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DNA/RNA Analysis of Phytoplankton by Flow Cytometry

In the last two decades, flow cytometry has been widely used in oceanography to estimate the abundance of the different populations composing the picophytoplankton and to obtain general information based on their cell size and pigment contents. Nucleic acid stains are used by aquatic biologists to enumerate heterotrophic bacteria, and more recently, viruses, by flow cytometry. For this type of application, the stoichiometry of staining is not critical since the dye is used as a simple coloring tool. The following basic protocols describe methods that allow a more precise characterization of the whole genome of very small organisms. These methods have several major applications, including the precise determination of the genome size and ploidy level (see Basic Protocol 1) and cell cycle analysis (see Basic Protocol 2) of phytoplanktonic cells.

One further step in the characterization of phytoplanktonic populations is the use of fluorescent oligonucleotide probes targeted to 18S rRNA that permits discrimination of specific taxa in the heterogeneous natural communities of picophytoplankton (Basic Protocol 3).

DETERMINING GENOME SIZE AND PLOIDY LEVEL OF PHYTOPLANKTON

BASIC PROTOCOL I

During the last two decades, numerous studies have focused on the measurement of genome size and ploidy level of land plants by flow cytometry (Galbraith et al., 1983; Brown et al., 1991). These analyses, which were performed directly on nuclei isolated from fresh plant tissues, have been adapted for marine purposes. In general, estimation of genome size is best performed on isolated nuclei. Nevertheless: when a complex membrane composition prevents the release of nuclei from algae or when isolated nuclei are not stable over time, these analyses can be performed on fixed cells.

Little information is available on the genome size and the base composition of marine algae (Veldhuis et al., 1997). In addition to being an important item of information by

Table 11.12.1 Dyes Used for Determination of Genome Size and Cell Cycle Analysis of Planktonic Cells

Dye	MW	Absorption maximum (λ_A; nm)	Fluorescence maximum (λ_F; nm)	Working conc.	Final conc.
Ethidium bromide	394	518	605	0.1 mg/ml	5 µg/ml
Propidium iodide	668	535	617	0.1 mg/ml	5 µg/ml
Mithramycin	1085	433	570	1 mg/ml	30 µg/ml
Chromomycin A	1 183	420	560	I mg/ml	30 µg/ml
DAPI	350	357	451	0.1 mg/ml	1 µg/ml
Hoechst 33342	652	365	502	0.1 mg/ml	1 µg/ml
TOTO- 1	1303	514	533	1 µM	30 nM
YOYO-1	1271	491/450[a]	509/550a[a]	1 µM	30 nM
TO-PRO- 1	645	515	531	1µM	30 nM
YO-PRO- 1	629	491/450[a]	509/550[a]	1 µM	30 nM
PicoGreen	NA[b]	480	520	l/10	l/1000
SYBR Green I	NA[b]	494	521	l/100	1/10,000

[a]Number before slash is according to manufacturer; number after slash is according to Hirons et al. (1994).
[b]NA, molecular weight not available from manufacturer.

Contributed by Dominique Marie, Nathalie Simon, Laure Guillou, Frédéric Partensky, and Daniel Vaulot

Current protocols in Cyfomerfy (2000) 11.12.1-11.12.14

itself, genome size can be used to discriminate species that have similar morphological features, a common occurrence in both nano- and picoplankton (Partensky and Vaulot, 1989; Boucher et al., 1991; Simon et al., 1994; Vaulot et al., 1994; Veldhuis et al., 1997). Determining ploidy level in cultured populations is also critical for assessing the sexual cycle of eukaryotic microalgae (Vaulot et al., 1986; Le Gall et al., 1993).

Nucleic acid stains are useful for assessing the genome size of algal cells, but no universal staining procedure exists because of the large number of nucleic acid dyes and the wide taxonomic diversity of algae. The intercalary dyes, such as propidium iodide or ethidium bromide, bind with both RNA and DNA and allow precise measurement of nucleic acid content. However, they interfere with the emission spectrum of chlorophyll, and thus cannot be used directly on whole cells. The G-C-specific dyes mithramycin and chromomycin A are excited by violet wavelengths (453 to 457 nm) and are often helpful for marine species. The W-excited dyes, such as DAPI or Hoechst 33342, are currently used for the study of A-T-rich species. In addition to genome size they can be used along with the G-C-specific stains for the determination of the percent G-C (Le Gall et al., 1993; Simon et al., 1994).

A new generation of dyes made available by Molecular Probes bind both DNA and RNA and present the advantages of high quantum yield and of excitation at 488 nm, a wavelength available on small flow cytometers equipped with an air-cooled laser. Some of them, such as TOTO- 1, YOYO- 1, TO-PRO- 1, YO-PRO- 1, or PicoGreen, are sensitive to the ionic strength and composition of seawater. To deal with this problem, samples can be diluted in low hypotonic buffer in order to minimize the interaction of culture medium or seawater (Marie et al., 1996). If samples cannot be-diluted, the most sensitive dye usable for the cell cycle analysis of photosynthetic microorganisms is SYBR Green I, which has a much stronger affinity for double-stranded DNA than for RNA (Haugland, 1996; Marie et al., 1997), and is not sensitive to ionic strength.

