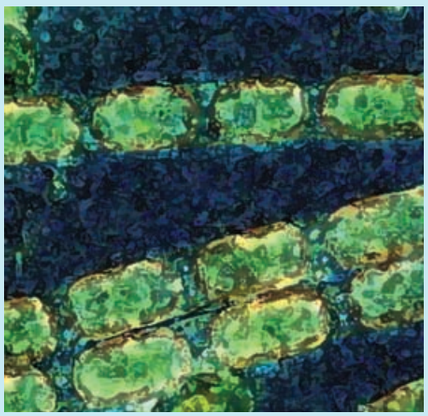

CHAPTER 17

PHYTOPLANKTON CELL COUNTING BY FLOW CYTOMETRY

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1.0. INTRODUCTION

On many occasions, cultures can be easily monitored and counted by optical microscopy (see Chapter 16). However, researchers have recognized early on the need to automate cell counting. Automated cell counting is usually much faster than optical microscopy counting, and it minimizes errors associated with human counting. Because many more cells can be counted, the statistical significance of the data is considerably improved. Furthermore, other cellular parameters can also be determined, for example, cell volume or DNA content. Finally, the smallest phytoplankton (picoplankton, <2 to 3 μm) cannot be distinguished from bacteria when examined using a light microscope (e.g., *Prochlorococcus*), but they can be counted using automated techniques. However, automated cell counting also has its problems and limitations. Instruments are relatively expensive, starting at U.S. $20,000 for simple counters and reaching up to U.S. $300,000 for the most sophisticated flow cytometers. Some of the high-end flow cytometers can only be used by highly trained personnel. Finally, because cells are measured blindly, proper controls are necessary to ensure that the signal measured does not result from nontarget particles (detritus, contaminants).

In the 1970s, the introduction of the Coulter Counter (now marketed by Beckman Coulter) for phytoplankton counting (Sheldon and Parsons 1967, Sheldon 1978) constituted the first major advance toward automated phytoplankton counting. Particles in solution are drawn through a small aperture, separating two electrodes between which an electric current flows. As each particle passes through the aperture, it displaces its own volume of conducting liquid, briefly increasing the impedance of the aperture. This signal is converted into a voltage pulse. By counting the number of pulses for a given volume passing through the aperture, one obtains an estimate of the particle concentration. The equivalent spherical volume of each cell can also be estimated from the amplitude of the pulse. Although widely used to count large phytoplankton cells in cultures, the Coulter Counter has several limitations. First, even with the smallest aperture available, it is technically very challenging to count cells smaller than 1 to 2 μm, making this technique not applicable to picoplankton. Second, because a single cell parameter (cell volume) is determined, it is difficult to discriminate phytoplankton cells from other particles such as bacteria, detritus, and even air bubbles or to work with mixed cultures containing, for example, several phytoplankton species with overlapping sizes. Another instrument that uses a light beam to measure particle size is the HIAC counter (Pacific Scientific Instruments). It is less widespread than the Coulter Counter but is particularly well adapted to the continuous monitoring of cultures (Malara and Sciandra 1991, Sciandra et al. 2000). Because a single parameter is measured, it suffers from the same type of limitation as a Coulter Counter.

Although flow cytometry (FCM) was developed more than 30 years ago, it is only in the mid-1980s that it was first applied to phytoplankton analysis both in culture (Trask et al. 1982, Olson et al. 1983, Yentsch et al. 1983) and in the field (Olson et al. 1985). Since then, it has been increasingly used by aquatic (mostly marine) biologists for the analysis of small particles (Veldhuis and Kraay 2000) as well as phycologists (Collier 2000). Its major advantage over the Coulter Counter is that it simultaneously records several parameters for each event, allowing for the discrimination between cells and detritus. It is also particularly well adapted to the analysis of picophytoplankton, which are difficult or tedious to analyze with traditional methods such as epifluorescence microscopy. These tiny organisms are present in all aquatic environments at concentrations up to 5×10^5 cell·mL^{-1}. Direct analysis provides information on the abundance, cell size, and pigment content of the major photosynthetic picoplankton groups (cyanobacteria and eukaryotes). Although well suited for picophytoplankton, FCM can also be used for cultures of larger phytoplankton up to a size of 100 to 200 μm; beyond this size, custom modifications or special instruments must be used (Dubelaar et al. 1989). Moreover, some instruments are able to physically sort cells to identify them or bring them into culture (see Chapter 7). The use of benchtop instruments on board ships has helped to improve our knowledge of the geographical distribution and population dynamics of the picoplankton in relation to its physical and chemical environment (Partensky et al. 1999). FCM has been little used in freshwater research, although this trend seems to be reversing (Crosbie et al. 2003). Highly sensitive nucleic acid–specific stains such as TOTO-1, YOYO-1, and the SYBR Green family have also made possible the detection and enumeration of heterotrophic bacteria (Li et al. 1995, Marie et al. 1997) or more recently of viruses (Marie et al. 1999). The application of FCM extends also to physiological analyses (e.g., DNA analysis) (Vaulot et al. 1986) and to phylogenetic analyses with the help of fluorescent molecular probes (Simon et al. 1995).

In this chapter, we detail the use of FCM for marine algae culture work. Most of the protocols described can

be applied equally well to field samples and to freshwater organisms. In the latter case, only obvious modifications, such as replacing seawater wherever mentioned by freshwater, are required (Lebaron et al. 2001, Crosbie et al. 2003).

