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What Flow Cytometry Can Tell Us About Marine Micro-Organisms – Current Status and Future Applications

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1. Introduction

Born in the field of medicine for the analysis of mammalian cell DNA, flow cytometry (FCM) was first used in microbiology studies in the late 1970s thanks to optical improvements and the development of new fluorochromes (Steen & Lindmo, 1979; Steen, 1986). Its initial applications in clinical microbiology are dated to the 1980s (Steen & Boyne, 1981; Ingram et al., 1982; Martinez et al., 1982; Steen 1982; Mansour et al., 1985), and, by the end of that decade, FCM had also become popular in environmental microbiology (Burkill, 1987; Burkill et al., 1990; Yentsch et al., 1983; Yentsch & Pomponi, 1986; Yentsch & Horan, 1989; Phinney & Cucci, 1989). Today, it is a powerful and commonly used tool for the study of aquatic micro-organisms. FCM has thus become a precise alternative to microscopic counts, increasing the number of both the micro-organisms detected and the samples that can be analyzed. The advantages of FCM include single-cell detection, rapid analysis (5000 cells per second or more), the generation of multiple parameters, a high degree of accuracy and statistically relevant data sets.

The significance of flow cytometry can be summarized as the measure (-metry) of the optical properties of cells (cyto-) transported by a liquid sheath (flow) to a light source excitation (most often a laser) (Shapiro, 2003).

FCM facilitates single cell analyses of both cell suspension, such as eukariotic and prokariotic cells, and “non cellular” suspension, such as microbeads, nuclei, mitochondria and chromosomes.

A typical flow cytometer is formed by different units: the light source, the flow cell, the hydraulic fluidic system, several optical filters, a group of photodiodes or photomultiplier tubes and, finally, a data processing unit (Veal et al., 2000; Longobardi, 2001; Shapiro, 2003; Robinson, 2004; Diaz et al., 2010).

In a flow cytometer, individual cells pass in a single file within a hydrodynamically focused fluid stream. Single cells are centered in the stream so that they intercept an excitation source, meaning that scatter and/or fluorescence signals can be collected and optically separated by dichroic filters and detectors. The data collected are then converted into digital information. Finally, software displays data as events along with their relative statistics.

The light scattering properties are detected as FALS (forward angle light scatter) and RALS (right angle light scatter). FALS, collected in the same direction as the incident light (0-13° conic angle with respect to the incident point of the laser), is measured in the plane of the laser beam and provides information on cell size, while RALS is usually measured at 90° (70-110° conic angle) to the beam and provides data on cell granularity or the internal structure of the cell (Hewitt & Nebe-Von-Caron, 2004) (Fig. 1).

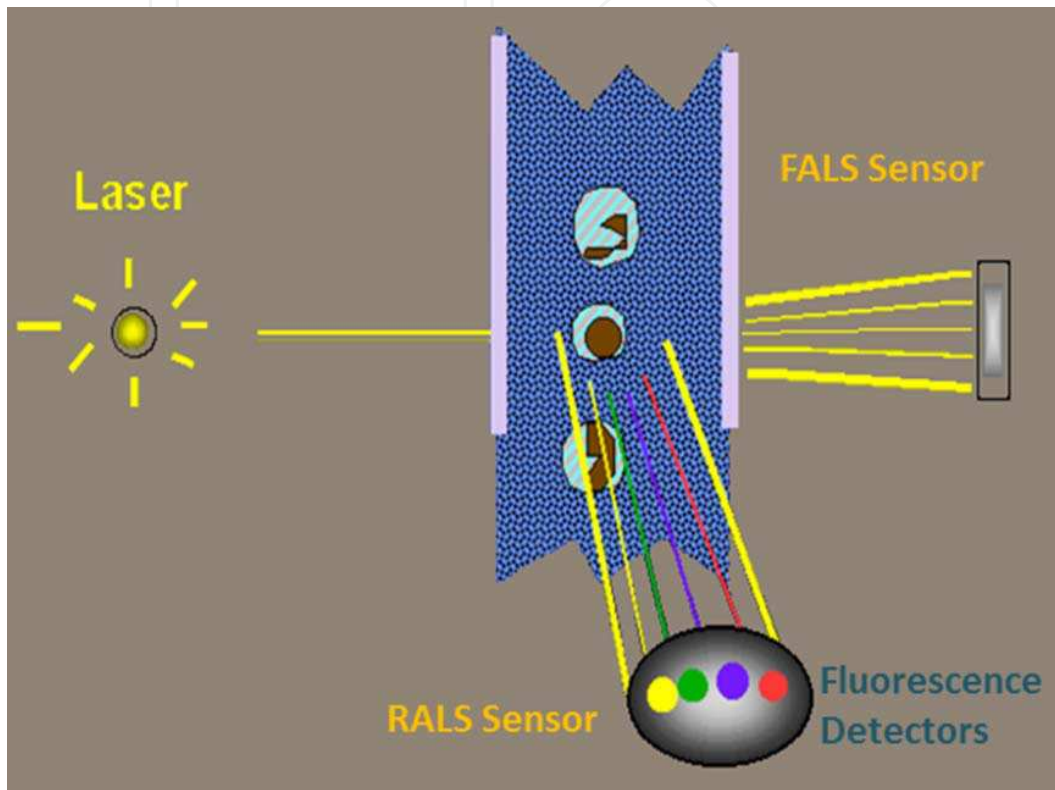


Fig. 1. Light fractions scattered and fluorescence by an excited single cell

Together with the FALS and RALS data, fluorescent information can also be collected, which includes signals from autofluorescence or induced fluorescence.

Each single value can be amplified, and stored events are commonly represented in a monoparametric histogram or biparametric dot plot. One-parameter histograms represent the number of cells or particles per channel (y-axis) versus the scattering or fluorescence intensity (x-axis). Dot plots are the most common graphic representations of the relative distribution of different cell populations.

Regions and gates can be made to better separate and analyze populations of interest. Furthermore, on the basis that the dyes used to stain cells have overlapping emission spectra, the compensation is normally made to reduce interference.

While basic instruments may only permit the simultaneous collection of two or three fluorescence signals, the more complex and expensive research instruments mean that it is possible to obtain more than 14 parameters (Winson & Davey, 2000; Chattopadhyay et al., 2008) depending on the laser equipment utilized. Selection of the lasers will depend on the range of wavelengths needed for the excitation of the selected fluorochromes.

Some flow cytometers have the ability to physically separate different sub-populations of interest (cell sorting) depending on their cytometric characteristics (stream-in-air), thus permitting the recovery and purification of cell subsets from a mixed population for further applications (Bergquist et al., 2009; Davey, 2010).

In natural samples in particular, a very important advantage of FCM is the opportunity to analyze micro-organisms with minimal pre-treatment and without the need for cultivation steps, also taking into account that the most of natural bacteria are resistant to cultivation (Fig. 2). Furthermore, FCM is particularly well-suited for the investigation of natural picoplankton. This is because of their small size ($<2\ \mu\text{m}$; Sierbuth, 1978), which renders the analysis thereof difficult by more traditional methods. Particularly due to the rapidity with which data can be obtained, flow cytometry has been routinely used over the last few decades for the analysis of different types of micro-organisms in marine samples (Porter et al., 1997; Yentsch & Yentsch, 2008; Vives-Rego et al., 2000; Wang et al., 2010). It is now commonly accepted as a reference technique in oceanography.