Materials

Cell suspension (l-5 x 10^5 cells/ml)
Nucleus isolation buffer (NIB; see recipe; add 5 µl of 1 M sodium bisulfite per ml buffer immediately before use and filter through 0.2-µm pore-size filter)
Internal reference (i.e., suspension of nuclei or cells from species for which genome size is known, at -1 x 10^5 cells/ml)
1% (w/v) RNase A (type IA, Sigma) in distilled water (heat 10 min at 90°C to degrade contaminating DNase)
Nucleic acid-specific stain (see Table 11.12.1 for concentrations)

0.2-µm pore-size filters for plastic syringes
10 µm nylon mesh
Flow cytometer with laser emitting UV, violet, and blue lines (also see Critical Parameters)

1. Add 10 to 50 µ1 cell suspension to 1 ml nucleus isolation buffer (NIB).

The nuclei will be released by hypotonic shock andwill remain stable in NIB.

If isolated nuclei cannot be obtained, fix the culture with 1% paraformaldehyde.

2. Add 10 µ1 internal reference and mix the sample by vortexing.

The measurement of DNA content for an uncharacterized marine species is obtained by comparing the mean fluorescence of this species with that of a speciesfor which the genome size is known (Fig. 11.12. **1).** This reference must *be added before staining.*

If one wants to compare two strains to determine whether they have the same genome size, it is critical to work with a mixture of the strains as well as with each strain separately.

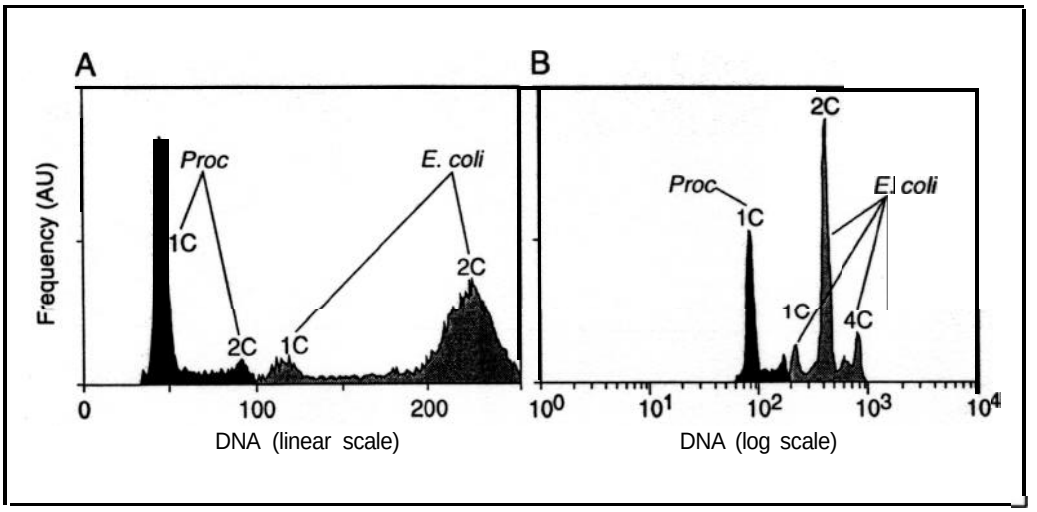

Figure 11.12.1 Estimation of the genome size of the photosynthetic prokaryote *Prochlorococcus (Proc),* using a culture of *E. coli* pretreated with rifampicin as internal reference. Data are presented on both linear (A) and logarithmic (B) scales.

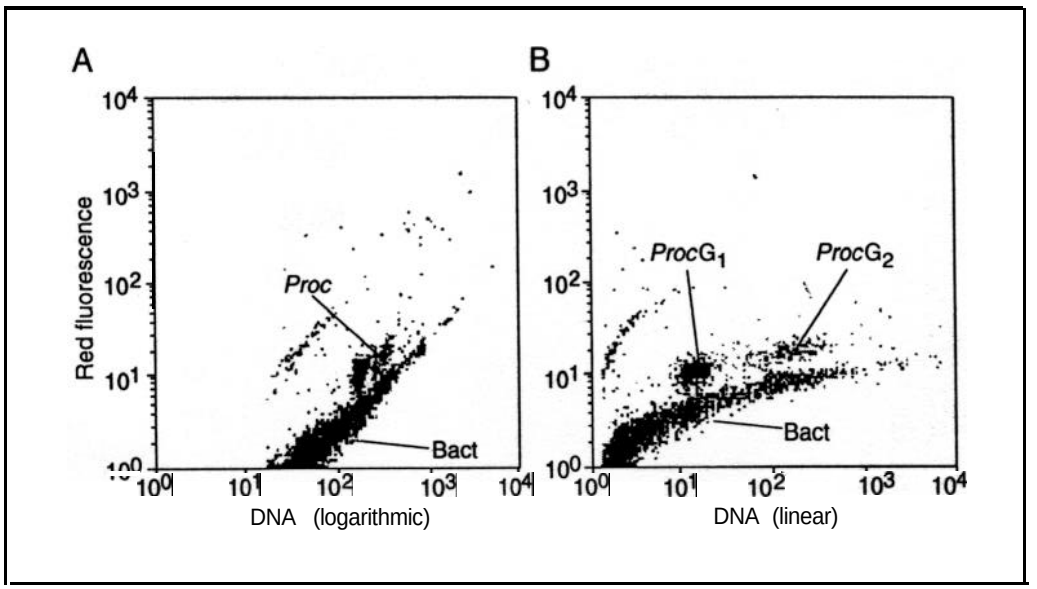

Figure 11.12.2 Green versus red fluorescence obtained for a sample collected at 65 m in the Pacific Ocean (5°S by 15O°W) during the OLIPAC cruise, and stained with SYBR Green I. The green fluorescence signal was recorded on both logarithmic (A) and linear (B) scales.