2.0. PRINCIPLES OF FLOW CYTOMETRY

2.1. General Principles

FCM measures cells in liquid suspension. Cells are aligned hydrodynamically by an entrainment fluid (sheath fluid) into a very narrow stream, 10 to 20 µm wide, onto which one or several powerful light sources (arc mercury lamp or laser) are focused. Each time a particle passes through the beam, it scatters light; angular intensity depends on the refractive index, size, and shape of the particle. Moreover, if the particle contains a fluorescent compound whose absorption spectrum corresponds to the excitation source (e.g., blue light for chlorophyll), it emits fluorescence at a higher wavelength (e.g., red light for chlorophyll). These light pulses are detected by photodiodes or more often by photomultipliers and then are converted to digital signals that are processed by a computer. Measurement rates vary between 10 and 10,000 events per second. On the more sophisticated instruments, it is then possible to physically sort cells of interest based on any combination of the measured parameters (see Chapter 7).

2.2. Fluidics

Flow cytometers are equipped with a tank supplying the sheath liquid (buffer, distilled water, seawater) that carries the cells through the instrument; a second tank collects the waste fluid. Cell suspensions are injected or pushed through a capillary into a sheath fluid stream. Under laminar flow conditions, the sheath liquid aligns the cells into a narrow centered stream. The illumination of cells can be performed in the air, just outside a nozzle through which the sheath fluid exits, or in a quartz cuvette through which the sheath fluid flows. The latter solution increases the detection sensitivity, which is required for picophytoplankton. The flow rate must be adjusted depending on the cells of interest to keep laminar flow conditions and to control the number of events to be analyzed per unit time.

2.3. Optics

When a particle passes through the excitation beam, light can be reflected or refracted. In most flow cytometers, the light scatter detectors are located at 180° (forward scatter or FSC) and at 90° (side scatter or SSC) with respect to the light source. Both parameters are related to cell size, but the side scatter is more influenced by the cell surface and internal cellular structure (Morel 1991, Green et al. 2003).

Many fluorescent molecules can bind to a wide range of cytochemical compounds such as proteins, lipids, or nucleic acids. Each fluorescent dye is characterized by its excitation and emission spectra. Flow cytometers are usually equipped with a laser emitting at a single wavelength (488 nm). Therefore, only fluorescent molecules excited at that particular wavelength can be used. If multiple excitation wavelengths are available, then the choice of the fluorochromes is much wider.

The flow cytometer is equipped with highly sensitive photomultiplier tubes that are able to measure and amplify the brief pulse of light emitted by the cells. When a cell intersects the excitation beam, the emitted light is collected by a lens and passes through a series of filters that remove the excitation light, allowing only the emission light to be detected. With several photomultipliers, multiple wavelength emission ranges can be collected (e.g., orange and red fluorescence for algal cells).

2.4. Electronic and Software Processing

To be usable, analog data from the photomultipliers must be converted to digital form, that is, to a number on a scale ranging, for example, from 1 to 256 (2^8) corresponding to 8-bit conversion. To avoid saturation of the conversion circuitry, only events of interest must be converted. Therefore, the operator needs to select one or several signals (called *discriminators* or *triggers*) and must set thresholds for each discriminator. When the value of one of the discriminator signals is larger than the corresponding threshold, all signals from the triggering particle are converted. Choosing adequate discriminators and thresholds is critical to correctly record the cells of interest, especially when working with very small cells or particles. As an example, to record chlorophyll fluorescing microalgae, it is best to choose red fluorescence as the discriminator and to select a threshold that is high enough so optical and

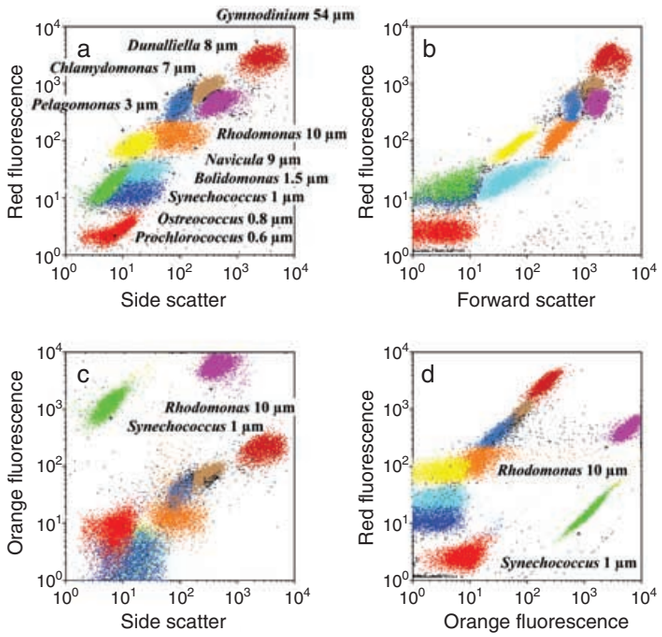


FIGURE 17.1. Cytograms obtained with a mixture of eight photosynthetic eukaryotes and two cyanobacteria in culture, analyzed using a FACSort flow cytometer using the natural fluorescence of phycoerythrin (orange) and chlorophyll (red). (See Table 17.1 for culture origin.)

electronic noise are left out but that is low enough so no cells are missed.

Digital data are then transmitted to a computer that displays and records the results. They will appear on the screen as mono-parametric histograms or bi-parametric cytograms (Fig. 17.1). Data can be further processed to discriminate specific cell populations and estimate their cell concentration and average cellular parameters using software provided with the instrument or public domain programs such as WinMDI (facs.scripps.edu/software.html) or CytoWin (www.sb-roscoff.fr/Phyto/cyto.html). For more sophisticated analyses, such as cell cycle deconvolution, specialized commercial software is available (e.g., from Verity Software House: www.vsh.com).

2.5. Available Instruments

A few companies occupy the FCM market. These include Becton Dickinson (www.bd.com), Beckman Coulter (www.beckman.com), Dako Cytomation (www.dakocytomation.com), and Partec (www.partec.de). Available instruments fall into three categories, which are covered in the following sections.