Knowledge of seawater microbial diversity is important for understanding community structure and patterns of distribution. In the ocean water column, organisms $<200\ \mu\text{m}$ include a variety of taxa, such as free viruses, autotrophic bacteria (cyanobacteria, which include the group known formerly as prochlorophytes), heterotrophic bacteria, protozoa (flagellates and ciliates) and small metazoans (Legendre et al., 2001), all of which have different morphological, ecological and physiological characteristics.

Heterotrophic and autotrophic bacteria, viruses and autotrophic picoeukaryotes represent marine picoplankton ($2-0.2\ \mu\text{m}$), while the larger fraction of micro-organisms is divided into nano-plankton ($20-2\ \mu\text{m}$) and micro-plankton ($200-20\ \mu\text{m}$).

Among these taxa, bacteria are very important because they play a crucial role in most biogeochemical cycles in marine ecosystems (Fenchel, 1988), taking part in the decomposition of organic matter and the cycling of nutrients. Bacteria are also an important source of food for a variety of marine organisms (Das et al., 2006), and their activity has a major impact on ecosystem metabolism and function. Both autotroph and heterotroph micro-organisms constitute two fundamental functional units in ecosystems, where the former generally dominate eutrophic systems and the latter generally dominate oligotrophic systems (Dortch & Postel, 1989; Gasol et al., 1997). An extensive body of literature has documented the great importance of the activity of algae in terms of the size of picoplankton in the global primary production of aquatic ecosystems (Craig, 1985; Stockner & Antia, 1986; Stockner, 1988; Callieri & Stockner, 2002). Picocyanobacteria are a diverse and widespread group of photosynthetic prokaryotes and belong to the main group of primary producers (Castenholz & Waterbury, 1989; Rippka, 1988). Picoeukaryotes, meanwhile, are a diverse group that is widely distributed in the marine environment, and they have a fundamental role in aquatic ecosystems because of their high productivity. Like bacteria, marine viruses are thought to play important roles in global and small-scale biogeochemical cycling. They are also believed to influence community structure and affect bloom termination, gene transfer, and the evolution of aquatic organisms. Viruses are the most numerous 'lifeforms' in aquatic systems, being about 15 times more abundant than total of bacteria and archaea. Data from literature seem to indicate that the abundance of marine viruses is linked to the abundance of their hosts, so that changes in the prokaryotic host populations will affect viral abundance (Danovaro et al., 2011).

Given that the vast majority of the biomass [organic carbon (OC)] in oceans consists of micro-organisms, it is expected that viruses and other prokaryotic and eukaryotic micro-organisms will play important roles as agents and recipients of global climate change (Danovaro et al., 2011).

Accordingly, the accurate determination of micro-organism abundance, biomass and activity is essential for understanding the aquatic ecosystem. Consequently, the aim of this review is to provide a general overview of the applications of flow cytometric techniques to studies in marine microbiology.

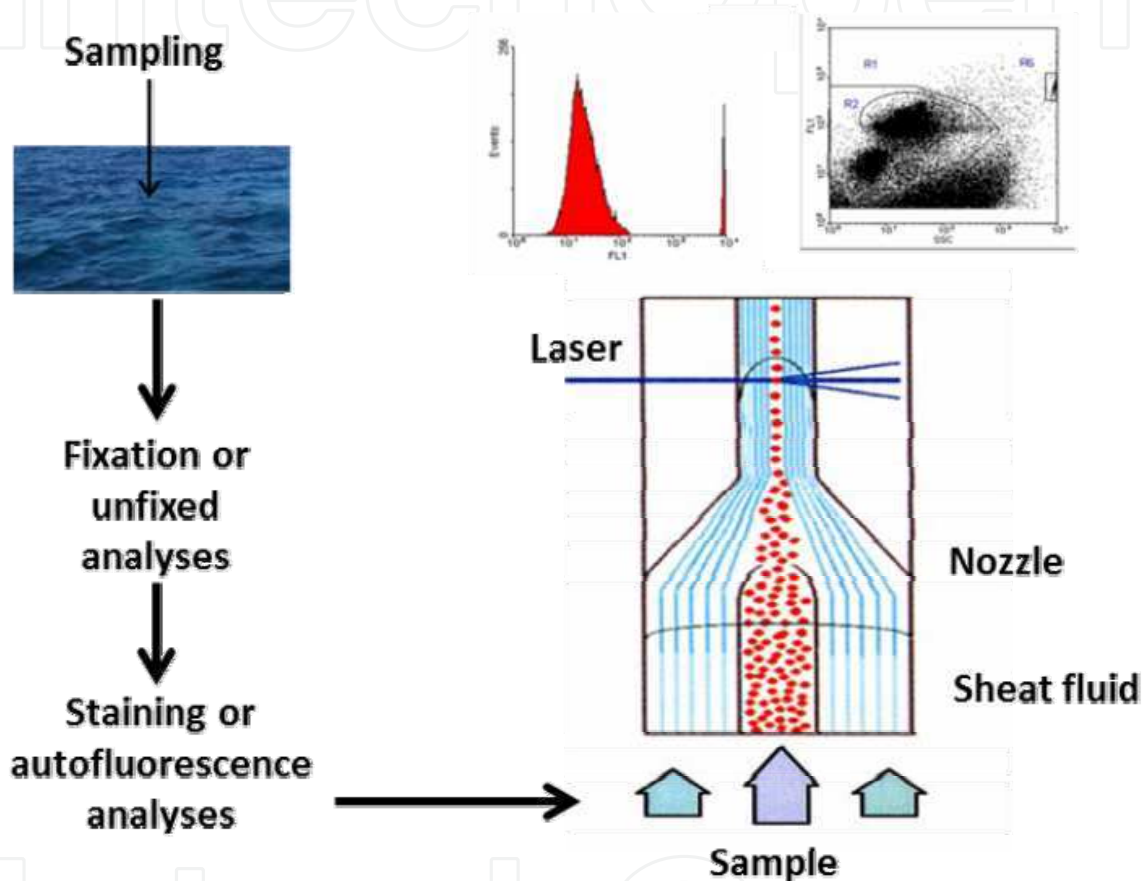


Fig. 2. Scheme of the main step: from sampling to the flow cytometric data

2. Autofluorescence analyses

The opportunity to measure fluorescence by flow cytometry is a key aspect in microbial ecology, since light-scattering characteristics alone are not usually enough to uncover much detail about either the taxonomic affinities or the physiological status of micro-organisms (Davey & Kell, 1996). Phytoplanktonic micro-organisms are an ideal subject for flow cytometric analysis because they are naturally autofluorescent by virtue of their complement of photosynthetic pigments. Most of these pigments can absorb the blue light of the 488 nm line of an Argon laser, meaning that they can be distinguished because of their unique fluorescence emission spectra. Standard filter arrangements in a dual laser system (488 and 633 nm lasers) can distinguish and quantify chlorophyll fluorescence (red ex, em > 630 nm), phycoerythrin (PE) fluorescence (blue ex, em 570 nm) and phycocyanine (PC)

fluorescence (red ex, em >630 nm) (Callieri, 1996; Callieri & Stockner, 2002). Accordingly, flow cytometric data collected from natural phytoplankton assemblages can be used to identify and classify phytoplankton based on scattering characteristics (size) and fluorescence (pigmentation) (for an example, see Figure 3).

