to be applied to natural samples where the cell population of interest (a given species or genus for example) is part of a complex community. The latter application will require improvement in the sample preparation protocol to avoid cell loss and in the fluorescent reporters used to increase positive to negative signal ratios.

Cells are discriminated on SSC versus green fluorescence (FITC) cytograms. *Emiliania huxleyi* (Prymnesiophyceae) has been chosen as an example for in *situ* hybridization with a probe specific for the Prymnesiophyceae (FITC-PRYMN0l, Fig. 6) and as a control, with a probe specific for the division Chlorophyta (FITC-CHLO0l, Fig. 7) that should not label these cells. Fluorescent microspheres (0.95 μm beads) were added as internal reference.

Monoparametric distributions of the green fluorescence relative to FITC, are superimposed on Fig. 8. Data were collected independently for the autofluorescence, the negative (FITC-CHLO0l), the positive (FITC-PRYMN0l) and specificity (FITC-PRYMN0l / PRYMN0l) controls.

Materials

Equipment

- FACSort flow cytometer
- Hybridization oven
- Centrifuge for 15 ml tubes
- Microcentrifuge
- Pipetmen and tips for 1 to 1000 µl
- 1,5 ml eppendorf tubes
- I5 ml tubes

Solutions

PBS
- Phosphate Buffered Saline (Sigma P 3688) pH 7.4

Permeabilising agents
- Paraformaldehyde 10% pH 7.2 (see above for preparation)
- 70% Ethanol in PBS, filtered through 0.2 pm

Hybridization buffer
- NaC10.9 M
- Tris HCl (pH 7.8) 20 mM
- SDS 0.01 %
- Formamide X % (concentration ranging from 0 to 50% has to be determined experimentally)

Probes

- Detailed protocol for the design and labeling of taxon specific oligonucleotide probes in Amman et al.[22]
- FITC labeled probes are kept at -80°C in 50 µl aliquots in distilled water. Working stocks are 50 ng/µl.
- Labeled probe used as examples:
  - FITC-CHLO0l specific for Chlorophyte algae
  - FITC-PRYMN0l specific for Prymnesyophyceae algae
- Unlabeled probe: Unlabeled-PRYMN0l

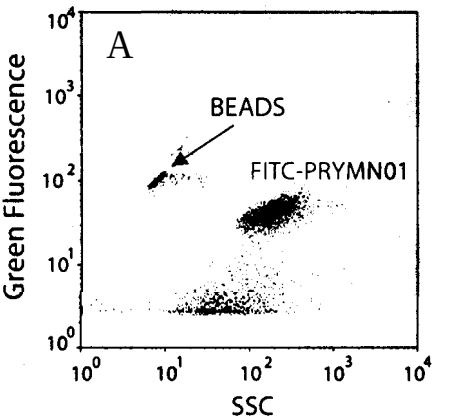
Procedure

Whole-cell hybridization

1. For each species prepare4 pre-labeled 15 ml tubes in order to measure:
   - Autofluorescence (no probe)
   - The fluorescence conferred by the specific probe (e.g. FITC-PRYMN0l)
   - The fluorescence conferred by the non-specific probe (e.g. FITC-CHLO0l)
   - The fluorescence conferred by a mixture of the specific probe labeled and unlabeled (e.g. FITC-PRYMN0l + PRYMN0l)

Note: If the concentration of the target population is low (10^3,10^4 cells/ml) in the original sample, concentration procedures (ultrafiltration or centrifugation) should be used prior to fixation.

Fig. 6



Fresh paraformaldehyde (less than one week old) has to be used.

2. In each tube add 5 ml of an exponential culture of picoeukaryotes at a concentration of about 10^5 cells/ml
3. Add 500 µl of paraformaldehyde 10% in each tube. Incubate for 1 h at 4°C.
4. Spin down the cells at 4000 xg for 3 min at 4°C
5. Remove the supernatant and immediately resuspend the cells in 500 µl of cold (-20°C) ethanol:PBS (70:30, vol:vol).

