Volume 8, Issue 2, 2019, 558 - 566 ISSN 2284-6808
Letters in Applied NanoBioScience
www.NanoBioLetters.com
https://doi.org/10.33263/LIANBS82.558566
Original Review Article Open Access Journal
Received: 15.04.2019 / Revised: 25.05.2019 / Accepted: 15.06.2019 / Published on-line: 18.06.2019

Updates on the applications of flow cytometry in microbiology

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ABSTRACT
Flow cytometry (FCM) was first developed for medical and clinical applications such as hematology and oncology. Although these areas still account for the vast majority of publications on this technique, during the past few years it has been also introduced in other areas, such as optimization and monitoring of biotechnological and environmental processes, pharmacology, toxicology, bacteriology and virology. In the food and drinks industries, the time required for conventional microbiology tests can lead to substantial delays in product release to the market. FCM has been used in conjunction with viability markers for rapid counting of yeast, molds and bacterial cells, including foodborne pathogens and microbial contaminants, in food products as well as for monitoring and improving the final products quality. FCM is an excellent tool, still unexplored in clinical microbiology, allowing for detection of cellular and non-cellular components in different clinical specimens, the study of antimicrobial activity, allowing for rapid and direct antimicrobial susceptibility testing and for the investigation of resistance mechanisms. Recent FCM developments important for addressing questions in environmental microbiology include the study of microbial physiology under environmentally relevant conditions, the development of new methods to identify active microbial populations and to isolate previously uncultured microorganisms and of high-throughput autofluorescence bioreporter assays. Moreover, the technological advancements will make possible the miniaturisation and automation of FCM devices, allowing to revolutionize their applications in the near future. The purpose of this minireview is to update the current applications of FCM in different fields of applied microbiology, and to highlight the main advantages and pitfalls for each of them.
Keywords: *flow cytometry, food microbiology, microbial cells detection, antimicrobial susceptibility, antibiotic resistance*.

1. INTRODUCTION

Flow cytometry (FCM) provides rapid analysis of multiple characteristics of single cells. The acquired FCM data is both qualitative and quantitative. The analyzed characteristics include cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular proteins [1].

In the past FCM instruments were big and complex equipment that were found only in academic centers. Advances in technology make it possible for hospitals to employ them in their current diagnostic instruments. Nowadays, FCM machines are smaller, cheaper, more user-friendly and more adequate for high volume operations.

In clinical settings, FCM is applied for the analysis of different specimens, i.e. blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine and solid tissues. FCM was first developed for medical and clinical applications such as hematology and oncology. Although these areas still account for the vast majority of publications on this technique, during the past few years it has been introduced in other areas, such as bioprocess monitoring, pharmacology, toxicology, environmental sciences, bacteriology and virology [2]. Currently, the high-throughput multi-parameter FCM is exploited to obtain single cell data and use them for understanding and optimization of biotechnological and environmental processes.

2. GENERAL PRINCIPLES

FCM is the measurement (-metry) of cellular (cyto-) properties (physical or chemical) as they are moving in a fluid stream (flow) through a stationary set of detectors. FCM measures optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). Physical properties, such as size (represented by forwarding angle light scatter) and internal complexity (represented by right-angle scatter) can be used for detecting certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state [3, 4]. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes (table 1), each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously. Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more [5, 6, 7].

Inside a FCM instrument, cells in suspension are drawn into a stream developed by a surrounding fluid sheath that determines laminar flow. Cells or particles are passing

individually through an interrogation point where a beam of monochromatic light, usually from a laser, intersects the cells. The emitted light is scattered in all directions and is collected by the optic components that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis [1, 4]. The resulting information usually is displayed in histogram or two-dimensional dot-plot formats [1].

3. FCM APPLICATIONS IN MICROBIOLOGY

Throughout the last decade, a growing number of papers describing various applications of FCM in the field of microbiology have been published and a book devoted to the subject has been recently published, [8]. FCM is a potentially valuable analytical method in microbiology. Unlike culture base techniques which involve the study of microbial populations, FCM offers the possibility to rapidly analyze large numbers of individual microorganisms by several parameters, been able to detect and quantify differences between populations. Culture base techniques used for the analysis of microbiological samples are time consuming; they are not able to detect non-culturable microorganisms and they do not provide real-time information on the physiological status of the organism *in situ* [9].

FCM is largely used in food industry for optimization and monitoring of microbial industrial processes [10, 11], for the detection of microbial contaminants in food products [12] and also it has applications in environmental research [13]. But although in clinical laboratories the application of the flow cytometric technique is well established in hematology and the use of this method in routine clinical microbiology is still rare [14].