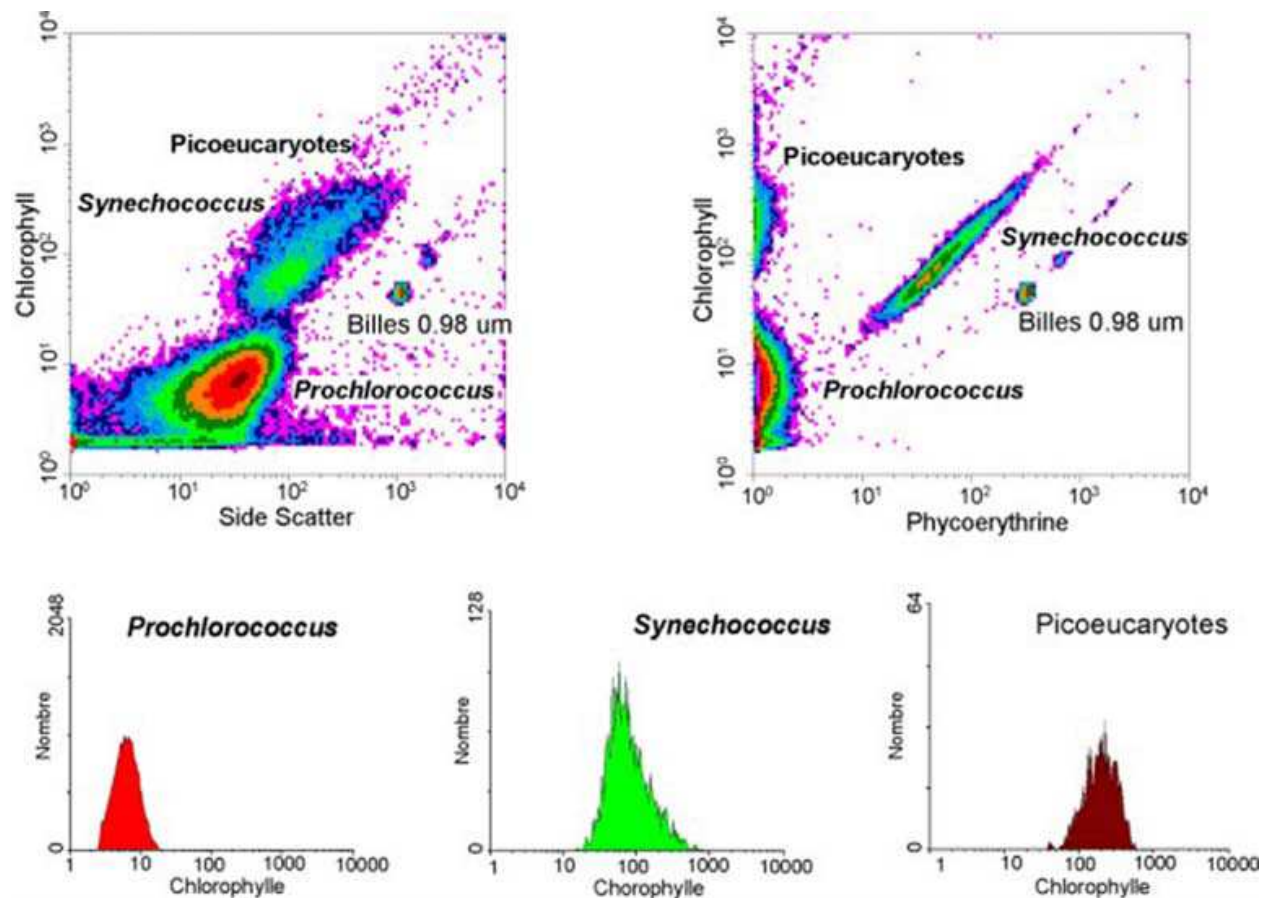


Fig. 3. Autotrophic picoplankton by flow cytometry. Image provided by Daniel Vaultot, CNRS, Station Biologique de Roscoff, France

The use of flow cytometry in aquatic microbial ecology increased our knowledge of the structure of phytoplankton assemblages (Olson et al., 1993). Based on flow cytometric analyses, phytoplankton are typically divided into Cyanobacteria (*Synechococcus*, *Prochlorococcus*) and small (pico-) and large eukaryotes. They are also able to define the distributions and dynamics of each group (e.g. Olson et al., 1990; Campbell et al., 1994; Li, 1995; Lindell & Post, 1995; Partensky et al., 1996; Campbell et al., 1997). The phycoerythrin (PE)-containing *Synechococcus* can be distinguished from *Prochlorococcus*, which are similar in size, but do not produce the 'orange' fluorescence that is typical of phycoerythrin. Eukaryotic phytoplankton, meanwhile, are distinguished based on their larger scatter and chlorophyll fluorescence signals.

The application of flow cytometry to marine samples led to the discovery of a primitive, prokaryotic picocyanobacteria of the Prochlorophyta group (Chisholm et al., 1988), with divinyl chlorophyll-*a* (chl-*a*) as the principal light-harvesting pigment and divinyl chlorophyll *b* (chl-*b*), zeaxanthin, alfa-carotene and a chl-*c*-like pigment as the main accessory pigments (Goericke & Repeta, 1993).

In some cases, the larger cells may be further distinguished based on their scattering characteristics (coccolithophorids) or the presence of both PE and chlorophyll (cryptophytes) (Olson et al., 1989; Collier & Campell, 1999).

Many authors have reported the distributions and dynamics of each photosynthetic group in the water column in different marine environments (Li, 1995; Campbell & Vaultot, 1993; Vaultot & Marie, 1999). As both cyanobacteria and picoeukaryotes are widely distributed in the marine environment, they play a fundamental role in aquatic ecosystems because of their high productivity.

Cyanobacteria are a diverse group of unicellular and multicellular photosynthetic prokaryotes (Castenholz & Waterbury, 1989; Rippka, 1988); they are often referred to as blue-green algae, even though it is now known that they are not related to any of the other algal groups.

Seasonal patterns of picoplankton abundance have been observed in many studies, revealing a strong relation with water temperature. A study on picophytoplankton populations conducted by Alonso and colleagues (2007) in north-west Mediterranean coastal waters showed a peak during the winter for picoeukaryotes, and peaks in spring and summer for *Synechococcus*. Meanwhile, *Prochlorococcus* was more abundant from September to January.

Zubkov et al. (2000) found that *Prochlorococcus* spp. were the dominant cyanobacteria in the northern and southern Atlantic gyres and the equatorial region, giving way to *Synechococcus* spp. in cooler waters. *Synechococcus* cells also become more numerous and even reach blooming densities near the tropical region affected by the Mauritanian upwelling. Finally, the concentrations of Picoeukaryotes tend to be at their height in temperate waters.

The small coccoid prochlorophyte species, *Prochlorococcus marinus*, were found to be abundant in the North Atlantic (Veldhuis & Kraay, 1990), the tropical and subtropical Pacific Ocean (Campbell et al., 1994), the Mediterranean (Vaultot et al., 1990) and in the Red Sea (Veldhuis & Kraay, 1993).

A monitoring study conducted in the Central Adriatic Sea (authors' unpublished results) revealed the presence of cyanobacteria, pico-eukariotes and nano-plankton (Fig. 4), while prochlorococcus were absent throughout the entire year.