2.5.1. Benchtop Analyzers

Benchtop analyzers are small instruments widely used in medicine for blood analysis of antigen-responding cells. These instruments usually possess a single excitation wavelength at 488 nm, delivered by a small air-cooled laser. Possible choices are the FACS family (FACScan, FACSort, FACSCalibur) from Becton Dickinson, the EPICS XL from Beckman Coulter, or the CyAn from DakoCytomation. Some of these instruments are fitted with limited sorting capacity (FACSort, FACSCalibur). Their reduced footprint and moderate price make them perfect for ship-board analysis and small laboratories. They do not require highly trained operators, and used instruments can be easily purchased.

2.5.2. High-Speed Sorters

High-speed sorters are, in general, fairly large instruments that use several lasers delivering both ultraviolet (UV) and visible excitation lines. The more powerful water-cooled lasers require special open or closed water cooling circuits, as well as three phase power lines. Their capacity to excite many dyes makes them more versatile, allowing several components to be measured

simultaneously within the same cell (e.g., DNA and proteins). The sorting capacity of these high-end instruments reaches up to 20,000 cells per second. These include the FACS Vantage from Becton Dickinson, the EPICS Altra from Beckman Coulter, and the MoFlo from DakoCytomation. Because of their cost, the need for a dedicated room, and the complexity of their operation (usually requiring a special technician), they are much less widely used. Moreover, their sensitivity is, paradoxically, often lower than that of the smaller benchtop analyzers, because they use a jet-in-air nozzle design.

2.5.3. Custom Instruments

Some researchers have devised their own instruments, for example, based on a microscope (Olson et al. 1983) or modified existing flow cytometers to improve sensitivity (Dusenberry and Frankel 1994) or measurement size range (Cavender-Bares et al. 1998). More recently, flow cytometers have been specifically designed for in situ applications such as the continuous monitoring of phytoplankton in marine waters. These include the CytoBuoy (www.cytobuoy.com) (Dubelaar and Gerritzen 2000) and the FlowCytobot (Olson et al. 2003).

2.5.4. Choice of Instrument for Algal Cultures

All commercial instruments are suitable to analyze algal cultures. For picoplanktonic organisms or for virus detection, the use of a very sensitive flow cytometer, such as a benchtop FACS (Becton Dickinson), is critical.

3.0. Counting Phytoplankton by Flow Cytometry Using Natural Optical Properties

Phytoplankton possess fluorescing photosynthetic pigments (see Chapter 20) that can be used to discriminate cells from heterotrophic organisms and nonliving particles. The most common fluorescing pigments are chlorophyll, phycoerythrin, and phycocyanin. The latter two are phycobiliproteins that are typical of cyanobacteria, cryptophytes, and rhodophytes. Both chlorophyll and phycoerythrin are well excited with the common 488-nm excitation line and fluoresce at 690 nm (red) and 570 nm (orange), respectively. In contrast, phycocyanin is excited at 620 nm and emits at 640 nm. Therefore, it can be detected only with a red-emitting laser. Counting phytoplankton does not require any pretreatment of the samples, but if samples cannot be counted immediately, they must be preserved by aldehydes (formaldehyde or glutaraldehyde) and stored deep frozen, either in a –80°C freezer or in liquid nitrogen.

3.1. Measurement of Cell Concentration

Phytoplankton abundance is best obtained using fresh unfixed samples; fixation will always result in some degree of cell disruption and cell loss (Vaulot et al. 1989). Samples can be kept, however, at 4°C in the dark for up to 12 hours with only minimal change in abundance or optical parameters (Jacquet et al. 1998).

3.1.1. Materials

1. Phytoplankton cultures
2. 0.95-µm fluorescent microspheres diluted at $10^5 \cdot mL^{-1}$ in distilled water (e.g., Polysciences)
3. 0.2-µm filtered seawater
4. Micropipettes and tips for 10 to 1000 µL
5. Chronometer
6. Flow cytometer equipped with a 488-nm argon laser (e.g., benchtop FACS)

3.1.2. Culture Dilution

If two (or more) cells pass simultaneously through the excitation beam, or if two cells are too close to each other, and the second cell arrives while the instrument is busy recording the first cell, then the two cells are recorded as a single event (see Chapter 7). This phenomenon is called *coincidence*, and it results in an underestimate of the cell concentration. Coincidence threshold is best determined empirically. For example, it is possible to analyze a range of cell concentrations at a given sample flow rate. The coincidence threshold corresponds to the maximum cell concentration beyond which the number of recorded cells is not linearly related to the sample concentration (Gasol and Del Giorgio 2000). As an example, for instruments of the benchtop FACS family, coincidence for picoplankton cells begins at more than 800 cells per second. To prevent coincidence, it is necessary either to reduce the flow rate (e.g., from high to medium on a FACS) or to dilute cultures before analysis.

3.1.3. Instrument Settings

Phytoplankton acclimates to changes of photon-flux densities by changing pigment content, leading to a decrease in chlorophyll fluorescence per cell as light increases (Sosik et al. 1989). Fluorescence range for a given strain is very wide for *Prochlorococcus* (up to fifty-fold) and less pronounced for eukaryotes (fivefold to tenfold only). The intensity of the other cellular parameters such as scatter can also vary with light conditions and available nutrients. For example, as nutrients become limiting, scatter typically decreases in intensity. Thus, the voltage of the photomultipliers must be adjusted accordingly, depending on the size and fluorescence of the organisms of interest.

Typical settings for picoplankton and nanoplankton cultures on a benchtop FACS flow cytometer are as follows: forward scatter (FSC) = E00, side scatter (SSC) = 400, green fluorescence (FL1) = 650, orange fluorescence (FL2) = 650, and red fluorescence (FL3) = 550. All parameters are set on logarithmic amplification and the trigger is set on the red fluorescence.

3.1.4. Sample Analysis

We describe here a general procedure that can be used either for fresh or preserved samples.