Currently, there are a range of biological reagents kits restricted to simple analyses, for example to assay the total or viable numbers of microorganisms present. But, there are also technologies available to selectively label specific types of microorganisms. For example, fluorochrome-conjugated antibodies can be used to label microorganisms according to expression of particular antigens, fluorescent *in situ* hybridization to label according to phylogeny and fluorogenic enzymatic substrates to label according to the expression of specific enzyme activities [9].

Tabel 1. Some of the fluorescent molecules used to study microorganisms by flow cytometry (modified from., [15, 16].

Dye	Substrate	Applications
SYTOX Green	DNA-RNA	**Viability, DNA quantification**
Propidium Iodide (PI)	DNA-RNA	**Viability, DNA quantification, Cell cycle studies**
Ethidium bromide	DNA-RNA	**DNA quantification, Cell cycle studies**
SYTO 13	DNA-RNA	**Viability, DNA quantification, Cell cycle studies**
Hoechst 33258/33342	DNA (GC pairs)	**Cell cycle studies**
Mithramycin	DNA	
Pyronine Y	RNA	**RNA quantification**
Fluorescein isothiocyanate (FITC)	Proteins	**Microbe detection**
Texas Red	Proteins	
Oregon Green Isothiocyanate	Proteins	
Antibodies labeled with flurochromes	Antigens	
Fluorescently labeled oligonucleotides	Nucleotide sequences	
Indo-1	$Ca2^+$	**$Ca2^+$ mobilization**
Fura-2	$Ca2^+$	
Fluor-3	$Ca2^+$	
BCECF	pH	**Metabolic variations**
SNARF-1	pH	
DIOC6(3)	Membrane potential	**Metabolic variations**
Oxonol [DiBAC4(3)]	Membrane potential	
Rhodamine 123	Membrane potential	
Lectins	Membrane oligosaccharides	**Cell wall composition, Microbe detection**
Substrates linked to fluorochromes	Enzyme activities	**Metabolic activity**
Nile Red	Lipids	**Yeast metabolic state**
Red Fun-1	Vacuolar enzyme activity	
Calcofluor white	Chitin and other carbohydrate polymers	**Fungal detection**

4. FCM IN FOOD INDUSTRY

FCM is an analysis tool of great importance in food development and food spoilage. It offers several potential advantages as a method for the detection of bacteria in food samples. FCM is rapid, can be done quantitatively, can be automated and produces real time as well as archival data, all of which offer important information for rapid detection systems as well as critical control point monitoring. In addition, FCM equipped with cell sorting capability can be used to isolate cells or bacteria of interest for further analysis or confirmatory culture [17].

Brewing.

The brewing industry has long had an interest in applying FCM to the microbiology of the brewing process. The measurement of the DNA content of yeast cells was one of the first major applications of flow cytometry in brewing [19, 20, 21, 22] and is still one of the main applications of this method in this field. Yeasts are widely used to produce commercially important compounds such as pharmaceutical agents, enzymes for food industry, bioethanol, and chemicals, including organic acids. Stress factors like nutrient deficiency and extracellular acidification can affect the physiology of the yeast cells and thus the industrial fermentation yield. pH_i is a crucial determinant of the overall physiology of *S. cerevisiae* cells and affects product synthesis and quality. FCM was used to monitor the kinetics of the pH homeostasis of *S. cerevisiae* cultures grown to a stationary phase in different growth conditions. Populations with pH homeostasis behaving differently after ethanol or glucose addition and after exposure to weak acid stress and glucose pulse were identified [23]. The DNA content of yeast cells provides information about the cell cycle and is thus a powerful tool to assess the effects of propagation parameters on the yeast cell cycle and yeast physiology. FCM assays were developed for assessing the physiological status of the yeast cells during fermentation process [24, 25]), for determination of yeast viability and cell number in a brewery [26-32], predictions of flocculation ability of brewer yeasts, separation of the prototroph yeast fusants [33], assessment of *Saccharomyces cerevisiae* vitality [34, 35, 36], detection of spoilage microorganisms [24, 37], the influence of beer process conditions on beer stability [38], identification of haploid strains of industrial brewer's yeast, fermentation process control [20, 39, 40]. The use of FCM for age assessment of a yeast population and its application in beer fermentation was reviewed in the publication [41].

Winery.