Other authors (Marie et al., 2006) have underlined the similarity of the distribution of picoeukariotes to that of total chlorophyll-*a* in the Mediterranean Sea, with maximum concentrations reaching around 2×10^2 cell/ml.

Shi and co-authors (2009) have characterized photosynthetic picoeukaryote populations by flow cytometry in samples collected in the south-east Pacific Ocean, registering abundances from 6×10^2 to $3,7 \times 10^4$ cell/ml. Meanwhile, 18S rRNA gene clone libraries were constructed after flow sorting.

3. Total cell counting

Total cell counting is one of the most important functions of flow cytometry. The rapidity and accuracy of the data obtained overcome the limitations (e.g. time-consuming, subjectiveness linked to the operator) of other techniques such as epifluorescence microscopy.

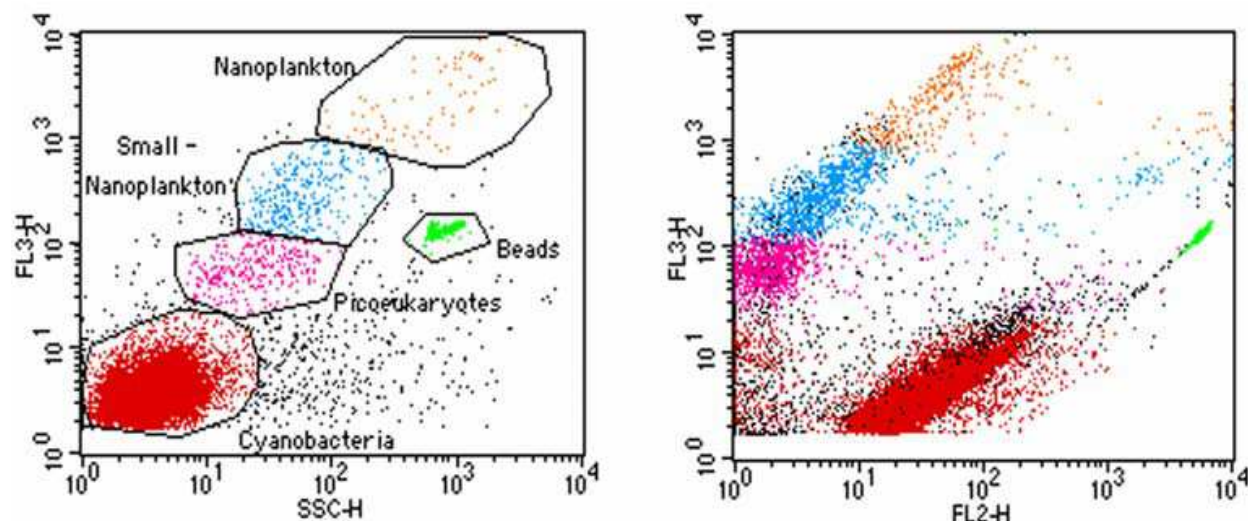


Fig. 4. Example of dot plot showing autothrophic micro-organisms in the Central Adriatic Sea

Flow cytometric countings can be determined with high statistical confidence. Some flow cytometers are equipped with volumetric counting hardware that enables the absolute cell count to be made through a predefined volume. Yet most cytometers do not have this equipment, and, in such circumstances, cell counting is performed by: 1) the addition of synthetic counting beads; 2) the calibration of the flow rate; and 3) weighing the sample before and after conducting any analyses. The addition of precounted beads is now also possible with commercially available beads for “absolute counting” (e.g. Coulter Flowcount beads, Cytocount counting beads, DakoCytomation, and Trucount tubes by Becton Dickinson). Accompanying datasheets provide the exact number per μl of beads to use (Cantineaux et al., 1993; Brando et al., 2000, Manti et al., 2008). The number of cells per microlitre is obtained by the following formula:

$$\text{Number of cells} = (\text{cell events}/\text{beads events}) * (\text{bead number}/\mu\text{l}) * \text{Dilution Factor}$$

Other methods have proposed the use of standard beads (Polysciences latex beads), as well described by Gasol and del Giorgio in 2000. Briefly, the beads have to be counted every day and must be sonicated to avoid aggregation.

Flow rate calibration can be performed by weighing a tube containing water, processing various volumes, estimating the time needed for each volume to go through and then reweighing the tube. This makes it possible to calculate the mean of the flow rate per minute (Paul, 2001).

The third method is comprised of estimating volume differences: the volume of the sample is measured by a micro-pipette before and after the run through the flow cytometer. However, these measurements are not as precise as those obtained using weight differences.

The flow cytometric counting of non-fluorescent cells is possible through the staining of nucleic acids (or other cellular components) with fluorescent dyes. There are commercially available probes that allow the direct counting of marine bacteria, such as, for example, the nucleic acid dyes Syto-9, Syto-13 (Lebaron et al., 1998; Vives-Rego et al., 1999), SYBR Green I

and II (Lebaron et al., 1998; Marie et al., 1997), Pico Green (Sieracki et al., 1999; Marie et al., 1996), TO-PRO 1, and TOTO-1 (Li et al., 1995). Their use permits the separation of cells from abiotic particles and background signals in a water sample. An initial selection step is represented by the threshold, usually in the typical channel fluorescence (e.g. green fluorescence when SYBR Green I is used). In order to better visualize cells, a dot plot containing the scatter signal (FCS or SSC) against fluorescence signals (green or red fluorescence) is recommended.

Figure 5 shows a marine sample stained with SYBR Green I and analyzed by a FACScalibur flow cytometer (Becton Dickinson).

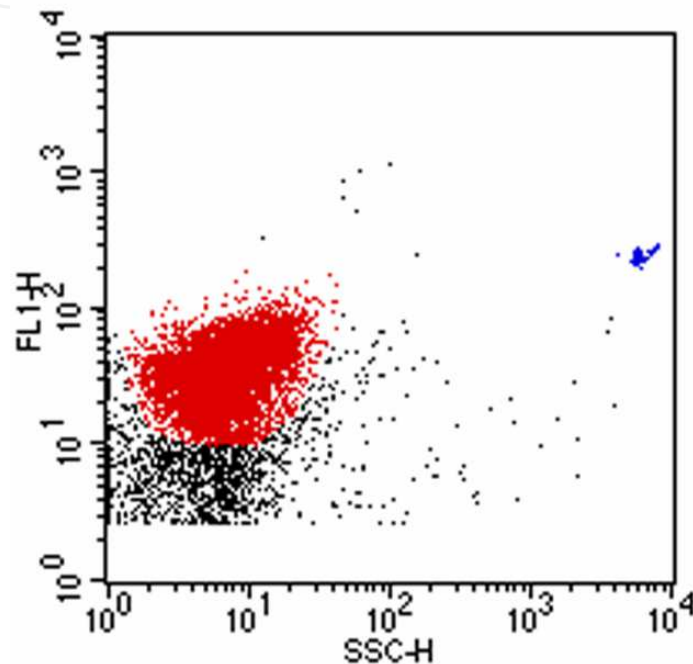


Fig. 5. Dot plot SSC vs. FL1 showing bacteria population stained with SYBR Green I

The affinity of the cyanine dyes, TOTO-1 and YOYO-1, and their monomeric equivalents, YO-PRO-1 and TO-PRO-1, decreases significantly with increasing ionic strength, meaning that their use is inappropriate for the analysis of seawater samples (Marie et al., 1996). Other dyes, such as the SYBR Greens I and II, SYTOX Green and the SYTO family, are less dependent on medium composition and can therefore be used to count marine bacteria (Marie et al., 1999b; Lebaron et al., 1998). As SYBR Green I (SYBR-I) has a very high fluorescence yield, its use is recommended for enumerating bacteria from marine samples (Table 1).

