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## Applications of Flow Cytometry to Clinical Microbiology

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### 1. Introduction

Microbiology in general and clinical microbiology in particular have witnessed important changes during the last few years. Traditional methods of bacteriology and mycology require the isolation of the organism prior to identification and other possible testing. In most cases, culture results are available in 48 to 72 h. Virus isolation in cell cultures and detection of specific antibodies have been widely used for the diagnosis of viral infections (Weinstein, 2007). These methods are sensitive and specific, but, the time required for virus isolation is quite long and is governed by viral replication times. Additionally, serological assays on serum from infected patients have often most limits in specificity and sensitivity. Life-threatening infections require prompt antimicrobial therapy and therefore need rapid and accurate diagnostic tests. Procedures which do not require culture and which detect the presence of antigens or the host's specific immune response have shortened the diagnostic time. More recently, the emergence of molecular biology techniques, particularly those based on nucleic acid probes combined with amplification techniques, has provided speediness and specificity to microbiological diagnosis. These techniques have led to a revolutionary change in many of the traditional routine tests used in clinical microbiology laboratories.

The current organization of clinical microbiology laboratories is now subject to increased use of automation exemplified by systems used for detecting bacteremia, screening of urinary tract infections, antimicrobial susceptibility testing and antibody detection. To obtain better sensitivity and speed, manufacturers continuously modify all these systems. Nevertheless, the equipment needed for all these approaches is different, and therefore the initial costs, both in equipment and materials, are high.

Indeed, in recent years microbiological techniques have been increasingly complemented by technologies such as those provided by flow cytometry.

We have gotten used to consider the flow cytometry applicable only in the field of hematology, then associate it with clinical microbiology makes it even more mysterious.

Over the past forty years we have witnessed several attempts of application of the flow cytometry to microbiology, with good results but also with many difficulties.

In particular, the problems encountered relate the difficulty of measuring microbes by flow due to their small size and point towards the development of instrumentation that has managed to overcome this limitation of standard instrumentation used for routine flow cytometry in different fields from microbiology.

The aim of this chapter is to provide a complete overview of the applications of flow cytometry in microbiology, referring mainly to what is published in the literature. Will be presented innovative methods and practical examples of applications of flow cytometry in different areas of microbiology following the scheme outlined in paragraphs listed below.

The authors report in paragraph “References” articles that offer important points of discussion to make useful chapter to the various professionals in the targeted book.

## 2. Flow cytometry and microbiology

Flow cytometry is a powerful fluorescence based diagnostic tool that enables the rapid analysis of entire cell populations on the basis of single-cell characteristics (*Brehm-Stecher, 2004*). Flow cytometry (FCM) could be successfully applied in bacteremia and bacteriuria, for rapidly microorganism’s detection on the basis of its cytometric characteristics. Although FCM offers a broad range of potential applications for susceptibility testing, a major contribution would be in testing for slow-growing microorganisms, such as mycobacteria and fungi.

This technique could also be applied to study the immune response in patients, in detection of specific antibodies and monitor clinical status after antimicrobial treatments.

In the last years of the 1990s, the applications of FCM in microbiology have significantly increased (*Fouchet, 1993*).

Earlier works had demonstrated the applicability of dual-parameter analysis (light scattered vs fluorescence coupled to cellular components as protein and DNA or auto-fluorescence) to discriminate among different bacteria in the same sample.

FCM has also been used in metabolic studies of microorganisms (es. autofluorescence due to NADPH and flavins as metabolic status markers), in DNA’s analysis, protein, peroxide production, and intracellular pH, for count of live and dead bacteria and/or yeasts, and for the discrimination of gram-positive from gram-negative bacteria on the basis of the fluorescence emitted when the organisms are stained with two fluorochromes.

Also it offers the possibility to investigate in yeasts and bacteria the respective gene expression (*Alvarez-Barrientos, 2000*).

## 3. Applications of flow cytometry to clinical microbiology

FCM is an analytical method that allows the rapid measurement of light scattered (intrinsic parameters: cell size and complexity) and fluorescence emission produced by suitably illuminated cells (fluorochromes). The cells, or particles, are suspended in liquid and produce signals when they flow individually through a beam of light, and the results represent cumulative individual cytometric characteristics. An important analytical feature of flow cytometers is their ability to measure multiple cellular parameters (analytical flow

cytometers). Some flow cytometers are able to physically separate cell subsets (sorting) based on their cytometric characteristics (cell sorters).