1. Turn on both the instrument and the computer.
2. Prepare the sheath fluid. Because cell scatter (especially FSC) is dependent on the nature of the sheath fluid (Cucci and Sieracki 2001), it is necessary to match the sheath and sample fluids. For example, for marine samples, 0.0.2-µm pore-size filtered seawater can be used as sheath fluid. In this case, it is best to remove any inline sheath filter, which becomes easily contaminated and quickly tends to release particles.
3. If samples were fixed and frozen (see Section 3.2.3), then thaw them at room temperature or at 37°C.
4. Transfer about 1 mL (minimum of 250 µL) of the culture into a flow cytometer tube.
5. Add 10 µL of the microsphere solution containing about 10^5·beads mL^{-1} as an internal reference.
6. Select (low, medium, or high) and calibrate the flow rate (see Section 3.1.5).
7. Set the discriminator on the red (chlorophyll) fluorescence with a threshold of 50.
8. Insert the sample tube in the instrument sample holder, and after about 15 seconds (to allow the flow rate to stabilize), start data acquisition. Typical analysis of a culture lasts 2 to 3 minutes with a delivery rate of 50 to 100 µL·min^{-1}.
9. Record the duration of analysis, which is necessary to estimate the cell concentration (see Section 3.1.6.).

3.1.5. Flow-Rate Calibration

On most commercial flow cytometers, it is not possible to deliver a specific sample volume or to precisely set the sample flow rate. Therefore, the sample flow rate must be determined by the operator for accurate cell concentrations.

Often, a solution of fluorescent microspheres with a known concentration (determined by epifluorescence microscopy) is used for the measurement of the flow rate. Because the electrostatic charges of the beads make them stick onto the plastic tubes (particularly in seawater), this modifies their initial concentration, and we do not recommend this method (still, we always add fluorescent beads to our samples to check flow stability and to normalize cell scatter and fluorescence; see Section 3.1.6). We use instead the procedure described below that works on flow cytometers from the benchtop FACS family that can be adapted to most instruments. This calibration should be repeated several times a day.

1. Select the same sample flow rate used for analysis.
2. Fill a tube with the same liquid as samples (e.g., seawater for marine phytoplankton).
3. Measure the volume of the sample (or weigh precisely the tube containing the sample).
4. Remove the outer sleeve of the injection system. The sheath fluid will drop down the sample needle.
5. Wait until a droplet has fallen, and then before the next one forms, place the sample tube and close the sample arm in the running position.
6. Simultaneously start the chronometer.
7. Run the sample for at least 10 minutes.
8. Remove the sample tube and simultaneously stop the chronometer.
9. Measure (or weigh) the remaining volume.

The rate (R), expressed in microliters per minute, is given by the following formula:

$$R = (V_i - V_f)/T, \quad (1)$$

where V_i = initial volume (µL), V_f = final volume (µL), and T = the time (minutes). The use of a scale leads to better precision for the determination of the flow rate:

$$R = (W_i - W_f)/(T * d), \quad (2)$$

1

OK as set

where W_i = initial weight (mg), W_f = final weight (mg), T = time (minutes), and d = density of the liquid used for calibration (distilled water = 1.00, seawater = 1.03).

3.1.6. Data Analysis

Phytoplankton cells cover a wide range of size and fluorescence properties (see Fig. 17.1). Therefore, data are always collected using logarithmic amplifications and recorded as list-mode files, which allows detailed offline analysis. In practice, 20,000 to 100,000 events are collected for microalgae and up to 150,000 for bacteria or viruses. List-mode files are then analyzed using either the instrument software or publicly available programs.

The different populations are discriminated using a combination of parameters: scatters (FSC and SSC) and fluorescences (usually red and orange). Figure 17.1 illustrates data obtained for a mixture of 10 phytoplankton species (8 eukaryotes and 2 prokaryotes) (Table 17.1) ranging in size from 0.6 to 60 µm and how the different cultures can be discriminated by using combination of parameters. For example, cyanobacteria (*Synechococcus*) and Cryptophyceae (*Rhodomonas*) can be discriminated from other eukaryotic algae (red algae excluded) based on their high orange/red fluorescence ratio linked to the presence of phycoerythrin (see Fig. 17.1d).

Absolute cell concentration for each population is computed as follows:

$$C_{pop} = T * N_{pop} / R * (V_{total} / V_{sample}), \quad (3)$$

Correct to (with subscripts as set) :
Cpop = Npop * (Votal/Vsample)/(R * T)

where C_{pop} = concentration of population (cells·µL^{-1}), N_{pop} = number of cells acquired, T = acquisition time (minutes), R = sample flow rate (µL·min^{-1}) as determined for the sample series (see Section 3.1.5), V_{total} = volume of sample plus additions (fixatives, beads, etc.) (µL), and V_{sample} = volume of sample (µL).

To compare different samples, cell parameters are normalized to parameters obtained for 0.95 µm of fluorescent microspheres added as internal reference. The mean value of each parameter (for the different populations) is divided by the mean value of the parameter for the beads:

$$X_{rel} = X_{pop} / X_{beads}, \quad (4)$$

where X_{pop} is the average value of a cell parameter (scatter or fluorescence) for a given population, and X_{beads} is the same parameter for the reference beads. Both X_{pop} and X_{beads} must be expressed as linear values (not channels) after conversion from the logarithmic recording scale.

3.2. Cell Fixation and Preservation

If samples cannot be analyzed immediately, they must be fixed and then frozen in liquid nitrogen and stored at –80° C or in liquid nitrogen until analysis. Physical treatments such as centrifugation and classic or tangential filtration must be avoided because they induce vari-

TABLE 17.1 Cultures referred to in this chapter. The RCC column corresponds the reference number of the culture in the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/collect.html).