Zubkov and colleagues (2000) determined the total number of picoplankton in marine samples using the fluorochromes TOTO-1 iodide and SYBR Green I. These dyes bind strongly to nucleic acids, but SYBR Green 1 penetrates cell membranes, whereas it is necessary to use detergent to aid the penetration of TOTO-1 into cells (Li et al., 1995; Marie, et al., 1996; Marie, et al., 1997). The number of bacteria found in subsamples stained by SYBR Green I were the same as the TOTO-1 counts for the same samples. The results obtained were evidence that the intensity of fluorescence with SYBR Green 1 was greater than with TOTO-1; at the same time, SYBR Green I improved the recognition of cells with low

staining, helping the separation of their signal from the background noise level. This confirms that SYBR Green is more adaptable for the analysis of marine bacteria.

In a study reported by Gregori and colleagues (2001), SYBR Green II expresses a higher selectivity for RNA, with a quantum yield of 0.54, while also maintaining a strong affinity for double-stranded DNA, with a quantum yield of 0.36, about half that of SYBR Green I.

In 1999, Gasol and co-workers published a study on a comparison of different nucleic acid dyes and techniques, such as flow cytometric and epifluorescence microscopy. They found that Syto13 counts correlate well with DAPI and SYBR Green I counts, generating slightly lower fluorescence yields than those of the other fluorochromes. This was particularly true in seawater, meaning that, without dismissing the potential of other stains, this fluorochrome is a viable alternative to the total counting of marine planktonic bacteria.

Alonso and co-authors published (Alonso et al., 2007) a monthly study in Blanes Bay, which revealed that the abundance of heterotrophic prokaryotes (ranging from $0,5 \times 10^6$ to $1,5 \times 10^6$ cell/ml) roughly followed the pattern of Chl-a.

In general, heterotrophic bacterial abundances followed the distribution of total picophytoplankton, revealing seasonal changes in their distribution, as reported for the subtropical northern Pacific Ocean (Campbell & Vaulot, 1993; Zubkov et al., 2000).

Lasternas and colleagues (2010) produced results from a cruise on the Mediterranean Sea during the summer of 2006. The composition and viability of pelagic communities were studied in relation to nutrient regimes and hydrological conditions. It was found that the picoplankton fraction dominated the pelagic community across the study region, with bacterioplankton being the most abundant (mean \pm SE $7,73 \pm 0,39 \times 10^5$ cells/ml) component.

4. Detection of viruses

Viruses control microbial and phytoplankton community succession dynamics (Fuhrman & Suttle, 1993; Suttle, 2000; Castberg et al., 2001; Weinbauer, 2004; Weinbauer & Rassoulzadegan, 2004; Sawstrom et al., 2007; Rohwer & Thurber, 2009). They also play an important role in nutrient (Wilhelm & Suttle, 1999) and biogeochemical cycling (Fuhrman, 1999; Mathias et al., 2003; Wang, et al., 2010).

Initial studies of viruses in aquatic environments were performed using either transmission electron microscopy (TEM) (Bergh et al., 1989; Borsheim et al., 1990; Sime-Ngando et al., 1996; Field, 1982) or epifluorescence microscopy (EFM) (Hennes & Suttle, 1995; Chen et al., 2001; Danovaro et al., 2008). The use of EFM combined with the development of a variety of highly fluorescent nucleic acid specific dyes soon became the accepted study method, because it involved faster and less expensive technology. Nowadays, viruses (especially bacteriophages) are still typically counted by EFM using fluorochromes such as SYBR Green I, SYBR Green II, SYBR Gold or Yo- Pro I (Xenopoulos & Bird, 1997; Marie et al., 1999a,b; Shopov et al., 2000; Hewson et al., 2001a,b,c; Chen et al., 2001; Middelboe et al., 2003; Wen et al., 2004; Duhamel & Jacquet, 2006). These techniques are selective for viruses that are infectious to a specific host, but they are very time-consuming.

In 1999, however, Marie and colleagues (Marie et al., 1999a,b) successfully proposed the use of flow cytometry for the analysis of viruses in the water column. Other authors then

applied FCM to virus studies (Marie et al., 1999a,b; Brussaard et al., 2000; Chen et al., 2001; Jacquet et al., 2002a,b).

The protocol proposed by Marie and colleagues in 1999 included the use of SYBR Green I to stain virus nucleic acids. This protocol was revised and optimized by Brussaard in 2004.

Viruses are too small in particle size (less than 0.5 micron) to be discriminated solely on the basis of their light scatter properties using the standard, commercially available, benchtop flow cytometers. As most flow cytometers are not designed for the analysis of these small and abundant particles, attention to detail must be paid to obtain high quality data. It is, therefore, crucial to determine the level of background noise with the use of an adequate negative control such as a 0.2 µm pore-size filtered liquid of a comparable composition.

Brussaard (2004) has shown that a variety of viruses of different morphologies and genome sizes could be detected by flow cytometry. Indeed, flow cytometry (FCM) data suggested that two virus groups (V-I and V-II) were present in natural water samples (Marie et al., 1999; Wang et al., 2010).

In their research, Wang et al. (2010) revealed a viral abundance ranging from 7.06×10^6 VLP ml⁻¹ to 5.16×10^7 VLP ml⁻¹, with the average being 2.47×10^7 VLP ml⁻¹. The V-II group was the dominant viroplankton, and had lower DNA compositions than the V-I group.

5. DNA content

The use of nucleic acid dyes for the detection of bacterioplankton cells revealed a tendency to cluster into distinct fractions based on differences in individual cell fluorescence (related to the nucleic acid content) and side and forward light scatter signals. There were at least two major fractions: cells with a high nucleic acid content (HNA cells) and cells with a low nucleic acid content (LNA cells) (Robertson & Button, 1989; Li et al., 1995; Marie et al., 1997; Gasol et al., 1999; Troussellier et al., 1999; Zubkov et al., 2001; Lebaron et al., 2001; Sherr et al., 2006) (Fig. 6). In a recent study, Bouvier and co-authors (2007) underlined that despite the large presence of these clusters in aquatic ecosystems (fresh to salt water, eutrophic to oligotrophic environments), there is still no consensus among scientists about their ecological significance.

The results obtained by Bouvier and others (Bouvier et al., 2007) support the notion that it is more likely that the existence of these two fractions in almost all of the bacterioplankton assemblages is the result of complex processes involving both the passage of cells from one fraction to another as well as bacterial groups that are characteristic of either HNA or LNA fractions.

The findings by Zubkov et al., (2007), which were based on the results of fluorescence *in situ* hybridization, revealed that 60% of heterotrophic sorted bacteria, with low nucleic acid content, were comprised of SAR11 clade cells.

The SAR11 clade has the smallest genome size among free-living bacteria (Giovannoni et al., 2005), and they are also the most abundant class of the bacterial ribosomal RNA genes detected in seawater DNA by gene cloning.