3. If fixed samples are used, pretreat samples with RNase A: Add 10 µl of 1% RNase A for every 1 ml of sample and incubate for 30 min at 37°C.
4. Filter through 10 µm nylon mesh to eliminate cellular debris or cell clumping.
5. Add the nucleic acid-specific stain (Table 11.2.1). Incubate 15 to 30 min on ice, in the dark.
6. Set up the flow cytometer using distilled water as the sheath fluid. Set the discriminator for the fluorescence corresponding to the dye used.

Measurement of DNA content is performed with both linear and logarithmic amplification (Fig. 11.X2.2).

7. Run the sample. Collect both linear and log fluorescence.

BASIC PROTOCOL 2

CELL CYCLE ANALYSIS OF PHYTOPLANKTON

Flow cytometry has been used extensively in the past to determine the cell cycle of different phytoplanktonic species in culture, and more recently in the field. In addition to providing a basic understanding of the relationships between cell cycling and environmental factors such as nutrient levels or light (Vaulot et al., 1994), the determination of the percentage of cells within the different phases of the cell cycle also provides a very elegant way to assess the division rate of phytoplankton in oceanic waters (Carpenter and Chang, 1988; Vaulot et al., 1995). Cell cycle analysis of marine species is performed on fixed cells.

Materials

0.05% (w/v) glutaraldehyde/ l% (w/v) paraformaldehyde
Cell suspension (1-5 x 10^5 cells/ml)
1% (w/v) RNase A (type IA, Sigma) in distilled water (heat 10 min at 90°C to degrade contaminating DNase)
1 M potassium citrate
SYBR Green I working solution (dilute commercial stock solution from Molecular Probes 1: 100 in distilled water)
lx 10^5 beads/ml suspension of 0.95-µm fluorescent microspheres (Polysciences)
Flow cytometer equipped with a laser emitting at 488 nm (also see Critical Parameters)

1. Fix the sample by adding 100 µl of 0.05% glutaraldehyde/ l% paraformaldehyde to 900 µl of cell suspension. Incubate for up to 15 min.

   If samples cannot be run immediately after fixation. they must be deep frozen in liquid nitrogen and stored at -80°C for delayed analysis. Frozen samples should be thawed at 37°C.

2. Add 10 µl of 1% RNase A for every 1 ml of sample. Incubate 30 min at 37°C.

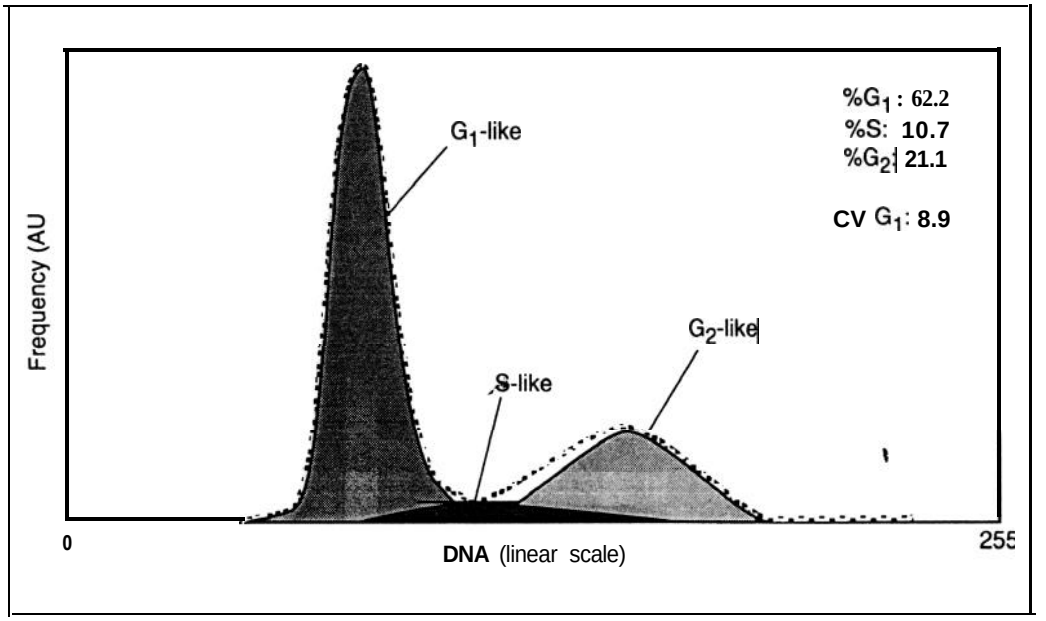

Figure 11.12.3 Cell cycle analysis using MultiCycle, on a natural sample of *Prochlorococcus* collected in the Pacific Ocean (Cast 94, 65 m). The coefficient of variation of the G_1-like peak is an indicator of the quality of the DNA staining.

3. Add 10 µl SYBR Green I working solution (final concentration corresponds to a 10,000-fold dilution of the commercial solution) and 30 µl 1 M potassium citrate. Incubate 15 min at room temperature in the dark.
4. Add 10 µl of a 1 x 10^5 beads/ml suspension of 0.95-µm fluorescent microspheres per 1 ml of sample.
5. Set up the flow cytometer using either distilled water or 0.2-µm-filtered sea water as sheath fluid.
6. Connect a T-connector to the output of the PMT collecting green fluorescence. Reconnect the analog-to-digital converters (ADCs) for green and orange to the connector.