RCC	*Class*	*Taxon*	*Size (µm)*
1	Chlorophyceae	*Chlamydomonas* sp.	7
6	Chlorophyceae	*Dunaliella tertiolecta* Butcher	8
22	Chrysophyceae	*Picophagus flagellatus* Guillou et Chrétiennot-Dinet	2
29	Cyanophyceae	*Synechococcus* sp.	1
80	Bacillariophyceae	*Navicula transitans* Cleve	9
89	Dinophyceae	*Gymnodinium sanguineum* Hirasaka	60
100	Pelagophyceae	*Pelagomonas calceolata* Andersen et Saunders	3
116	Prasinophyceae	*Ostreococcus tauri* Courties et Chrétiennot-Dinet	0.8
238	Bolidophyceae	*Bolidomonas mediterranea* Guilou et Chrétiennot-Dinet	1.5
286	Pelagophyceae	*Ankylochrysis lutea* Billard	6
350	Cryptophyceae	*Rhodomonas baltica* Karsten	10
407	Cyanophyceae	*Prochlorococcus* sp.	0.6

able losses. Because phytoplanktonic cells are discriminated on the basis of scatter and pigment fluorescence, the fixation procedure must preserve these properties. Classic methods such as formalin (a generic term that describes a solution of 37% formaldehyde gas dissolved in water usually containing 10 to 15% methanol) or Lugol's iodine fixation are generally inadequate because they modify cell shape or drastically affect fluorescence. Alcohol fixation will extract lipophilic pigments and lead to a loss of autofluorescence. Formaldehyde fixation (1% final concentration) is the best method, because in our experience, it minimizes cell loss. Moreover, solutions of formaldehyde are buffered and do not strongly modify the pH level of seawater samples. Formaldehyde is obtained by heating paraformaldehyde powder (that has no fixation properties) in distilled water or phosphate-buffered saline (PBS). Fixation with formaldehyde can be supplemented with glutaraldehyde 0.05% (final concentrations), particularly when cell cycle analysis is performed.

However, formaldehyde solutions are neither easy to prepare nor stable over time. Therefore, for inexperienced operators, or when there is any doubt on the quality of the formaldehyde to be used (e.g., during an important cruise), we recommend replacing formaldehyde with a commercial solution of glutaraldehyde at 0.1% (final concentration). This will lead to slightly higher cell loss but is clearly preferable to fixation with bad formaldehyde, which leads to a lot of background noise (cell debris, small particles), making flow cytometric analysis impossible.

3.2.1. Materials

1. Paraformaldehyde powder (Sigma P-6148)
2. Glutaraldehyde 25% aqueous solution (Sigma G-6257)
3. Sodium hydroxide in pellets
4. Cryovials (e.g., Nunc)
5. Pipettes and tips
6. 0.2-µm pore-size syringe filters
7. Paper filter

3.2.2. Preparation of 10% Formaldehyde

1. Note: If not confident with this procedure, use only glutaraldehyde.
2. Under a fume hood, add 10 g of paraformaldehyde powder to 70 mL of boiling distilled water.
3. Mix vigorously for at least 2 hours under the fume hood.
4. Add progressively small amounts of sodium hydroxide (0.1 M).
5. Agitate until the solution becomes clear.
6. Add 10 mL of 10% PBS.
7. Adjust the pH to 7.5.
8. Bring final volume up to 100 mL with distilled water.
9. Filter through paper filter.
10. Filter again through 0.2-µm pore-size syringe filters.
11. Aliquot to 15-mL tubes and store at –20°C.
12. Use unfrozen aliquoted formaldehyde solutions for not more than 1 week.

3.2.3. Fixation Procedure

1. Add 1% of formaldehyde or 0.1% of glutaraldehyde or a mixture of both (1% and 0.05%, respectively) to the sample.
2. Mix by vortexing rapidly.
3. Incubate for at least 15 minutes at room temperature.
4. If samples cannot be analyzed immediately, then they must be quickly frozen using liquid nitrogen. They can then be stored at –20°C for a short period (several days) but must be kept at µ80°C or in liquid nitrogen for long-term storage, because storage at –20°C beyond 1 week will result in rapid sample degradation.

3.3. Fluorescent Dyes

Fluorescent dyes that recognize specific molecules within cells extend considerably the application of FCM. Among these dyes, the most useful are probably nucleic acid stains. They are extremely diverse and can be used to detect contaminating bacteria or viruses (Marie et al. 1997) and to measure cell viability (Brussaard et al. 2001, Veldhuis et al. 2001) or cell cycle progression (Vaulot et al. 1986). A wide range of nucleic acid–specific dyes synthesized and manufactured by Molecular Probes (www.probes.com), such as YOYO-1, PicoGreen, or SYBR Green-I (Li et al. 1995, Marie et al. 1997), are now available and replace the UV-excited dyes, DAPI or Hoechst 33342, initially used for this purpose (Monger and Landry 1993, Button and Robertson 2001). Other markers that could be used but have received limited application for phytoplankton include protein stains such as SYPRO (Zubkov et al. 1999) or cellular activity stains such as FDA (Brookes et al. 2000).

3.3.1. Analysis of Heterotrophic Eukaryotes and Bacterial Contaminants

The analysis of heterotrophic cells requires a fixation step by aldehydes, as mentioned earlier, and the use of nucleic acid–specific stains. The affinity of the cyanine dyes (TOTO-1, YOYO-1) and their monomeric equivalents (TO-PRO-1, YO-PRO-1) decreases significantly with ionic strength, which makes them inappropriate for direct analysis of seawater samples (Marie et al. 1996). Other dyes such as SYBR Green-I (SYBR-I), SYBR Green-II, and SYTOX Green are less dependent on the composition of the medium and can be used for the enumeration of marine bacteria. Because SYBR-I has a very high fluorescence yield, we recommend this dye to enumerate bacteria in marine samples, although SYTO-9 may provide better results for freshwater samples (Marie et al. 1997, Lebaron et al. 1998).