Fluorochromes can be classified according to their mechanism of action: those whose fluorescence increases with binding to specific cell compounds such as proteins (fluorescein isothiocyanate [FITC]), nucleic acids (propidium iodide [PI]), and lipids (Nile Red); those whose fluorescence depends on cellular physiological parameters (pH, membrane potential, etc.); and those whose fluorescence depends on enzymatic activity (fluorogenic substrates) such as esterases, peroxidases, and peptidases. Fluorochromes can also be conjugated to antibodies or nucleotide probes to directly detect microbial antigens or DNA and RNA sequences (see Table 1).

Several articles of literature propose flow cytometry as rapid diagnostic tool in the fight against infection (*Alvarez-Barrientos, 2000*). In fact this methodology can be used in the isolation of microbes and their identification, in the determination of antibodies to a particular pathogen in different stages of the disease and in direct detection of essential microbial components such as nucleic acids and proteins directly in clinical specimens (tissues, body fluids, etc.) and for evaluation of effectiveness of antimicrobial therapy in general.

Recently, the Sysmex UF-100 flow cytometer has been developed to automate urinalysis. *Penders et al.* have valuated this instrument to explore the possibilities of flow cytometry in the analysis of peritoneal dialysis fluid and have compared the obtained data with those of counting chamber techniques, biochemical analysis and bacterial culture (*Penders, 2004*); while *Pieretti et al.* have applied this technology at diagnosis of bacteriuria, for example (*Pieretti, 2010*).

### 3.1 Direct detection of bacteria, fungi, parasites, viruses

Several studies are reported in the literature concerning the use of flow cytometry to determine the presence of bacteria, viruses, parasites, etc, in a biological sample. In this section we describe the techniques used for this purpose.

Microorganisms are small and they are very different in structure and function, and both these factors lead to technological and methodological problems in studying them.

Conventionally, microorganisms are studied at the population scale because cultures of microbes are considered to be uniform populations which can be adequately described by average values. However, the availability of tools such as flow cytometry and image analysis which allow measurements to be made on individual cells has changed our perception of microbes within both the laboratory and the natural environment. Only a small proportion of the diversity of microorganisms has been identified and a smaller proportion still has been characterized through laboratory studies.

Microbes cannot be investigated without technological assistance, meaning that methods such as microscopy and flow cytometry with appropriate fluorochromes are essential for the acquisition of both qualitative and quantitative information. Although these methods have become conventional tools in microbial cell biology and in the analysis of environmental samples, their use in investigations of bacteria is limited by the physical constraint of optical resolution. Application of cell markers is also a challenge, simply because the cells have only

Applications	Substrate	Dye
<b>Viability <sup>a</sup></b> <b>DNA quantification <sup>b</sup></b> <b>RNA quantification <sup>c</sup></b> <b>Cell cycle studies <sup>d</sup></b>	DNA-RNA	<i>SYTOX Green <sup>a,b</sup></i> <i>Propidium Iodide (PI) <sup>a,b,d</sup></i> <i>Ethidium bromide <sup>b,d</sup></i> <i>SYTO 13 <sup>a,b,d</sup></i>
	DNA (GC pairs)	<i>Hoechst 33258/33342 <sup>d</sup></i>
	DNA	<i>Mithramycin <sup>d</sup></i>
	RNA	<i>Pyronine Y <sup>c</sup></i>
<b>Microbe detection</b>	Proteins	<i>Fluorescein isothiocyanate (FITC)</i>
		<i>Texas Red</i>
		<i>Oregon Green Isothiocyanate</i>
	Antigens	<i>Antibodies labeled with flurochrome</i>
	Nucleotide sequences	<i>Fluorescently labeled oligonucleotides</i>
<b>Ca2<sup>+</sup> mobilization</b>	Ca2 <sup>+</sup>	<i>Indo-1</i> <i>Fura-2</i> <i>Fluor-3</i>
<b>Metabolic variations</b>	pH	<i>BCECF</i> <i>SNARF-1</i>
<b>Antibiotic susceptibility</b> <b>Metabolic variations</b>	Membrane potential	<i>DIOC<sub>6</sub>(3)</i> <i>Oxonol [DiBAC<sub>4</sub>(3)]</i> <i>Rhodamine 123</i>
<b>Cell wall composition</b> <b>Microbe detection</b>	Membrane oligosaccharides	<i>Lectins</i>
<b>Metabolic activity</b>	Enzyme activities	<i>Substrates linked to fluorochrome</i>
<b>Yeast metabolic state <sup>e</sup></b> <b>Fungal detection <sup>f</sup></b>	Lipids	<i>Nile Red <sup>e</sup></i>
	Vacuolar enzyme activity	<i>Fun-1 <sup>e</sup></i>
	Chitin and other carbohydrate polymers	<i>Calcofluor white <sup>f</sup></i>

Table 1. Features of same fluorescent molecules used in flow cytometry (modified from Alvarez-Barrientos 2000)

a thousandth of the volume of a normal blood cell and correspondingly small amounts of cellular constituents. This is the reason why multicolor approaches in bacteria with small cell volumes will not work, as the close spatial interaction of the dyes prevents quantitative analysis (Muller, 2009).