   Cell cycle analysis of phytoplankton must be performed with both logarithmic and linear amplifications (Fig. 11.12.2). The logarithmic scale is used to discriminate the populations ***of interest. The linear scale is required to perform cell cycle analysis (Fig. 11.12.3). On flow cytometers such as the FACS series, it is not possible to record both signals. To overcome this dificulty, a T-connector is set on the output of the green (FLI) photomultiplier (PMT); then the output of the orange PMT is disconnected. The analog-to-digital converters (ADC) of the green and orange are then reconnected to the T-connector The green ADC is used to collect the logarithmic signal, and the orange ADC to collect the linear signal of*** the ***fluorescence of the DNA-SYBR Green I complex.***

7. Put the discriminator on the red fluorescence.

   Typical settings on the authors FACSort flow cytometer are FSC = EO1, SSC = 450, FL1 = 660, FL2 (linear amplification X10), FL3 = 650for Procholorococcus cell cycle analysis.

8. Run the sample for 4 min at low rate (<50 µl/min).

IDENTIFYING PHYTOPLANKTON USING WHOLE-CELL HYBRIDIZATION WITH TAXON-SPECIFIC FLUORESCENT OLIGONUCLEOTIDE PROBES

BASIC PROTOCOL 3

The vast amount of sequence data obtained during the last 10 years for the small subunit of ribosomal RNA has led to the development of signature sequences as a phylogenetic determinative tool in microbial ecology. With this method, target species are detected and their phylogenetic position is determined by the binding of a fluorescently labeled oligonucleotide probe to a homologous RNA sequence in the cells. The use of flow cytometry to determine the hybridization signal allows both quantification of the intensity of the hybridization signal and rapid screening of many isolates.

Materials

Phytoplanktonic cells of interest
Culture medium for marine phytoplankton (sea water with appropriate nutrients; see ***http://ccmp.bigelow.org/CI/CI_OI .html)***
10% (w/v) paraformaldehyde stock (store at -20°C)
70:30 (v/v) ethanol/PBS, -20°C (see **APPENDIX 2A** for PBS)
Hybridization buffer (see recipe) of optimal formamide concentration
50 ng/µl fluorescein isothiocyanate (FITC)-labeled probe to be tested (see Critical Parameters and UNIT ***8.3)***
Washing buffer (see recipe) with the same stringency as the hybridization buffer
Phosphate-buffered saline (PBS; ***APPENDIX 2A)***pH 9.0
1 x 10^5 beads/ml suspension of 0.95-µm fluorescent microspheres (Polysciences)
Hybridization oven, or any oven or incubator whose temperature can be precisely tuned
Flow cytometer equipped with 488-nm argon laser (also see Critical Parameters)

Fix and permeabilize cells

1. Centrifuge the phytoplanktonic cells, selecting appropriate time and g-force for the species used. Resuspend the cell pellet in a small volume (1 ml) of fresh culture medium for marine phytoplankton.

   Final concentration should be at least 10^7 cells/ml. Centrifugation may lead to cell loss; therefore appropriate centrtfugation conditions have to be tested initially for each species.

2. Add 10% paraformaldehyde stock (fresh or freshly thawed) to a final concentration of 1% and incubate 1 hr on ice.

3. Vortex cells and centrifuge at 4°C using the appropriate conditions for the cells of interest.

4. Resuspend the cell pellet in cold (-20°C) ethanol:PBS.

   Care should be taken from this stage on when centrifuging and resuspending cells. To prevent cell loss, pellets should not be disturbed.

5. Vortex vigorously and centrifuge at 4°C.

   Vortexing is important in order to prevent cell aggregation.

Hybridize and wash cell

6. Resuspend the cells in hybridization buffer preheated at 46°C (or other optimal temperature; see Critical Parameters). Vortex vigorously and split the cell suspension into 20-µl aliquots.

   See ***Critical Parameters for discussion of how to optimize the stringency (formamide concentration) of the hybridization buffer:***

7. For each aliquot, add 1 µ1 of the labeled probe to be t&ted (working stock at 50 ng/µl).

   For each experiment, two controls should be prepared. One (autofluorescence) consists of a cell suspension with no probe. The other (negative control) consists of cells hybridized with a non-target probe (non-specific staining). For example, Chlamydomonas concordia hybridized with the probe CHLO02 is 4 times more fluorescent than with the PRYM02 probe. Conversely, the intensity of greenfluorescence of Prymnesium patelliferum is 4 times higher when hybridized with the PRYM02 probe than with the CHLO02 probe (Fig. I I. 12.4).

8. Incubate 3 hr at 46°C (or optimal temperature).

9. Resuspend cells in 50 µl preheated (46°C or optimal temperature) washing buffer.

10. Incubate 15 min at 46°C (or optima1 temperature).

11. Stop hybridization by adding 500 µl ice-cold PBS, pH 9.0. Keep samples on ice in the dark until analysis.

Analyze cells by flow cytometry

12. Add 10 µl of a 1 $\times 10^5$ beads/ml suspension of 0.95-µm fluorescent microspheres per 1 ml of sample.

13. Set up flow cytometer. Use forward light scatter and side scatter to look at size and refractive index distribution of the cells as well as red fluorescence to look at the chlorophyll fluorescence intensity after cell treatment. Collect all parameters, including green fluorescence, using logarithmic amplification.

    Discrimination of the cells from particles will be ob tained by setting the discriminator on the red fluorescence photomultiplier: After treatment chlorophyll-containing cells will retain some redfluorescence, even though chlorophyll is degraded.