3.3.2. Materials

1. 0.2-µm pore-size filtration units for plastic syringes
2. 0.95 µm fluorescent microspheres (see Section 3.1.1)
3. DNA-specific stains such as SYBR Green-I (all stock solutions *except* SYBR-I must be prefiltered onto 0.2 µm or less to avoid contamination)
4. Flow cytometer equipped with a 488-nm argon laser
5. Glutaraldehyde 25% aqueous solution and/or formaldehyde 10% (see Section 3.2)

3.3.3. Sample Preparation

1. If samples are live, add either 1% formaldehyde or 0.1% glutaraldehyde (final concentrations) and wait 20 minutes to allow a good fixation.
2. If samples have been preserved and frozen, thaw them at 37°C.
3. Dilute the sample in 0.2-µm pore-size filtered seawater if necessary (see Section 3.1.2).
4. Add the SYBR-I at a final dilution of 1:10,000 of the commercial solution.
5. Incubate for 15 minutes at room temperature and in the dark.
6. Add 10 µL of a suspension of 0.95 µm fluorescent microspheres at a concentration of 10^5 beads·mL^{-1} in 1 mL of sample.

3.3.4. Data Acquisition

1. Turn the flow cytometer and computer on.
2. Prepare the sheath fluid (distilled water can be used as sheath fluid, but for natural seawater samples, 0.2-µm pore-size filtered seawater is preferred).
3. Calibrate the flow rate (see Section 3.1.5).
4. Set the discriminator to green (SYBR-I) fluorescence with a threshold of 150.
5. Set logarithmic amplification for all parameters.
6. Typical settings on our FACSort flow cytometer are as follows: FSC = E01, SSC = 450, FL1 = 550, FL2 = 650, and FL3 = 650.
7. Run the sample. The flow rate and the cell concentration must be adjusted to avoid coincidence. Typically, we analyze samples for 1 to 2 minutes at a delivery rate of 25 to 50 µL·min^{-1} and the number of events is kept below 1,000 per second by sample dilution, so the total number of recorded events is about 100,000.

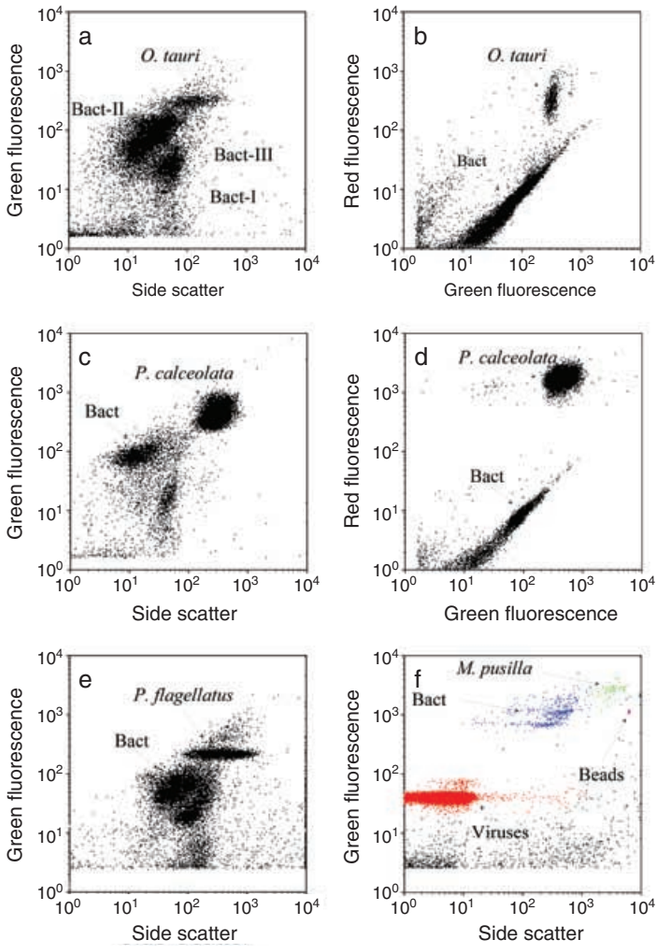
3.3.5. Data Analysis

The distribution of bacteria in cultures of *Ostreococcus tauri* Courties et Chrétiennot-Dinet (Fig. 17.2a,b) and *Pelagomonas calceolata* Andersen et Saunders (Fig. 17.2c,d) are illustrated, as well as the detection of the heterotrophic eukaryote *Picophagus flagellatus* Guillou et Chrétiennot-Dinet (Fig. 17.2e). In natural seawater samples and cultures, the use of SYBR-I allows the discrimination of two or three different bacteria clusters (Fig. 17.2a) that correspond to different taxonomic groups (Zubkov et al. 2001).

Some samples contain a lot of small particles and debris, which increase the level of background noise. This can induce coincidence or lead to the generation of large list-mode files. In such cases, the discriminator threshold must be increased to reduce the number of events seen by the flow instrument, or a "bitmap" window (nonrectangular region) can be defined that includes the population of bacteria so only the events in this area are recorded.

3.4. Analysis of Viral Infection

Because viruses can induce rapid decay of algal cultures, it is sometimes necessary to analyze cultures to evaluate the level of infection (see Chapter 22). The study of viroplankton initially required techniques like transmission electron microscopy (TEM), which are time consuming and allow the analysis of only a limited number of samples. During the 1990s, the use of nucleic acid–specific dyes detected by epifluorescence microscopy (EFM) improved our knowledge of viruses (Hennes and Suttle 1995) (see Chapter 22). More



Please make sure that arrows pointing to populations are visible in final figure.

FIGURE 17.2. Analysis of heterotrophic eukaryotes, bacteria, and viruses in algal cultures after staining with SYBR-I (green fluorescence). (See Table 17.1 for culture origin.) **(a,b)** Bacterial contaminants of *Ostreococcus tauri* (Prasinophyceae). Three populations of bacteria are visible. **(c,d)** Bacterial contaminants of *Pelagomonas calceolata* (Pelagophyceae). **(e)** *Picophagus flagellatus*, a heterotrophic Chrysophyceae, in co-culture with bacteria. **(f)** Detection of viruses in a *Micromonas pusilla* (Butcher) Manton et Parke (Prasinophyceae) culture. (Fluorescent 0.95-µm microspheres were added in the samples.)

recently, FCM has been successfully used for the analysis of viruses in solution, using the nucleic acid–specific dye SYBR-I (Marie et al. 1999).