Mueller and Davey (2009) have proposed a bibliometric analysis of flow cytometric studies in last forty-years in which appear that the role of flow cytometry in microbiology is steadily increasing.

A survey was made of the Web of Science database of the Institute for Scientific Information counting all papers whose topic database field contained the words flow and cytometr\* (es. citometry, citometric, etc) plus one or more of the following words: bacteri\*, microorganism, procaryot\* or yeast. The percentage of flow cytometry papers in general shows a steady growth after the 1990s, and in particular 8% of flow cytometry articles includes studies of microbes.

Earlier works had demonstrated the applicability of dual-parameter analysis to discriminate among different bacteria in the same sample. One parameter was light scattered (size), and the other was either fluorescence emission from fluorochromes coupled to cellular components (protein and DNA) or autofluorescence, or light scattered acquired from another angle. For example dual-parameter analysis of forward light scatter and red fluorescence signals (FSC-H vs FL3-H) allowed the discrimination between two species of *Candida*, as *Candida lusitaniae* and *Candida maltosa*, based on different fluorochrome staining backgrounds. These yeast species are indistinguishable by monoparametric analysis of forward light scatter or red autofluorescence.

In addition it is possible the quantification of different protein amounts (measured as FITC fluorescence) to distinguish different microorganisms (bacteria and/or yeasts) present in mixed cultures by histogram representation (FITC fluorescence vs number of events); or use dual-fluorescence to discrimination of specific fungal spores. For example, Alvarez-Barrientos et al. (2000) have proposed Calcofluor fluorescence vs PI fuorescence for detection of *Aspergillus*, *Mucor*, *Cladosporium*, and *Fusarium*. In particular, Calcofluor binds chitin in the spore wall, while PI stains nucleic acids. However, the use of several fluorochromes for direct staining or through antibody or oligonucleotide conjugates plus size detection is the simplest way to visualize or identify microorganisms by FCM.

The simple and rapid assessment of the viability of a microorganism is another important aspect of FCM. The effect of environmental stress or starvation on the membrane potential of bacteria has been studied by several groups using fluorochromes that distinguish among nonviable, viable, and dormant cells.

FCM has also been used in metabolic studies of microorganisms using autofluorescence due to NADPH and flavins as metabolic status markers. Other authors studied DNA, proteins, peroxide production, and intracellular pH, detection of live and dead bacteria and fungi, detection of gram-positive and gram-negative on the basis of the fluorescence emitted when the organisms are stained with two fluorochromes, and gene expression.

FCM has been extensively used for studying virus-cell interactions for cytomegalovirus (CMV), herpes simplex virus (HSV), adenovirus, human immunodeficiency virus (HIV), and hepatitis B virus (HBV).



### 3.1.1 Bacteria

Pianetti *et al.* (2005) compared traditional methods (spectrophotometric and plate count) used in bacteria counting cells with FCM for the determination of the viability of *Aeromonas hydrophila* in different types of water. They studied the presence of a strain of *Aeromonas hydrophila* in river water, spring water, brackish water and mineral water.

Flow cytometric determination of viability was carried out using a dual-staining technique that enabled us to distinguish viable bacteria from damaged and membrane-compromised bacteria. The traditional methods showed that the bacterial content was variable and dependent on the type of water. The plate count method is a widely used technique for determining the bacterial charge, but it supplies information related only to viability and growth capacity; while the absorbance method have a sensitivity who appears to be correlated with microbiological culture density.

The flow cytometric nucleic acid double-staining protocol is based on simultaneous use of permeable fluorescent probes (SYBR Green dyes) and an impermeable fluorescent probe (PI) and can distinguish viable, membrane-damaged, and membrane-compromised cells.

The results obtained from the plate count analysis correlated with the absorbance data. In contrast, the flow cytometric analysis results did not correlate with the results obtained by traditional methods; in fact, this technique showed that there were viable cells even when the optical density was low or no longer detectable and there was no plate count value. According to their results, flow cytometry is a suitable method for assessing the viability of bacteria in water samples. Furthermore, it permits fast detection of bacteria that are in a viable but nonculturable state, which are not detectable by conventional methods.