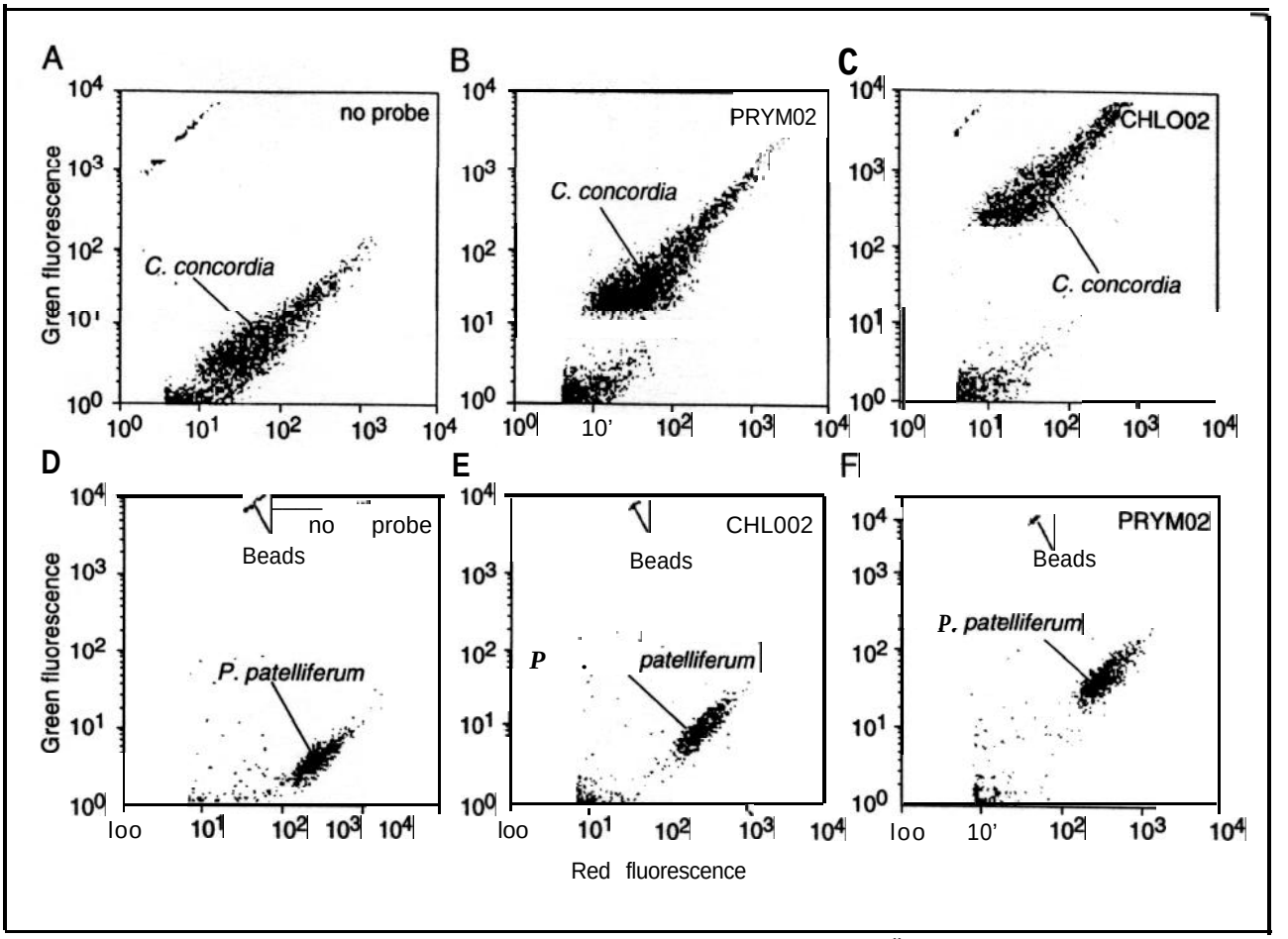

Figure 11.12.4 Cytograms showing the distribution of *Chlamydomonas concordia* (Chlorophyta; A, B, and C) and *Prymnesium patelliferum* (Prymnesiophyceae; D, E, and F) hybridized with no probe (A and D), the probes PRYM02 (specific for the class Prymnesiophyceae; B and F), and CHL002 (specific for the division Chlorophyta; C and E). Note the presence of cell aggregates for *Cblamydomonas concordia.* This may be avoided by intense vortexing in the hybridization buffer prior to incubation.

REAGENTS AND SOLUTIONS

Use *deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see* SUPPLIERS *APPENDIX.*

Hybridization buffer

0.9 M NaCl
20 mM Tris-HCl, pH 7.2
0.0 1% (w/v) sodium dodecyl sulfate (SDS)
10% to 50% deionized formamide
Store up to 1 year at -20°C

See Critical Parameters for optimization of formamide concentration (stringency).

Nucleus isolation buffer (NIB)

30 mM $MgCl_2$
20 mM sodium citrate
120 mM sorbitol
55 mM HEPES
5 mM EDTA
0.1% (v/v) Triton X- 100
Adjust to 7.5

continued

Store at -20°C

Add 5 µl 1 M sodium bisulfite per ml buffer just before the experiment (sodium bisulfite is not stable over time)

Washing buffer

Prepare 20 mM Tris-HCl, pH 7.2 (APPENDIX 2A) containing 0.01% (w/v) SDS and a concentration of NaCl which determines the stringency. Prepare washing buffer with the same stringency as the hybridization buffer (see recipe above). For hybridization buffers with O%, 10%, 20%, 30%, 40%, and 50% formamide, respectively, prepare washing buffers with 0.9, 0.45, 0.225, 0.112, 0.056, and 0.028 M NaCl.