Many authors have presented data about the presence of HNA and LNA, not only in marine environments, but also in freshwater (Boi et al., in prep.) and in lakes (Stenuite et al., 2009).

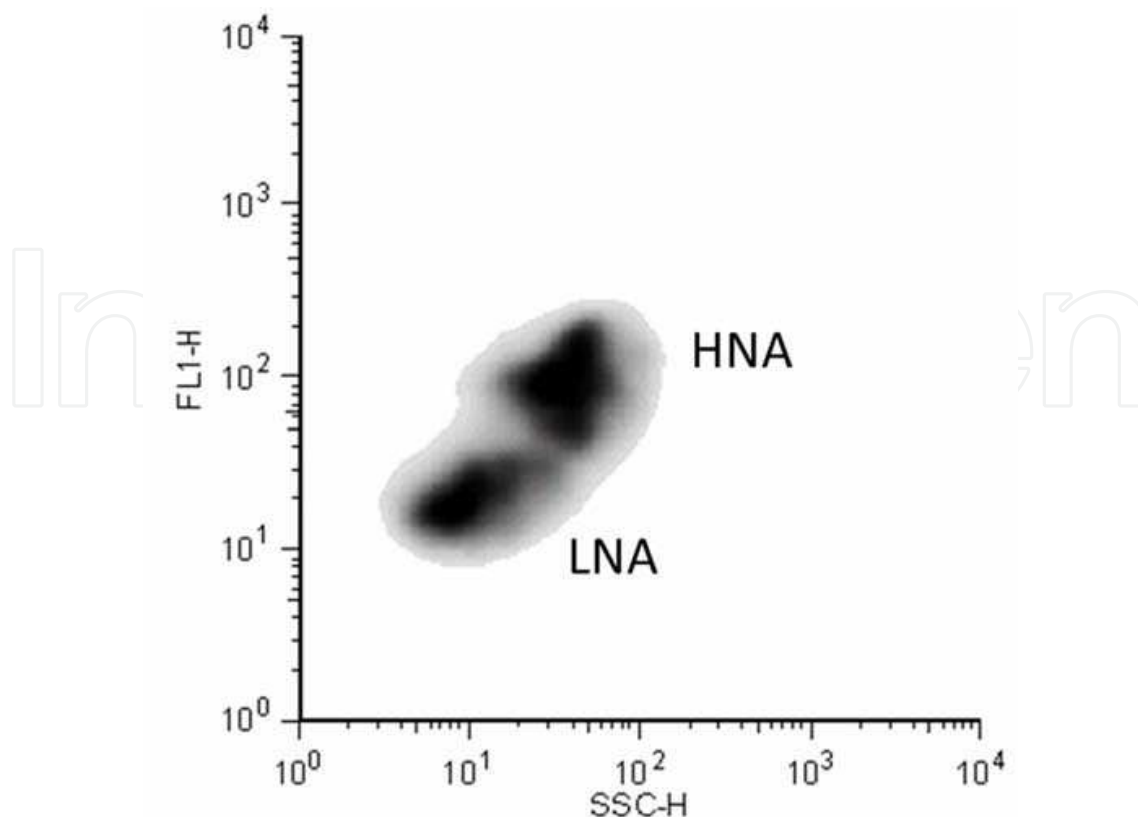


Fig. 6. Dot plot SSC vs. FL1 showing HNA and LNA cells stained with SYBR Green I

6. Physiological states

There is a wide and extensive variety of stains used in combination with FCM, with different degrees of specificity (Collier & Campell, 1999). Numerous classifications are available according to several criteria (Davey & Kell, 1996; Vives -Rego et al., 2000; Shapiro, 2000).

The most valuable source lists on fluorescent probes for flow cytometry are the *Handbook of Fluorescent Probes and Research Chemicals* (Haugland, 1996) and the catalogue of Molecular Probes, Inc. (Eugene, OR, USA; www.invitrogen.com). The current edition, which is the 11th, lists a range of dyes with different spectral characteristics and high specificities for nucleic acids.

Some fluorochromes bind specifically to cell molecules (nucleic acids, proteins and lipids) while increasing their fluorescence. Others accumulate selectively in cell compartments, or modify their properties through specific biochemical reactions in response to changes in the environment, such as pH, membrane polarization (cyanines, oxonols) or enzymatic activity (fluorogenic substrates) (Fig. 7).

A number of commercial kits are available which allow microbiologists to enumerate and determine physiological states and Gram status (Davey et al., 1999; Haugland et al., 1996; Winson & Davey, 2000).

Knowledge of the living/non-living and active/inactive states of cell populations is fundamental to understanding the role and importance of micro-organisms in natural ecosystems. Several probes, or a combination thereof, have been used to assess bacteria

physiological states (Lebaron et al., 1998; Joux & Lebaron, 2000; Gregori et al., 2001). Among others, an interesting application of FCM in microbiology is the determination of viability, even if this is one of the most fundamental properties of a cell that is difficult to define and measure.

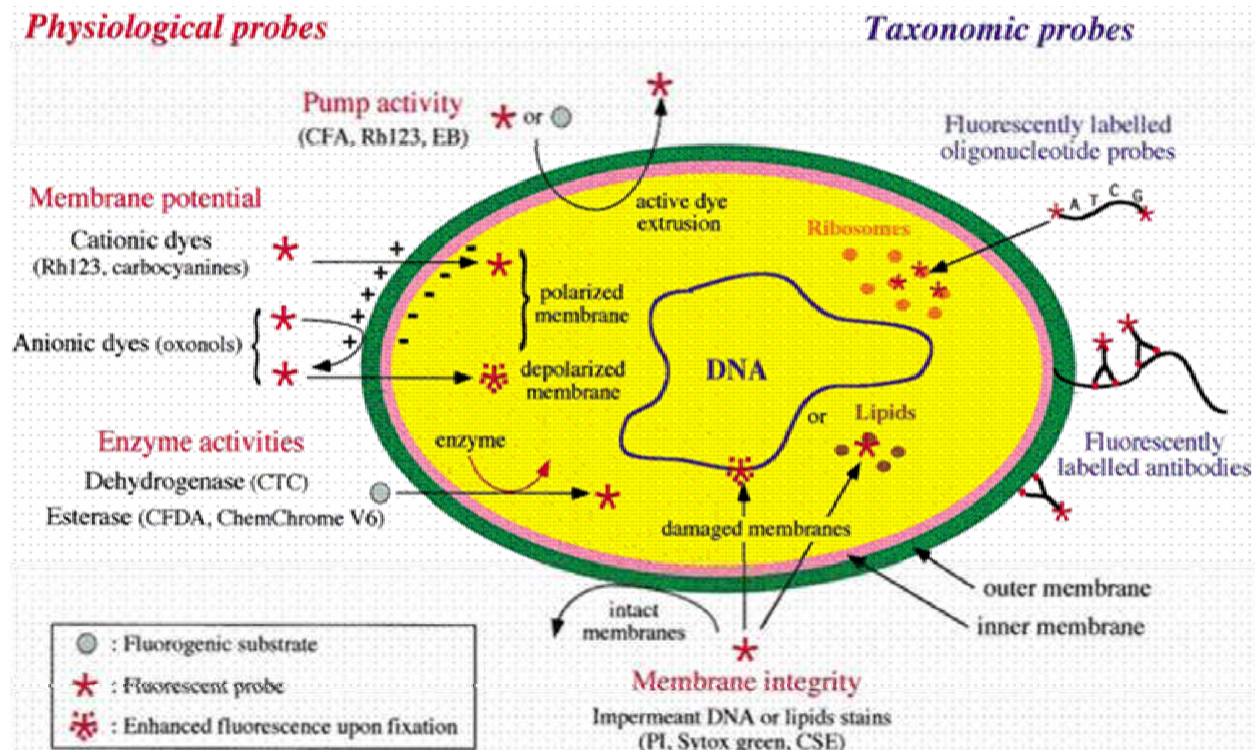


Fig. 7. Different cellular target sites for physiological and taxonomic fluorescent dyes from Joux & Lebaron, 2000