The preparation of the samples for the analysis of viruses is similar to that of heterotrophs, although a certain number of precautions must be taken. No significant difference has been found for virus enumeration performed on samples fixed with formaldehyde, glutaraldehyde, or a mixture of both aldehydes. For virus samples that are freshly fixed (i.e., have not been frozen), or for recalcitrant material, it is necessary to heat the samples for 10 minutes at 80°C in the presence of a detergent such as Triton X-100 (0.1% final concentration). Because a large fraction of virus particles can pass through 0.2 µm pore-size filters, 0.2-µe-size filtered seawater cannot be used to dilute the samples. The best solution to minimize the background noise is to dilute samples in Tris-EDTA buffer (Tris 10 mM, EDTA 1 mM). Different buffers have been tested, but Tris-based buffers give the best results, probably because Tris has free amines that interact with aldehydes. Distilled water must be used as sheath fluid. Samples are then stained with SYBR-I at a dilution of 1/20,000 of the commercial solution.

Typical settings on a FACSort flow cytometer are as follows: FSC = E03, SSC = 600, FL1 = 600, FL2 = 650,

Remove 0.2-ue-size

This should read : ...through 0.2um pore-size filters,filtered seawater cannot be used...

and FL3 = 650. Discriminator is set on the green fluorescence (FL1) with a threshold value of about 100. Analysis must be performed with a suspension of about 2×10^5 to 2×10^6 viruses/mL (final concentration). To avoid generating large files, samples can be run for 1 or 2 minutes at a rate ranging from 10 to 30 $\mu L \cdot min^{-1}$.

Natural viroplankton displays a wide range of sizes, and these particles are often difficult to separate from background noise. However, viruses that contaminate cultures are usually simple to analyze (see Fig. 17.2f). Viruses are too small to be discriminated only by their SSC or FSC properties. Detection must, therefore, be performed using the green SYBR-I fluorescence (see Fig. 17.2f). Because FCM was not designed for the analysis of such small particles, care must be taken to obtain reliable data. If samples are too diluted, there is a loss in the emission signal of the nucleic acid–dye complex. If they are not diluted enough, coincidence occurs or the population of viruses is overlapped by background noise. For viruses, coincidence seems to occur at more than 600 events per second on a FACSort, that is, at a lower rate than for beads, bacteria, or small algae.

3.5. Counting Dead vs. Live Cells

It is sometimes necessary to evaluate the percentage of living and/or dead cells in a sample. Propidium iodide (PI) or SYTOX Green can penetrate into cells that have lost membrane integrity so that dead cells exhibit fluorescence. However, PI cannot be used with phytoplankton because its red fluorescence interferes with that of chlorophyll; SYTOX induces green fluorescence, which is more suitable (Veldhuis et al. 2001). Conversely, fluorescent dyes from the SYTO family (Molecular Probes), such as SYTO-9, or calcein-AM can penetrate into intact cells and induce live cells to fluoresce green (Brussaard et al. 2001).

4.0. MOLECULAR PROBES

Phytoplankton can be discriminated from other particles by FCM based on their natural scattering and fluorescence properties. However, these natural properties are not sufficient to separate lower level taxa (e.g., genera, species). Antibodies labeled with fluorescent markers have been used in this context (Peperzak et al. 2000), but their use remains limited because of the lack of specificity for polyclonal antibodies and the cost for developing monoclonal antibodies. Nucleic acid probes targeting ribosomal RNA (Amann et al. 1995) offer a much more flexible solution. Probes can be easily designed to target any phylogenetic level from the division to the species, and various probes are available for phytoplankton.

The probes used are generally oligonucleotides (15 to 30 bases). Different probe-labeling techniques are available. Probes may be directly labeled with a fluorochrome (Simon et al. 1995), or labeling may be indirect (Not et al. 2002). For indirect labeling, hybridization of the probes and labeling with the fluorochrome are realized in two steps, as in the tyramide signal amplification of fluorescent in situ hybridization (TSA-FISH) technique. Indirect labeling increases the intensity of fluorescence and thus raises the limit of detection and the signal/noise ratio (Not et al. 2002), which is critical for small cells. Recently, TSA-FISH has been successfully adapted for the identification and enumeration of phytoplankton cells by FCM (Biegala et al. 2003).

The most common fluorochrome used is fluorescein isothiocyanate (FITC) (excitation = 488 nm; emission = 525 nm), but other fluorochromes that have higher fluorescence yield, such as CY3 (excitation = 550 nm; emission = 570 nm) or CY5 (excitation = 650 nm; emission = 670 nm), are also suitable, provided that the flow cytometer can be set to the corresponding excitation and emission wavelengths. For phytoplankton, the combined use of FCM and molecular probes may be useful to assess culture identity or when cultures are not pure and it is difficult to distinguish the taxon of interest from the contaminants.

4.1. Probe Design and Labeling

A database of the oligonucleotide probes for cyanobacteria and protists is available at www.sb-roscoff.fr/Phyto/Databases/RNA_probes_introduction.php. Although probes have been designed against some of the major algal groups such as the Chlorophyta, Prymnesiophyceae (Simon et al. 1995, 2000), or some key genera such as *Phaeocystis* (Lange et al. 1996), considerable work remains to be done to cover all existing taxa. Probes can be designed from ribosomal DNA databases using a public domain software such as ARB (www.arb-home.de). Advice for the design of new taxa-specific probes is available in Amann et al. (1995). Probes may be purchased directly labeled, but cost may

be reduced by custom labeling of oligonucleotide probes with fluorochromes such as FITC or CY3 (Amann et al. 1995).