Prepare the wash buffer fresh before use.

COMMENTARY

Background Information

DNA quantification methodology has benefited a great deal from the combined advent of DNA stain technology and flow cytometry. This combination of methods is now routinely applied in such diverse fields as medicine (e.g., screening of cancer cells) and plant biology (e.g., determination of the ploidy of seeds). In phytoplankton research, the determination of DNA content and GC percentage may be useful too as characterization tools (see Anticipated Results).

Another potential application. of flow cytometry is in the study of phytoplankton dynamics. By measuring the distribution of cells in the different phases of the cell cycle at regular intervals during a light-dark cycle, it is possible to compute the growth rate of these populations (Carpenter and Chang, 1988). This method applies to natural populations without the need to incubate cells, a method which is prone to biases known as the "bottle effect."

Although DNA quantification offers much potential, the use of molecular tools appears by far to be the most promising approach in microbial ecology. The specificity of molecular probes can be modulated at will to target any taxon level from kingdom to species, offering the possibility of accurate identification of populations in complex samples (for review, see Amann et al,. 1995).

Critical Parameters

Data analysis

Data are acquired as listmode files and are analyzed with the CytoWin software (Vaulot, 1989; available at ***http://www.sb-roscoff.fr/Phyto/cyto. html)*** to obtain the monoparametric distribution of the population of interest. Cell cycle analyses are then performed on the linear green signal using MultiCycle (P. Rabinovitch) that will fit the linear DNA distribution (Fig. 1 1.12.3) and allow one to determine the percentage of cells in the different phases of the cell cycle: G_1, S, and G_2 (prokaryotes) or G_2M (eukaryotes).

For in situ hybridization, the ratio of the mean fluorescence of cells hybridized with a specific probe to the mean fluorescence of cells hybridized with a nonspecific probe is computed to evaluate probe specificity and sensitivity.

Genome analysis

DNA content is best assessed on fresh material. The nuclei are released by hypotonic shock, and can remain stable for at least one hour in nucleus isolation buffer (NIB). Depending on the species of interest, the composition of the NIB may need to be optimized. For example, addition of extra citrate gives isolated nuclei with more condensed chromatin. Increasing concentrations of detergent may help to remove membranes or cytoplasmic material attached to nuclei, which induce background fluorescence. Examination of nuclei by epifluorescence microscopy will help the operator to determine the best conditions.

With some eukaryotic algae, isolated nuclei cannot be obtained by this method because of the complex composition of membranes (e.g., for diatoms) or because nuclei are not stable and degrade very quickly. In such cases, the DNA content can be estimated on whole cells after treatment with alcohol (ethanol or methanol) to remove fluorescent pigments, followed by incubation with RNase. However, the presence of contaminating DNA (mitochondrial and chloroplastic) often leads to inaccuracy of genome measurement.

For prokaryotes, which have no nuclei, DNA content can be assessed after fixation. The use of paraformaldehyde is recommended.

Choice of the internal reference

The DNA content is measured by comparing the mean DNA fluorescence of the species to that of a standard. Many parameters can influence staining of isolated nuclei, such as the temperature of incubation, the stage of the culture, the concentration of chemicals like detergent or citrate, or the presence of seawater components (e.g., when samples are not concentrated enough to be diluted). The staining of the internal reference will be affected by these parameters in a way similar to that of the sample. This precludes the use of inert material such as fluorescent microspheres as the standard. The choice of the internal reference will also depend on the species to be measured. If the species and the standard have genome sizes that are too different, this leads to inaccuracy in the determination of the DNA content. However, genome size is known precisely only for a small number of microbial strains that have been entirely sequenced (e.g., *Synechocystis* FTC6803 or *E. coli*). If one needs a precise estimation of the DNA content of cells of interest, this constitutes a major drawback. Chicken red blood cells (CRBC) have been used to assess the genome size of ***Phaeocystis*** (Vaulot et al., 1994). However. CRBC have a very high DNA content (2.33 pg DNA/cell) compared to the smallest phytoplanktonic species. For example, ***Ostreococcus tauri*** has a genome size of 6.62 fg (10.2 Mbp per cell; Courties et al., 1998). Another problem with CRBC isthatthey degrade quickly if the samples are not diluted enough to minimize the effect of seawater.

When the genome size difference between the sample and the reference is large, one should use the data acquisition on logarithmic scale with the following formula to calculate the DNA content:

$$D = 10^{[(F-F_R)\times N/C]} \times D_R$$

where D = DNA content of the unknown species; D_R = DNA content of the reference species; F = mean channel of the unknown species; F_R = mean channel of the reference species; N = number of decades of the logarithmic amplifier; and C = number of channels used for the acquisition (256 or 1024). This formula requires precise determination of the number of decades of the amplifier used for collecting the fluorescence (Durand, 1999). It is critical not to rely on the value given by the manufacturer (2 to 4 decades), which is never accurate.

As an example, the authors have estimated the genome size of *Prochlorococcus* at 1.9 Mbp by comparing its DNA distribution, after staining with SYBR-I or YOYO-1, with that of *E. coli* (1 C = 4.66 Mbp) treated with rifampicin (Fig. 11.12.1). This antibiotic inhibits RNA synthesis and blocks the initiation of new replication sites on the DNA, but allows DNA replication to terminate and cell division to occur. As a result, most cells are blocked with a 1C and 2C genome size. The value measured for *Prochlorococcus* by flow cytometry matches very well with that established by DNA renaturation kinetics (1.89 Mbp; Rippka et al., submitted). This constitutes a good illustration of the potential use of flow cytometry to evaluate the genome size of small organisms.