Many approaches are based on membrane integrity, such as the Life/Dead kits (e.g. the LIVE/DEAD BacLight bacterial viability kit from Molecular Probes) that are based on the rely of the propidium iodide based assessment of dead cells. Usually, a combination of SYBR Green dyes or Sytox 9 and PI is used to analyze dead cell numbers.

Barbesti and co-authors (2000) proposed a protocol for the assessment of viable cells based on nucleic acid double staining (NADS). The NADS protocol uses, simultaneously, a permeant dye, such as SYBR Green (Lebaron et al., 1998), and an impermeant one, as propidium iodide (Jones & Senft. 1985; Lopez-Amoros, 1997; Sgorbati et al., 1996; Williams et al., 1998). The efficiency of the combined staining is magnified by the energy transfer from SYBR Green to PI when both are bound to the nucleic acids, as described by Barbesti and colleagues (2000). Both dyes can be readily excited with the blue light from the laser or arc lamp of relatively simple and portable flow cytometers; the green nucleic acid probes lead to energy transfer from SYBR Green to the red PI fluorescence in the case of double staining (Barbesti et al., 2000; Falcioni et al., 2008; Manti et al., 2008). In order to better distinguish dead from viable cells, a dot plot containing fluorescence signals (green *vs* red fluorescence) is recommended (Fig. 8). Membrane intact cells that are considered to be viable emit a green fluorescence that is only due to the incorporation of SYBR Green. Cells with a damaged membrane will enable PI to enter and to bind some nucleic acids, with a corresponding increase in red and a decrease in green fluorescence.

In 2001, Gregori and co-authors optimized the double staining protocol, comparing two dyes belonging to the SYBR Green family. SYBR Green II expresses greater selectivity for RNA, while keeping a strong affinity for double-stranded DNA of about half that of SYBR Green I. The authors thus concluded that using SYBR Green II on marine samples was better.

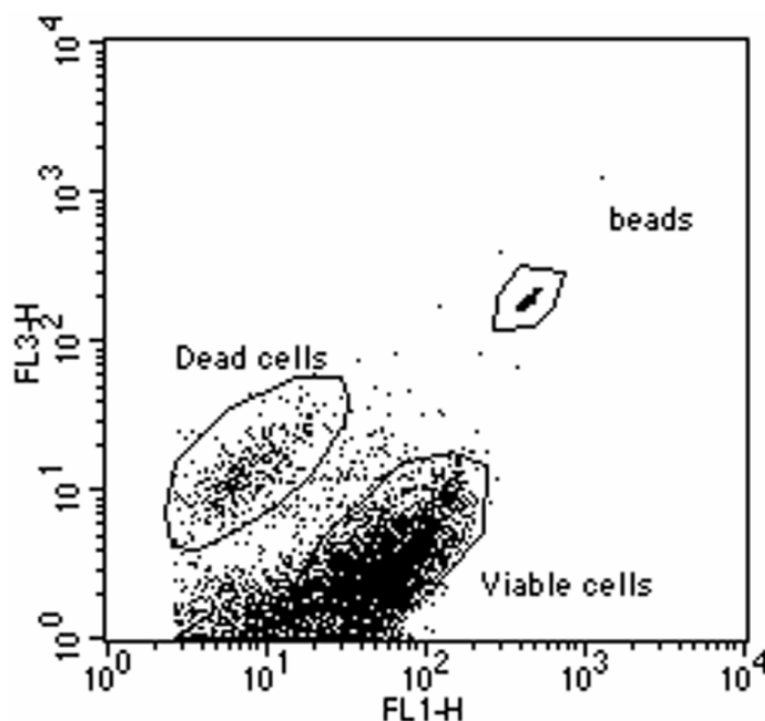


Fig. 8. Dot plot FL1 vs. FL3 of a marine sample stained with SYBR Green I and PI

Cell viability can be tested by assessing esterase activity or bacterial respiration. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in flow cytometry has been used to assess “active bacteria” in seawater (del Giorgio et al., 1997), and is referred to cells that have an active electron transport system and are capable of reducing the tetrazolium salt (CTC) (Table 1). Because CTC is reduced to a brightly fluorescent formazan, it is possible to enumerate respiring cells with great sensitivity, precision and speed.

While the use of this method has increased over the last few years (e.g. Sherr et al. 1999; Jugnia et al., 2000; Haglund et al., 2002), there have also been a number of studies that are highly critical of CTC as a means of distinguishing metabolically active cells (e.g. Ullrich et al., 1996, 1999; Karner & Fuhrman, 1997; Servais et al., 2001). Some authors have stated that CTC could be toxic for some bacteria, while in some cases the results obtained would underestimate the real activity of bacteria, especially in natural seawater (Gasol & del Giorgio, 2000). Although abundances of CTC+ cells in natural samples tend to be well correlated to measures of either bacterial production (e.g. del Giorgio et al., 1997; Sherr et al., 1999) or respiration (Smith, 1998), the proportion of total cells scored as CTC+ tends to be too low, generally less than 20%, and sometimes less than just a few percent (Smith & del Giorgio, 2003).

5 (and 6)-carboxyfluorescein diacetate (CFDA) was employed to detect esterase activity in living cells in seawater samples. CFDA is a non-fluorescent molecule, but upon intracellular

enzymatic cleavage produces a green fluorescent compound that can be detected by FCM (Gasol & del Giorgio, 2000) (Table 1). Some authors (Yamaguchi et al., 1994; Schupp & Erlandsen, 1987; Yamaguchi & Nasu, 1997) coupled 6CFDA with proidium iodide to distinguish active from inactive cell membranes. Accordingly, after 6CFDA-PI double staining, bacterial cells with esterase activity display only green CFDA fluorescence, while damaged cells show only red PI fluorescence.

	DYE	EX/EM	REFERENCE
NUCLEIC ACID PROBES	SYTO- 13	485-508/ 498-527	Andrade et al., 2003 Gasol et al., 1999 Gasol & del Giorgio, 2000 Alonso et al., 2007
	SYBR Green I/II	497-520	Lebaron et al., 1998, Gregori et al., 2001, Marie et al., 1997
	Propidium Iodide (PI)	536-623	Barbesti et al., 2000 Gregori et al., 2001
	TOTO-1	509-533	Guindulain et al., 1997; Zubkov et al., 2000
DEHYDROGENASE ACTIVITY	CTC	480/580-660	Gasol et al., 1995, Sherr et al. 1999, Servais et al., 2001; Pearce et al., 2007
ENZIMATIC ACTIVITY	CFDA	492/517	Gregori et al., 2001; Pearce et al., 2007

Table 1. Shows some available dyes used for the analysis of marine micro-organisms, their excitation and emission maximal wavelengths, along with some selected references

Another interesting application of FCM to microbiology requires the use of fluorochromes conjugated to antibodies or oligonucleotides for the detection of microbial antigens or DNA and RNA sequences to directly (Vives-Rego et al., 2000; Amann et al., 1990a; Marx et al., 2003; Temmerman et al., 2004) identify micro-organisms in natural ecosystems (Amann et al., 1990b; Amann et al., 2001; Wallner et al., 1997; Biegala et al., 2003).