Note: This step modifies the natural fluorescence properties of photosynthetic cells. Photosynthetic pigments are damaged by alcohol treatments

6. Transfer each sample in Eppendorf tubes.
7. Spin down the cells at 4000 x g for 3 min and resuspend them in 20 µl of hybridization buffer.

Note: At that stage, samples can be stored at -80°C until needed for hybridization experiments and analyses

8. Add 1 ul of the chosen probe (as defined in step 1) to a 20 µl aliquot of the cells suspension.

Note: Alternative detergent can be tested in the hybridization buffer such as:
Triton (0.01 to 0.1%) (Sigma T 9284).

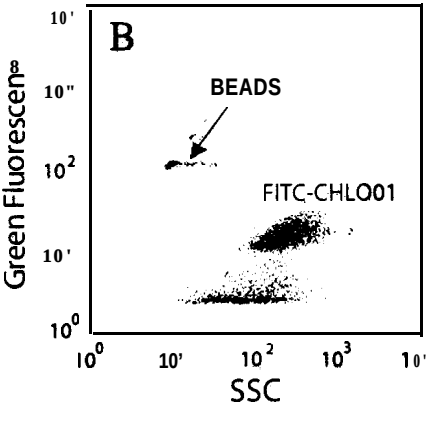


Fig. 7

Tergitol (0.05 to 0.1%) (Sigma NP-40).
CHAPS (0.1%) (Sigma C 5070).

9. Incubate 3 h in the dark at 46°C.

10. Stop the hybridization by adding 500 µl of ice cold PBS pH 9.0. Keep the samples on ice.

Note: After hybridization, a washing step might be needed to remove nonspecific staining

11. Analyze the samples within 24 h.

Analysis of samples

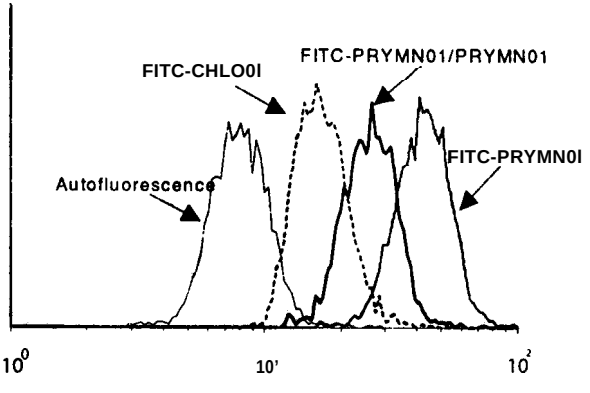
Note: After 24 h an increase of the autofluorescence of the cells is observed as well as a decrease of the intensity of the fluorescence corresponding to specific labeling.

Comments

Probe labeling

Fluorescein (FITC, Ex= 490nm, EM= 525nm) is currently used for probes labeling for in situ hybridization with algal cells. Purification of the oligonucleotides, is an essential step since remaining unlabeled oligonucleotides will compete with labeled probes and thus lower the fluorescence intensity of hybridized cells.

Fig. 8



Signal intensity

It has been reported that the intensity of the labeling depends on the physiological state of the cells, although this idea is controversial and may not be true for all groups. We observed that the intensity of the specific signal is optimal when cells are harvested in exponential growth stage. The intensity of the signal is also size dependent. For example for the chlorophytes *Chlamydomonas concordia* (8 to 12 pm) and *Micromonas pusilla* (1 to 3 pm) the ratio between specific and non-specific probe-conferred signals are 10 and 3 respectively. A range of controls are necessary to distinguish a truly specific signal from autofluorescence and non-specific signals. Cells which do not grow under optimal conditions, may have fewer ribosomes and therefore show reduced fluorescence signals.[11] Differences in the cell permeability for probes among different species could be another reason for unequal labeling of cells. We use the following positive and negative controls:

- A sample without any probe is used to record the autofluorescence and all other fluorescence values will be ratioed to autofluorescence.
- A positive control constituted by a specific probe: the PRYMN01 and CHLO01 probes are used as positive controls for Prymnesiophyceae and chlorophyte cultures respectively.
- A negative control constituted by a non-specific probe: the CHLO01 and PRYMN01 probes are used as negative controls for Prymnesiophyceae and chlorophyte cultures respectively.
- A specificity control is constituted by a mixture of FITC-labeled and unlabeled specific probe in equal or varying proportions. If the probe is really specific, then 18S rRNA target sites should be blocked by the unlabeled probe and the intensity of the green fluorescence of FITC should decrease when the concentration of unlabelled probe increases.[12]

Until now, the use of this technique both on cultured and natural seawater samples has been limited by problems linked to low signal intensity. Several solutions have been proposed such as the use of multiple probes[21] or the use of indirect labeling (e.g. biotin labeled probes in conjunction with fluorescently labeled streptavidin). In all cases, very strict controls are needed before establishing that a specific probe labels a given strain of cultured algae.

Subprotocol 4
DNA content and G-C% on isolated eukaryotic nuclei for taxonomy and ploidy studies

An other potential use of nucleic acid-specific dyes, is the determination of the DNA content and the G-C% of phytoplankton cells. This is performed on unialgal cultures. DNA quantification has to be done on isolated nuclei, without fixation, in order to obtain stochiometric binding of the dyes. Ploidy level can be analyzed either on isolated nuclei or on fixed cells, when nuclei cannot be isolated. The isolation ofnuclei, by osmotic pressure modification or by the action of detergents, presents two major advantages. First it facilitates the access of DNA to large stain molecules that normally cannot cross easily cellular membranes. Second, it removes the cytoplasm and avoids non-specific binding.

Addition of an internal reference is necessary to obtain a precise measurement of the DNA content of unknown species. Nuclei of reference and unknown cells must be mixed together prior to staining. The reference nuclei must be carefully chosen such that their DNA content are close to that of the planktonic cells. Chicken red blood cells (CRBC, 2.33 pg, 42.7% G-C) can be used for large algal cells. Algae with known DNA content and G-C% can be used when CRBC fall outside the range (e.g. *Phaeocystis* strain PCC 64 = 0.21 pg for the 1C,54% G-C[13]). The DNA-related fluorescence should be acquired on both linear and logarithmic scales. The linear scale is optimal to accurately estimate the DNA content. The logarithmic scale is useful when the unknown species and the internal reference display a large difference in terms of dye-fluorescence intensity (Fig. 9). The cytogram of log Side Scatter (SSC) versus log DNA-fluorescence, is used for the identification of isolated nuclei and to gate out the debris. The adequacy of the preparation and staining procedure is evaluated by the coefficient of variation (CV) of the Gl-like peak which must be as low as possible. Ideally, each sample should be run in duplicate, allowing for quality control of staining variability. Five to ten replicates should be performed for each species and averaged for reliable statistical results. The ratio of the peak positions of the sample to the standard is used for the calculation of the ploidy level. Routinely 5 000 to 20 000 events excluding noise are collected.

Three dyes are useful for such analysis. Propidium Iodide (PI) displays no base specificity and therefore is useful for assessing DNA content. Hoechst (or DAPI) dyes are A-T-specific while Chromomycin A3 (CA3) is G-C specific. Parallel staining of samples with these 3 dyes allows to estimate G-C% .[23] Propidium Iodide (PI, Ex=493 nm, Em=639 nm) binds to double-stranded nucleic acids by intercalation and has broad excitation bands both in UV and in blue-green regions that make it usable on the majority of the flow cytometers. PI is sensitive to the ionic strength and may be used in low hypotonic buffers. We have used it on whole cells, when analysis cannot be performed with HO342 or CA3. Staining is optimum at low concentrations ranging from 1 to 5 μg/ml.