Coefficient of variation

The quality of the cell cycle analysis is strongly dependent upon the coefficient of variation (CV) of the G_1 peak of the linear DNA distribution. A high CV (>10%) makes it very difficult to estimate the fraction of cells in the different phases of the cell cycle, especially the S-phase. Broad G_1-like peaks result from inadequate fixation procedures or from interaction between the stain and some components of the seawater. This can also be due to a concentration of stain that is either too low or too high, leading, respectively, to nonstoichiometric or nonspecific binding. Moreover, because many planktonic species have a small genome size, the dye concentration must be lowered compared to the quantities used with mammalian or plant cells. The usual practice of filtering stock solutions through 0.2-µm-pore-size filters may induce retention of dye such as SYBR Green I on the filter, thereby changing the concentration of the stock solution.

In situ hybridization

Limits of the technique: Current focuses in aquatic microbial ecology include the study of the diversity of planktonic organisms (bacteria, phytoplankton, and heterotrophic protists) as well as the structure and dynamic of the communities&d populations. Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes, combined with flow cytometry, is a promising new approach for the characterization of microbial communities. Optimized for the identification of bacteria (Wallner et al., 1993), this approach has been used for the analysis of bacteria in activated sludge (Wallner et al., 1997). It has also been used to sort and further analyze target species

using molecular methods such as gene amplification and sequencing (Wallner et al., 1997). Probes for eukaryotic phytoplankton have also been designed, and conditions of hybridization have been optimized for flow cytometry (Simon et al., 1995, 1997; Lange, 1996). Basic Protocol 3 may be used for the rapid screening of phytoplankton isolates. However, to date, no application of this method to natural marine phytoplankton communities has proven successful, mainly because the intensity of the hybridization signal is too low to discriminate one phytoplanktonic species from the rest of the community. This is probably due to the fact that cells in the marine environment have a reduced rRNA content, possibly because of suboptimal growth. To overcome this problem, new and brighter fluorochromes would be needed (Schönhuber et al., 1997).

Flow cytometer: Better discriminations between target and nontarget cells have been obtained using small flow cytometers, such as the FACSort equipped with air-cooled argon lasers delivering 15 mW, than with larger instruments, such as the Epics 545 equipped with a water-cooled laser delivering 1.2 W. The reason for this difference in signal/noise ratio may be linked to the optical configurations of these instruments.

Design and ordering of probes: The design of taxa-specific probes requires an extensive knowledge of the phylogeny of the target groups and is subject to a few rules which are reviewed in detail by Stahl and Amann (1991) and Amann (1995). To date, the SSU rDNA gene is the best candidate because (1) it includes signatures for species as well as phyla, (2) its database is very large, and (3) the targets in the cells are relatively abundant (one per ribosome). A few oligonucleotide probes targeted to phytoplankton taxa are now available, among which some were tested using whole-cell hybridization combined with flow cytometry (Table 11.12.2).

DNA probes may be purchased already labeled (e.g., at the 5' end with FITC) and purified from commercial companies. Alternatively, labeling and purification can be custom-made following the protocol described by Amann (1995).

Choice of species to be used for tests: Probes that are theoretically specific for a targeted group should be empirically tested on cells. At least one target species and one non-target species that is closely related to the target group should be chosen. The rRNA molecule of a non-target species should present at least one mismatch with the probe; however, more mismatches are better. It is necessary to grow and harvest cells under optimum conditions. To optimize the signal, cells should be harvested in mid-exponential phase. Cells may be stored at -80°C either as a paraformaldehyde-fixed suspension or in the hybridization buffer.

Preparation and optimization of hybridization buffers: Hybridization conditions depend upon the dissociation temperature (T_d) of the oligonucleotide and must be empirically optimized for each new probe. The temperatures of hybridization and washing buffers are routinely adjusted in order to achieve a good discrimination between target and non-target sequences. For whole-cell hybridization, formamide concentration or ionic strength can be adjusted while temperature is kept constant. The melting point of hybrids is lowered by -0.7°C for every 1% increase of formamide concentration in the buffer. With this strategy, optimal conditions may be determined within a day using a single oven and batches of target and non-target cells.

Preparation and storage of hybridization and washing buffers: Buffers with varying concentrations of formamide (i.e., l0%, 20%, 30%, 40%, and 50% for hybridization buffers with different stringency) and of NaCl (i.e., washing buffers) may be prepared in advance, divided into aliquots in l-ml microcentrifuge tubes, and stored at -20°C until needed.

Choice of species to be tested: Choose one target species and one non-target species that is most closely related to the target group-i.e., whose 18S rRNA possesses the minimum possible number of mismatches compared to the probe. The rationale is that non-target species with more mismatches will hybridizeeven less.

Tests with different hybridization stringencies: Proceed with the hybridization protocol using the full range of hybridization and corresponding washing stringencies and analyze the cells by flow cytometry. Plot the intensities of green fluorescence of both species against the percentage formamide used for the hybridization buffers. For subsequent use of the probe, choose the percentage formamide that best discriminates species. Ideally, using the optimal buffers, the intensity of fluorescence of the nontarget cells should not exceed the intensity of green autofluorescence of the cells, and target cells should show a relatively bright signal in the green. In practice, a light nonspecific labeling is often observed (Fig. 11.12.4).