Because it is rapid and easy to use, FCM can be considered a useful method for microbiological quality control in wineries and for the investigation of the growth dynamics of microorganisms in wine [42, 43, 44, 45]. The density and viability of the yeast inoculum are important factors for the fermentation process. These parameters have been measured using methylene blue staining of the yeast population, hemacytometer, microscope and culture based methods but these assays are time consuming and subject to error. FCM provides a rapid and accurate means to monitor the density and viability of yeast throughout the fermentation process and thus the fermentation process becomes transparent [18, 28, 42, 43, 44, 45]), as well as to detect the presence of spoilage organisms such as *Zygosaccharomyces*, *Dekkera* (*Brettanomyces*) and *Lactobacillus* [46, 47].

By monitoring the physiological state of *O. oeni* (measurements of intracellular pH, transmembrane potential and vitality,[30] concluded that FCM could be used to better control the malolactic fermentation. FCM was also applied in the monitoring of the viability and membrane damage of oenological strains of *Lactobacillus plantarum* along with the malic acid consumption in media containing ethanol prior to wine inoculation. The results have shown a significant decrease of membrane damage and improvement of strain viability in the harsh wine conditions [48]. FCM was employed also for investigation of Viable But Non Culturable (VBNC) state following *S. cerevisiae* SO_2 exposure. The results indicated that the yeast cells can survive in a VBNC state in synthetic wine for up to one month, suggesting that the use of sulfite for stabilizing different beverages should be assessed using other methods than plating methods [49].

Dairy products.

FCM appears as a very promising tool for the analysis of raw milk, starter culture and final products in the dairy industry. Current uses of FCM in dairy industry are including: characterization of cellular states in stress conditions (acidic, oxidative, osmotic, cold stress) [50], study of population dynamics in batch cultures, study of bacterial fitness to help optimizing processes, early detection of bacteriophages, membrane changes during cheese cooking, evaluation of antimicrobial effects of bacteriocins, enumeration of viable probiotics in commercial preparations, controlling foods organoleptic properties [19, 51, 52]. FCM technique was introduced 10 years ago in dairy industry for the evaluation of somatic cell load and total bacteria count in raw milk, which has revolutionized the grading of raw milk, producing rapid and accurate results [53, 54].

For probiotic products, it is important to ensure the presence of sufficient numbers of viable cells that will bring about beneficial health effects [52, 55, 56, 57]. A specific FCM method was developed to enumerate viable *Bifidobacteria* in commercial products by using double staining of antibody and viability probe probe [58].

Backery.

In backery, FCM is used for the development of new *Saccharomyces* strains. For example it was applied for selection of rare mating hybrids produced between an industrial baker's yeast strain and a laboratory yeast strain 59]. Investigations regarding the influence of extracellular acidification on cell physiology have shown stationary cells are better able to maintain their pH_i homeostasis independently of different extracellular pH values 60]. FCM enables pHi assessment with not only high sensitivity but also extraordinarily high speed in data acquisition and processing [23].

Biomass assay.

FCM proved to accurately estimate biomass dry weight in *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cultures containing very few algal cells i.e. laboratory studies and high throughput assays in microwell plates. Also, the study indicates that the FCM data could complement the optical density assays for improvement of the accuracy in biomass estimation [61].

Food safety.

Contamination of foods including fresh and processed meat with microbial pathogens has been responsible for disease outbreaks with major human and economic consequences. The detection of specific pathogenic bacteria within meats (and eggs), namely *Salmonella* spp., *E. coli* O157:H7 and *Campylobacter* spp. [62], is an important focus in the development of hazard analysis critical control point (HACCP) procedures for meat processing [17]. Food safety regulators, food producers, distributors, and retailers need effective microbiological methods with improved sensitivity, specificity, and speed for quality control purposes to eliminate or reduce product contamination by foodborne pathogens (American Type Culture Collection (ATCC), 2012). While traditional

microbiological methods have worked well for decades to detect and stop pathogen contamination in food, the speed of modern food production practices requires dramatic improvements in speed and accuracy of microbiological methods to assure the same safety standards [63].

Food-related applications of FCM include detection and enumeration of meat spoilage microorganisms [17, 64, 65, 66, 67].

The reproducibility of the results and the proven correlation with standard plate count method obtained in industrial conditions make FCM a good predictive method for product and process quality control [68].

However, in case of the presence of low levels of microorganisms in food samples, the detection can still take 18–20 h to obtain and the spoilage organism has to be disaggregated from the meat surface which, with some organisms forming a glycocalyx layer, is necessarily difficult [69].