The Hoechst bis-benzidimide dyes (HO342 and H0258) or DAPI are low cost non-intercalating dyes that have a high specificity for double-helical DNA, and bind preferentially to sequences with A-T bases. They emit blue fluorescence when excited by ultraviolet (UV) light at -350 nm. HO342 can be used with a large variety of marine species. It cannot be used to estimate absolute DNA content or to compare cells that differ in their proportion of A-T bases. Nevertheless, it can be used on entire cells for ploidy measurements, by comparing the peak positions of an internal standard and of the species of interest, or of a mixture of species. HO342 fluorescence emission can be enhanced by addition of citrate (10 to 50 mM) or sodium sulfite (1 to 5 mM). It may be used at very low concentrations, up to 1 μg/ml, on fixed phytoplanktonic cells and is preferred to its homologue HO258 or to DAPI, that generally present unspecific binding. We observed that RNase treatment improves both the intensity of the signal and the coefficient of variation of the G1-like peak of H0342-stained cells, especially for the prokaryotic fraction of picoplankton.

Chromomycin A3, like Mithramycin, is a non-intercalating fluorescent antibiotic that presents a strong affinity for the 2-amino group of guanine in DNA when complexed with magnesium. It is optimally excited at 457 nm and very poorly at 488 nm. Therefore it is not suitable for single wavelength lasers that equip most small flow cytometers. Stock solutions of CA3 containing $MgCl_2$ (100 mM) show no deterioration for months when stored at -20°C. CA3 can be used on aldehyde-fixed cells without interfering with chlorophyll emission. It also gives good results with

diatoms, after fixation by alcohols. It can be used over a wide pH range, from 6 to 8.5, with concentrations of 5 to 100 µg/ml, both in low and high hypotonic buffers, in presence of 30 mM magnesium. CA3 may be a potential health hazard and should be used carefully.

Flow cytometric analyses can be conducted on an EPICS 541 flow cytometer (Coulter, Hialeah, FL) equipped with a Biosense flow chamber and an Argon laser (Coherent). For each dye, the optical configuration of the EPICS 541 flow cytometer is illustrated in Fig. 10.

Fig. 9: *Phaeocystis* strain Rosko A was analyzed in presence of CRBC as internal standard after staining by the A-T-specific dye H0342. DNA fluorescence was collected as linear and logarithmic signals (Fig. 9). The linear fluorescence is necessary for the calculation of the ratio of modal DNA fluorescence between the species and the internal standard. The logarithmic signal allows to obtain a large dynamic range necessary to visualize a mixture of nuclei with very different DNA contents. Figure 11 shows the superimposition of histograms obtained independently for H0342, CA3 and PI. The amplification of the signal was adjusted for each dye to set the modal position of the internal reference (CRBC) to a fluorescence value of 70.

Materials

Equipment

- To quantify DNA content and GC%, analysis has to be performed with three different dyes, that have different excitation spectra. This requires the use of a tunable laser, that can be set in UV (353-357nm), violet (457nm) and blue (488 nm) lines that are available on argon laser
- Pipetmen and tips for 1 to 1000 µl
- 1.5 ml eppendorf tubes
- 10 µm nylon mesh

Reagents

- Hoechst 33342: stock solution 1 mg/ml in distilled water (Molecular Probes, H-1399)
- Chromomycin A3: stock solution 1 mg/ml in 100 mM magnesium chloride (Sigma, C 2659)
- Propidium Iodide: stock solution 1 mg/ml in distilled water (Sigma, P 4170)

Solutions

Nuclei isolation buffer
- Sorbitol 125 mM
- Potassium Citrate 20 mM
- Magnesium Chloride 30 mM
- Hepes: 55 mM
- EDTA: 5 mM
- Triton X-100: 0.1%