7. Bacteria identification with antibodies and nucleic acid probes (FISH)

Immunodetection techniques utilize the specificity of the antibody/antigen association as a probe for recognizing and distinguishing between micro-organisms. Parallel, immunological detection methods can provide quantitative data, including in relation to the sensitivity of the method used. The application of immunology in phytoplankton research started when Bernhard and co-authors (1969) developed antibodies against two species of diatoms, but it was in the 1980s that immunological techniques for species identification were actually applied in marine research. The first species investigated were prokaryotes (Dahl & Laake, 1982; Campbell et al., 1983); later Hiroish et al. (1988) and Shapiro et al. (1989) conducted studies on eukaryotic organisms.

The use of antibodies in combination with FCM is a powerful tool for the specific detection and enumeration of micro-organisms in medical, veterinary and environmental microbiology

(Cucci & Robbins, 1988; Porter et al., 1993; Vrieling *et al.*, 1993a; McClelland & Pinder, 1994; Vrieling & Anderson, 1996; Kusunoki et al., 1998; Chitarra et al., 2002). Antibodies also have a role to play in determinations of the physiological characteristics of cells; Steen and colleagues used fluorescently labelled antibodies as part of a flow cytometric method of antigenicity determination (Steen et al., 1982) that may vary according to growth conditions (Davey & Winson, 2003).

The availability of antibodies against bacteria is limited mostly to the research and identification of pathogens (e.g. Kusunoki et al., 1996; Kusunoki et al., 1998; McClelland & Pinder, 1994; Tanaka et al., 2000).

Barbesti and colleagues (Barbesti et al., 2000) performed bacterial viability measurement and identification tests using a Cy5-labelled monoclonal antibody combined with SYBR Green I and propidium iodide.

A recent study (Manti et al., 2010) conducted in natural seawater samples reports the immunodetection of *Vibrio parahaemolyticus* and an examination of the specificity and sensitivity of the polyclonal antibody used.

As described above for antibodies, oligonucleotides allow the detection and recognition of micro-organisms in a mixed population. The phylogenetic heterogeneity of micro-organisms can be studied with analyses of ribosomal RNA sequences. Fluorescence *in situ* hybridization (FISH) is based on the homology of an oligonucleotide probe with a target region in an individual microbial cell.

In natural samples, however, the signal derived from the use of labelled oligonucleotide probes is often undetectable because of the low rRNA content. Among other methods, FISH with horseradish peroxidase (HRP)-labelled oligonucleotide probes and tyramide signal amplification, also known as catalyzed reporter deposition (CARD), is especially suitable for aquatic habitats with small, slow growing, or starving bacteria (Diaz et al., 2007).

Oligonucleotide probes labelled (directly or indirectly) with fluorescent markers can be detected by epifluorescence and confocal microscopy, or by flow cytometry (Giovannoni et al., 1988; De Long et al., 1989; Amann et al., 1990a; 1990b; 2001; Pernthaler et al., 2001). Several publications have reported the combination of rapidity and the multi-parametric accuracy of flow cytometry, with the phylogenetic specificity of oligonucleotide FISH probes as a powerful emerging tool in aquatic microbiology (Yentsch & Yentsch, 2008; Hammes & Egli, 2010; Muller & Vebe-Von-Caron, 2010; Wang et al., 2010).

The combination of FCM and FISH has been successfully applied to describe microbial populations dispersed in a liquid suspension derived from different media (Lim et al., 1993; Joachimsthal et al., 2004; Rigottier-Gois et al., 2003; Barc et al., 2004; Lange et al., 1997; Wallner et al., 1993 and 1995; Miyauchi et al., 2007).

Only a few studies (Lebaron et al., 1997; Gerdtz & Luedk, 2006; Kalyuzhnaya et al., 2006; Yilmaz et al., 2010) have combined FISH and FCM for the analysis of aquatic microbial communities. The main limitation of combining CARD-FISH and FCM is that the former is commonly performed and optimized on a solid support (i.e. polycarbonate membrane filters; Pernthaler et al., 2002), while the latter requires liquid samples with a well dispersed suspension of single cells (Shapiro, 2000). Schonhuber and co-authors (1997) have bridged the two methodologies while working with liquid suspensions, although the proposed

permeabilization procedure was not ideal for the detection of large bacterial groups with different cell walls. Meanwhile, Biegala and colleagues (2003) successfully performed a CARD-FISH-FCM protocol for the detection of marine picoeukaryotes, while Sekar and co-authors (2004) proposed the enumeration of bacteria by flow cytometry identified by *in situ* hybridization.

A recent study (Manti et al., 2011) proposed an improved protocol for the flow cytometric detection of CARD-FISH stained bacterial cells, remarking on the importance of improving the identification and quantification of phylogenetic populations within heterogeneous, natural microbial communities.

8. Conclusions

Flow cytometry is a powerful technique with a wide variety of potential applications in marine microbiology. Due to its characteristics, FCM has contributed to the knowledge of free living planktonic microbial community structures and their distribution.

The employment of new techniques and probes normally used in other ecosystems or in clinical microbiology could enhance the field of application of flow cytometry and so the studies of marine assemblages.

Furthermore, modern flow cytometers also provide quantitative data and image analyses for the detection of microbial subgroups, thereby extending the field of flow cytometry applications (Andreatta et al., 2004; Olson & Sosik, 2007).

Last but not least, the development of a portable and cheap flow cytometer, and/or imaging system with a reliable interpretation may render the monitoring of microbial communities in marine ecosystems faster and efficient.

9. References

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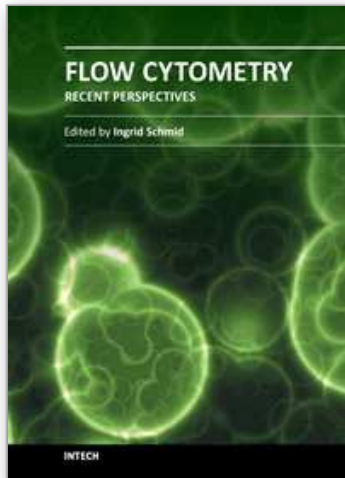
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Flow Cytometry - Recent Perspectives

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0626-5

Hard cover, 500 pages

Publisher InTech

Published online 13, June, 2012

Published in print edition June, 2012

"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

A. Manti, S. Papa and P. Boi (2012). What Flow Cytometry can Tell Us About Marine Micro-Organisms – Current Status and Future Applications, Flow Cytometry - Recent Perspectives, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0626-5, InTech, Available from: <http://www.intechopen.com/books/flow-cytometry-recent-perspectives/what-flow-cytometry-can-tell-about-marine-microorganisms-current-status-and-future-applications>

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