FCM has been used for detection of pathogens and for bacteriological quality control of milk [71, 72, 73, 74] and the physiological functions of the bacterial cells in pineapple and mango juice [70]. A Gram-staining technique combining staining with two fluorescent stains, Oregon Green-conjugated wheat germ agglutinin (WGA) and hexidium iodide (HI) followed by flow-cytometric detection were described. A clear differentiation between Gram-positive and Gram-negative bacterial populations in bulk tank milk was possible, thus the FCM can be used to analyze milk samples without precultivation [75]. The identification of pathogens by microsphere immunoassays using FCM offers the specificity provided by antibodies coupled with the speed and multiparametric analysis provided by FCM [15]. An example is the simultaneous identification of antibodies against *Brucella abortus* and *S. aureus* in milk samples. The FCM appears to be useful for large-scale brucellosis eradication programs. It offers the possibility of using one test to identify animals that are serologically positive for both *B. abortus* and *S. aureus* [76].

Biofilms represent an important problem in food industry, with food contamination, equipment failure, and public health aspects to consider. A recent application of FCM in food industry is the study of microbial biofilm developed on stainless steel surfaces. FCM was used for demonstration of the possibility of plasmid transfer in dual-species biofilms formed of *Pseudomonas putida*, as a model for food spoilage organisms and *Escherichia coli,* as a model for pathogenic organisms, thus highlighting the importance of biofilms in the food industry as hot spots for the acquisition of multiresistance plasmids [77].

A variety of rapid methods for detecting *E. coli* O157:H7 in food have been developed to augment or enhance conventional methods. Some of these are based on bioluminescence, FCM, immunology (e.g., enzyme-linked immunosorbent assay ELISA), fluorescent in situ hybridization (FISH), and quantitative real-time polymerase chain reaction (Q-PCR) [78].

In a validation study, for detection of *E. coli* O157:H7 in raw spinach the FCM method was equal to the bacteriological analytical method in sensitivity and far superior in speed [79]. A recently developed diagnostic tool is RAPID-B, which combines FCM, enhanced detection techniques and background reduction that functions accurately to provide results that are as precise as culture plates but that require substantially less time [62].

Studies have shown that RAPID-B can be used for rapid, economical, and stable detection of *E. coli* O157:H7 and *Shigella* contamination in the food industry [80,81].

5. FCM APPLICATIONS IN AQUATIC AND ENVIRONMENTAL MICROBIOLOGY

F. JOUX & P.LEBARON, [82], forecasted the development of FCM methods that would utilize combinations of physiological and taxonomic probes to detect and characterize target cells. Recent FCM developments important for addressing questions in environmental microbiology include: (i) the study of microbial physiology under environmentally relevant conditions, (ii) new methods to identify active microbial populations and to isolate previously uncultured microorganisms, and (iii) the development of high-throughput autofluorescence bioreporter assays [83].

Technical advances have simplified FCM instrument handling, improved cell sorting capabilities, and brought in multiparametric measurements, thus implying this prediction is rapidly fulfilling. Using two to three different fluorescent dyes that target specific biomolecules and physiological processes are possible to characterize microbial communities, in terms of physiological states of cells, their taxonomic positions, and expressed gene functions. Because of the multiparametric measurements, these features can be assessed simultaneously, and in the end target cells can be separated and isolated for further study [82].

Abundant populations of marine free-living prochlorophytes were first identified in water samples from the North Atlantic and Pacific with the help of FCM [83]. Since then, many other researchers have reported the presence of this group of prokaryotic picoplankton in the euphotic layer of the world's tropical and temperate oceans [84]. After isolation, culture, and genetic and ecophysiological characterization, they were named *Prochlorococcus marinus* [85]. FCM has been essential in the discovery of this organism [84].in the realization of its ubiquitous presence in the ocean [86, 87] and in the assignment of its quantitative role as a primary producer in different geographical locations. This was due to the unmatched capacity of FCM to analyse small cells in terms of sensitivity and time, and to the fact that both *Synechococcus* and *Prochlorococcus* showed a very characteristic signature when analyzed by FCM. Initially, it was used to discriminate and enumerate the different populations of phytoplankton, eukaryotic and prokaryotic autotrophs on the basis of their distinct pigments [88] and of heterotrophic bacteria [89] and viruses [90] using a variety of fluorescent dyes.