RNase mixture 0.1 g/l
- 50% RNase A
- 50% RNase B

Sodium bisulfite
- Stock solution 1 M in distilled water prepared daily and stored at 4°C

Preparation

RNase
- The mixture of RNase A and B is boiled for 10 min at 90°C to degrade any contaminating DNase

Alsever solution
- 72 mM NaCl
- 27 mM Sodium Citrate
- 114 mM Glucose
- 1% Triton X-100
- Adjust pH to 6.1 with Citric Acid 10%

CRBC (Chicken Red Blood Cells)
- Collect 1 ml of blood
- Add 3 ml of Alsever solution
- Mix for 1 min
- Centrifuge 1000 x g for 3 min
- Rince with 3 ml of Alsever solution
- Centrifuge 1000 x g for 3 min
- Resuspend in 3 ml of Alsever solution
- Store in small aliquots at -20°C.

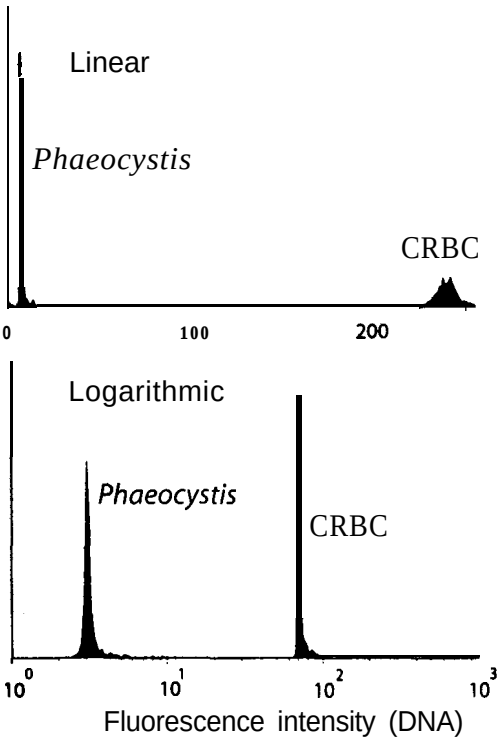
■ ❑ Procedure

Isolation of nuclei

1. Add 50 µl of sodium meta-bisulfite to 10 ml ofisolation buffer.

Note: Addition of a protectant such as sodium bisulfite or ß-mercapto-ethanol is recommended to avoid rapid degradation of

Fig. 9



isolated nuclei. Sodium bisulfite is used in place of ß-mercaptoethanol because of the high toxicity of the latter.
Solutions containing sodium bisulfite are unstable and must be prepared just before the experiment.

2. Prepare 3 separate Eppendorf tubes, one for each dye.

3. Add 2 to 100 µl of the cell suspension to the necessary volume of isolation buffer, such that the final volume is 1 ml.

Add internal reference (CRBCs)

4. Add 5 µl of the internal reference. If CRBC are used thaw an aliquot and dilute it 50-fold with Alsever solution.

Note: The internal reference and the sample maybe mixed before addition of the dye. The choice of the internal reference may depend on the nature of the sample (see Comments).

Staining

5. Add the stains, e.g. respectively 5, 40 and 30 µl/ml of stock solutions of Hoechst 33342, Chromomycin A3 or Propidium Iodide.

Note: RNase can be used with intercalary stains such as Propidium Iodide. Thus add 5 µl/ml of the RNase stock solution.