4.2. Cell Labeling

The cell labeling protocol was designed for the identification of cells but was not optimized for cell counting.

4.2.1. Materials

1. Hybridization oven set at 46°C
2. Microcentrifuge
3. Fixatives: formaldehyde (stock at 10%) (see Section 3.2.2) and ethanol
4. Hybridization buffer: 0.9 M NaCl, 20 mM Tris HCl (pH 7.8), 0.01 sodium dodecyl sulfate (SDS), 0% to 50% formamide. For every 1% increase in the concentration of formamide, the melting temperature (T_m) of the hybrid is reduced by 0.7°C. The percentage of formamide must be adapted for each probe to ensure a specific labeling (Amann et al. 1995).
5. Wash buffer: 0.028 to 0.9 mM NaCl, 5 mM EDTA, 0.01% SDS, 20 mM Tris-HCl, pH 7.5. The concentration of NaCl must be adapted for each probe so the stringency of the washing buffer is equivalent to the stringency of the hybridization buffer.
6. Resuspension buffer: PBS

4.2.2. Procedure

1. Cell fixation and permeabilization: Samples (5 mL of cell suspension at 10^5 cells/mL) should be fixed with formaldehyde (1% final concentration; glutaraldehyde should not be used because it prevents probe binding) and stored at 4°C for 1 hour. Cells are then pelleted (3 minutes, 4000 × g) and resuspended in a cold (–80°C) mixture (70:30, v/v) of ethanol and PBS (500 µL).
2. Hybridization: Cells are then pelleted again in an Eppendorf type of tube and resuspended in 20 to 100 µL of hybridization buffer. A 20-µL aliquot of the cell suspension is then incubated at 46°C for 3 hours with the oligonucleotide probe (2.5 ng·µL^{-1}). An aliquot without probe incubated in the same condition can serve as a negative control for autofluorescence. Hybridization is stopped by the addition of 1 mL of cold PBS at a pH of 9.0. Samples are then stored at 4°C until analysis with FCM.

4.3. Analysis of Hybridized Cells with Flow Cytometry

First calibrate the sample flow rate (see Section 3.1.5). Set the discriminator to green fluorescence (if the fluorochrome used is FITC). Set all parameters on logarithmic amplification (Fig. 17.3). Events are recorded in list mode. The flow rate and the cell concentration must be adjusted to avoid coincidence. Typically, we analyze samples for 1 to 2 minutes at a delivery rate of 25 to 50 µL·min^{-1} and the number of events is kept at less than 1,000 per second (by diluting samples that are too concentrated).

4.4. Limits and Troubleshooting

The protocol used for whole-cell hybridization involves several centrifugation steps that generally lead to the

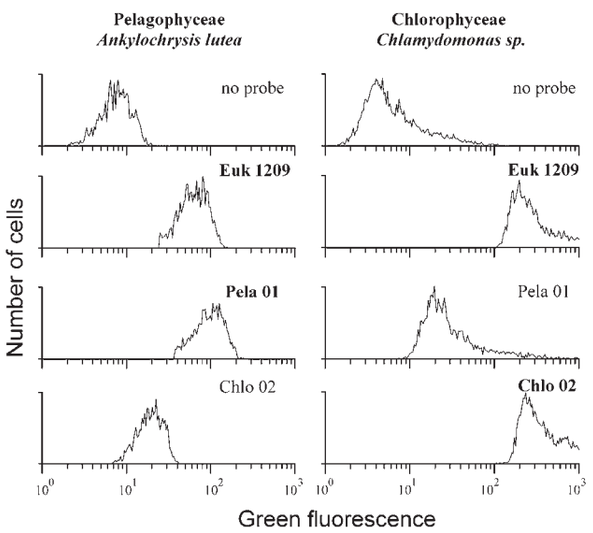


FIGURE 17.3. Flow cytometric analysis of fluorescence signals for whole-cell hybridization of exponentially growing *Ankylochrysis lutea* (van der Veer) Billard in Honda and Inouye (Pelagophyceae) and *Chlamydomonas* sp. (Chlorophyceae) with fluorescein isothiocyanate (FITC)–monolabeled probes. Both species are nanoplanktonic (diameter 7 to 10 µm). For each species, the distribution of green fluorescence intensity per cell is plotted on a three-decade log scale. The intensity of green fluorescence per cell was measured for cells incubated without probe (green autofluorescence, no probe) and in the presence of a general eukaryotic probe (Euk1209), a Pelagophyceae-specific probe (Pela 01), and a Chlorophyta-specific probe (Chlo 02). (Modified from Simon et al. 2000.)

formation of cell clumps and/or to cell losses due to the adhesion of the cells to the surfaces of the tubes. Such losses can be reduced by treating the tubes with surfactants, by adding surfactants to the cells, and by sonication (Biegala et al. 2003).

The protocol based on monolabeled probes is quite short and simple. It is best suited, however, for large microplankton and nanophytoplankton cells. For smaller cells (picoplankton), the intensity of fluorescence conferred by the probes is, in general, not sufficient to distinguish target from nontarget cells, especially if cultures are not in exponential growth phase. In this case, enzymatic amplification (TSA) may be needed because it increases 20 to 40 times the fluorescence intensity of target cells (Schönhuber et al. 1997, Biegala et al. 2003).

5.0. Conclusion

FCM is now a well-established technique to analyze phytoplankton both in the field and in culture. There has been very little change in instrument design, although novel flow cytometers such as the FACSAria (released in late 2002) that combine small footprint, high sensitivity, and very fast sorting could prove ideal for both culture (in particular for the isolation of novel strains) and field work. Most progress has come from the development of novel fluorochromes such as SYBR Green that allow routine and enumeration analysis of bacteria and viruses. The application of molecular probes that permit accurate cell identification will probably develop considerably soon as the number of available algal sequences, a prerequisite for probe design, increases.

6.0. Acknowledgments

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