Distinction between different physiological states is possible by utilizing FCM in conjunction with fluorescent dyes that measure biological parameters such as nucleic acid content, respiration rates, intracellularly enzyme activity, and cytoplasmic and outer membrane integrity Such differentiations in physiological activity are not well addressed by conventional plating techniques [82]. FCM techniques allowed a functional classification of the physiological state of microorganisms based on the presence or absence of an intact fully polarized cytoplasmic membrane and the transport systems across it, thus enabling

assessment of population heterogeneity. These techniques have been applied to characterize microbial cells exposed to various environmental conditions, including various pollutants [9192, 93, 94, 95, 96, 97, 98, 99], or water disinfection treatments [100] and quantify microbial growth in oligotrophic environments [101].
The independence from cultivation makes FCM an appealing technique for fast detection of the viability of pathogenic bacteria in the viable but nonculturable state, which can be present in the aquatic environment but not detectable by conventional methods. The wide distribution of aeromonads in different aquatic ecosystems underlines their capacity to adapt to environments with different trophic levels. Several studies have shown that the phenospecies *Aeromonas hydrophila* is prevalent in cleaner water, whereas *Aeromonas caviae* is prevalent in water with a high level of fecal pollution; the frequencies of the other phenospecies appear to be unrelated to environmental conditions. SYBR Green I coupled with propidium iodide allowed the assessment of the bacterial viability in water. FCM provided rapid direct cells counting in water samples without the need of cultivation, and therefore sample handling and possible contamination due to sample handling were reduced. Thus, FCM allowed the original characteristics of the water samples to be analyzed [102].

Legionella is a pathogenic bacterium that establishes and proliferates well in water storage and distribution systems. Worldwide it is responsible for numerous outbreaks of legionellosis, which can be fatal. Despite recent advances in molecular and immunological methods, the official, internationally accepted detection method for *Legionella* spp. in water samples (ISO 11371) is still based on cultivation. In a comprehensive comparative trial, 85 tap water samples were tested with both the FCM approach and the standard culture method [103]. No correlation and low strength of agreement was observed between culture and FCM results. In approximately 50% of the samples, FCM showed higher concentrations than culture (difference >0.5 log), but in 20% of samples, higher plate counts were observed. These were attributed to the major draw-backs of the current approach—the polyclonal antibodies targeting only the serogroups 1-12 and the inability to detect *L. pneumophila* in amoebal cells. The application of monoclonal antibodies specifically targeted to naturally grown pathogens and the availability of *Legionella* specific antibody-coated microbeads for immuno-magnetic separation (IMS) may significantly improve and simplify the procedure [103, 104]. Advances in the availability of suitable *Legionella* spp. or *L. pneumophila* specific monoclonal antibodies, optimally targeting epitopes of proteins or lipopolysaccharides that are well expressed in the VBNC state, and the combination of more than one viability assay, will significantly increase the potential of FCM for routine determination of *Legionella* concentrations in tap water samples [105].

Monitoring microbial dynamics in engineered and environmental aquatic ecosystems is a key step toward a better understanding of the driving forces and consequences of changes in bacterial concentrations and community composition [106]. Studies have indicated that automated *in situ* FCM analysis is feasible for the investigation of dynamic aquatic ecosystems at high temporal resolution during multiple days. Automated FCM allows for the combination of microbial data at a high temporal resolution with conventional online parameters, thus expanding the horizon toward a complete sensing approach., [106].
An excellent study demonstrating the dynamics of microbial growth at very low substrate concentrations in drinking water systems was performed by [107]. The researchers used a FCM approach to analyze total cell number in water samples from a drinking water pilot plant using SYBR1 Green staining. The instrument quantified cell numbers as low as 1000 cells/ml and highlighted cell growth or decline across different unit processes in drinking water treatment facilities [107]. FCM assessment of bacterial abundance is now routinely used for water samples [108, 109], but, it is also attractive to determine bacterial cell abundance in a range of other environments Recently, FCM proved to be a useful approach for determining bacterial abundances in soils, sediments and sludge [110].

6. CONCLUSIONS

FCM is rapidly becoming an essential tool in industrial biotechnology, food and pharmaceutical quality control, routine monitoring of drinking water and wastewater systems, and microbial ecological research in soils and natural aquatic habitats. FCM allows to distinguish between viable and dead cells using multiparametric data acquisition, analysis and cell sorting which is of great importance in food development and food spoilage. On the other side, the technological advancements will make possible the miniaturisation and automation of FCM devices, allowing to revolutionize their applications in the near future.

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8. ACKNOWLEDGEMENTS

The financial support of the project PN-III-P4-ID-PCE-2016-0921 - Rapid flow cytometric method for microbial detection and antibiotic susceptibility assay directly from clinical specimens is gratefully acknowledged.

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