6. Keep the sample on ice and wait for 5 min for Hoechst, 15 min for Chromomycin or Propidium Iodide.

7. Filter through 10 pm nylon.

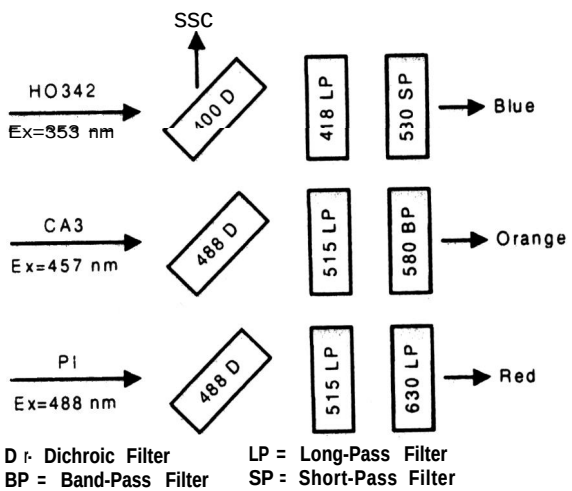


Fig. 10

8. Analyze on the flow cytometer at room temperature.

Note: Distilled water is used as sheath fluid.

Flow Cytometric analysis

Comments

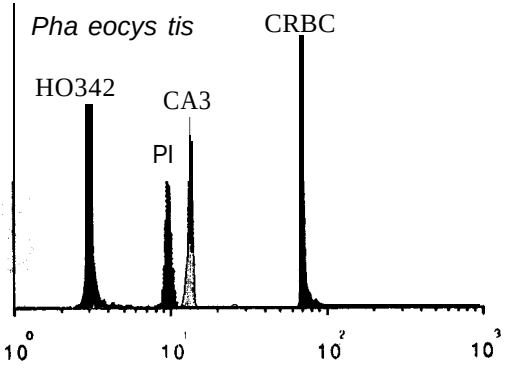
Internal standard

The absence of convenient biological standard for the estimation of the DNA content of marine species constitute a major problem. The main difficulty of the procedure is therefore to choose an internal standard. CRBC standards generally give coefficients of variation below 5%. Nevertheless, in some cases, we observed a rapid degradation of the internal standard, due to the high hypotonicity of the isolation buffer or to some chemical compounds contained in the initial sample

DNA quantification

In the example given in Fig. 9, the position of the 1C peak of *Phaeocystis* relative to CRBC was 0.043 with HO, 0.19 with CA3 and 0.133 with PI. These ratios differ significantly, indicating that the G-C% of *Phaeocystis* is quite different from that of CRBC. As the intercalating dye PI binds proportionally to the base-pairs content, the ratio between the peak position of the sample to the standard is used to estimate the genome size. HO342 and CA3 bind to sequences of respectively 5 consecutive

Fig. 11



A-T bases and 3 consecutive G-C bases.[23] described a non-linear relationship between changes in fluorescence intensity and base composition. As A-T% (or G-C%) increases, the HO342 (or CA3) signal increases in a non-linear manner. Godelle developed a curvilinear model that allowed the determination of the G-C%. Two simplified estimates of the base-composition of a unknown species can be obtained from a known reference as follows:

$$\text{A-T\%}_{\text{species}} = \text{A-T\%}_{\text{reference}} * (R_{HO342} / R_{PI})1/5$$

$$\text{G-C\%}_{\text{species}} = \text{G-C\%}_{\text{reference}} * (R_{CA3} / R_{PI})1/3$$

where:
R_{PI} = Intensity species / intensity reference obtained with PI
R_{HO342} = Intensity species / intensity reference obtained with HO342
R_{CA3} = Intensity species / intensity reference obtained with CA3

Isolation of nuclei

With phytoplanktonic cells, it is often difficult to isolate nuclei without damaging them (e.g. for diatoms). The method described above has been developed in order to obtain optimal results on a wide range of photosynthetic cells. However, slight modifications in the composition of the isolation buffer may be required for some species. For example higher detergent concentrations may help for some difficult samples. Observations with epifluorescence microscopy will help the operator to optimize of the protocol. With HO or DAPI, variations in light emission can be observed when dye concentration is too high (nuclei appear white), when nuclei are partially degraded (variable color) or when the pH is too acid (yellow fluorescence). Aberrant morphology of nuclei or entire cells are usually an indicator of inadequate buffer composition.

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