Table 11 .12.2 Oligonucleotide Probes Designed for the Detection and Identification of Phytoplankton Taxa by Whole-Ceil Hybridization"

Specificity	Probes	Oligonucleotide sequence (5' to 3')	References
Upper group level			
Eukaryotes	EUK1209R	GGGCATCACAGACCTG	Lim et al., 1993
	EUK502	ACCAGACTTGCCCTCC	Lim et al., 1993
	EUK309R	TCAGGCTCCCTCTCCGG	Lim et al., 1993
	EUKB	TGATCCTTCTGCAGGTTCACCTAC	Lim et al., 1993
Chlorophyta	CHLO01	GCTCCACGCCTGGTGGTG	Simon et al., 1995
Chlorophyta	CHLO02	CTTCGAGCCCCCAACTTT	Simon et al., in press
"Non-chlorophyta"	NCHLO0 1	GCTCCACTCCTGGTGGTG	Simon et al., 1995
Class level			
Pelagophyceae	PELA0l	ACGTCCTTGTTCGACGCT	Simon et al., in press
Prymnesiophyceae	PRYM0l	ACATCCCTGGCAAATGCT	Lange et al., 1996
	PRYM02	GGAATACGAGTGCCCCTGAC	Simon et al., in press
Genus level			
Phaeocystis spp. (Prymnesiophyceae)	PHAEO01	CGGTCGAGGTGGACTCGT	Lange et al., 1996
Species level			
Chrysochromulina polylepis (Prymnesiophyceae)	CPOLY0 1	GACTATAGTTTCCCATAAGGT	Simon et al., 1997
Paraphysomonas vestita	PVl	TAAAACCCATCCTATTATATC	Rice et al., 1997a, b
(nonphotosynthetic)	PV2	TTCCGTATGCCAGTCAGA	Rice et al., 1997a, b
(Chrysophyceae)	PV3	AGTATAAATATCACAGTCCGA	Rice et al., 1997a, b
	PV4	ATATAATCTTTTCGATGATGA	Rice et al., 1997a, b
	PV5	CCCATCCTATTATATCAGAAA	Rice et al., 1997a, b
Other (lineages, group of species)			
Chrysochromulina polylepis/Prymnesium parvum/P patelliferum/P calathiferum (Prymnesiophyceae)	CLADEl	GGACTTCCGCCGATCCCTAGT	Simon et al., 1997
Chrysochromulina species included in a clade (Clade 2) (Prymnesiophyceae)	CLADE2	AGTCGGGTCTTCCTGCATGT	Simon et al., 1997
Aureococcus, Pelagomonus, Pelagococcus, CCMP 1395 (belonging to the Pelagophyceae)	PELA02	GCAACAATCAATCCCAATC	Simon et al., in press

[a] All probes are specific for signature sequences on the SSU rRNA of their target organisms.

Troubleshooting

Genome analysis

Unstable fluorescence of nuclei (increase or decrease of the mean fluorescence of the isolated nuclei distribution) may be observed. Both increase and decrease of the mean fluorescence result from degradation of nuclei or from the effect of some chemicals that are released after hypotonic shock. An epifluorescence microscope is useful to determine if nuclei are degraded, and in such a case, the composition of the buffer must be optimized to stabilize the chromatin. To deal with the effect of chemicals, the presence of an internal reference is required as mentioned previously.

In situ hybridization

Potential problems include the following.,

1. No cells visible. The first possibility here is that the cells were lost during processing. The protocol requires several steps of spinning and resuspending cells. When removing supernatants, be sure not to disturb the pellet. The second possibility is that the signal of the cells

is lost within the noise. In order to avoid problems, always filter the buffers to be used.

2. ***Low signal intensity.*** Make sure that the cells are in mid-exponential phase before harvesting. The signal intensity is strongly dependent on the ribosome content, which drops by an order of magnitude in stationary phase.

Anticipated Results

Genome size and ploidy level

Very little information is currently available concerning the genome size and GC percentage of phytoplankton species, although these parameters are very useful for characterizing these ceils when more classical parameters such as morphology are not discriminative enough. For instance, by determining the genome size of 16 cultured strains of *Phaeocystis* spp. Lagerheim by flow cytometry Vaulot et al. (1994) have defined 6 different clusters. Similarly, using different DNA-specific dyes, Simon et al. (1994) have been able to discriminate several eukaryotic strains by flow cytometric measurement of their DNA content and GC percentage (Simon et al., 1994).

Cell ***cycle***

The analysis of the cell cycle of marine species was first designed to study the dynamics of dinoflagellate cells (Chang and Carpenter, 1991). Another nice application of this technique was the determination of the growth rate of *Prochlorococcus* at different depths of the equatorial and tropical Pacific (Vaulot et al., 1995; Liu et al., 1997). More recently, this technique was used to study the effects of the variations in cloud cover on *Synechococcus* populations in the surface waters of a Mediterranean bay et al., 1998).

In sit u ***hybridization***

The combination of whole-cell hybridization with flow cytometry was first tested on cultured bacteri (Amann et al. ,1990 ; Wallner et al., 1993) and later applied to bacteri in a natural samples (Pemthaler et al., 1997; Wall-- ner et al., 1997 Shönhuber et al., 1999). The same technique has been applied to detect phytoplankton phylogenetic groups (from th divi- e sion to the species level), and especially the smalles species in culture (the photosynthetic nano- and picoeukaryotes; Lange et al., 1996; Simon et al., 1995, 1997, in press).

Time Considerations

From the harvesting of the live cells to the analysis with flow cytometry, 5 hr are needed (including 3.25 hr of incubation), provided that buffers are ready to use.

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