Similar study was proposed to McHugh *et al.* (2007) who investigated FCM for the detection of bacteria in cell culture production medium, using a nucleic acid stain, thiazole orange, which binds to nucleic acids of viable and nonviable organisms. They analyzed different bacteria: Gram positive (*Microbacterium species*) and Gram negative (*Acinetobacter species*, *Burkholderia cepacia*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*) vegetative bacteria, and Gram positive spore former (*Bacillus cereus*).

Loehfelm T.W. (2008) proposed a new application of FCM: identification and characterization of protein associated to biofilm in *Acinetobacter baumannii*, an opportunistic pathogen that is particularly successful at colonizing and persisting in the hospital environment, able to resist desiccation and survive on inanimate surfaces for months (Kramer, 2006). The authors have identified a new *A. baumannii* protein, Bap, expressed on the surface of these bacteria that is involved in biofilm formation in static culture, and that is detectable with FCM applied the following settings: forward scatter voltage, E02 (log); side scatter voltage, 582 (log); FL1 voltage, 665 (log); event threshold, forward scatter 434 and side scatter 380.

Weiss Nielsen and collaborators (2011) proposed an interesting video-protocol for detection of *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* present in biofilm by flow cell system.

Tracy *et al.* (2008) described the development and application of flow-cytometric and fluorescence assisted cell-sorting (FACS) techniques for study endospore-forming bacteria. In particular, they showed that by combining flow-cytometry light scattering with nucleic acid staining it's possible discriminate, quantify, and enrich all sporulation associated morphologies exhibited by the endospore-forming anaerobe *Clostridium acetobutylicum*. By

light scattering discrimination they detect the temporal aspects of sporulation, accurately quantify the proportion of the population participating in sporulation, and sort cultures into enriched populations for subsequent analysis. By coupling with nucleic acid staining (SYTO-9 plus PI), they effectively discriminated between different sporulation-associated phenotypes, and by using FACS they were able to enrich for the various sporulation phenotypes.

### 3.1.1.1 Bacterial detection and live/dead discrimination by flow cytometry

Flow cytometry is a sensitive analytical technique that can rapidly monitor physiological states of bacteria (reproductively viable, metabolically active, intact, permeabilized) and can be readily applied to the enumeration of viable bacteria in a biological sample (Khan, 2010).

Accurate determination of live, dead, and total bacteria is important in many microbiology applications.

Traditionally, viability in bacteria is synonymous with the ability to form colonies on solid growth medium and to proliferate in liquid nutrient broths.

FCM makes specificity of different fluorochrome-labeled antibodies to binding at specific antigens present in the surface of microorganisms for their identification in short period of time (less than 2 h), but with the extent of availability of specific antibodies.

The first fluorochrome used to detect bacteria was ethidium bromide in association with light-scatter signal, and the second was propidium iodide (PI).

Live cells have intact membranes and are impermeable to dyes such as PI which only leaks into cells with compromised membranes, while thiazole orange (TO) is a permeant dye and enters all cells, live and dead, to varying degrees. With gram-negative organisms, depletion of the lipopolysaccharide layer with EDTA greatly facilitates TO uptake. Thus a combination of these two dyes provides a rapid and reliable method for discriminating live and dead bacteria. An intermediate or “injured” population can often be observed between the live and dead populations.

It is possible to create a gating strategy for bacterial populations (es. *Escherichia coli*) staining the sample with thiazole orange (TO) and propidium iodide (PI), and analyze FSC vs SSC dot plot. You can set liberally a region (R1) around the target population and another (R2) around the beads. Then you can analyze FL2 vs SSC dot plot setting another region (R3) around the stained bacteria. At this point you can observed FL1 vs FL3 dot plot gated on (R1 or R2) and R3, with regions set around the live, “injured” and dead bacterial populations.

Very interesting is the work that Khan *et al.* have proposed in 2010 on enumeration of viable but non-culturable and viable-culturable Gram-Negative Bacteria using flow cytometry.

The traditional culture methods for detecting indicator and pathogenic bacteria in food and water may underestimate numbers due to sub-lethal environmental injury, inability of target bacteria to take up nutrient components in the medium, and other physiological factors which reduce culturability; however, these methods are also time-consuming and cannot detect non-culturable (VBNC) cells. An issue of critical about microbiology is the ability to detect viable but non-culturable (VBNC) and viable-culturable (VC) cells by methods other than existing approaches. Culture methods are selective and underestimate the real population, and other options (direct viable count and the double-staining method using epifluorescence microscopy and inhibitory substance-influenced molecular methods)



are also biased and time-consuming. A rapid approach that reduces selectivity, decreases bias from sample storage and incubation, and reduces assay time is needed (Davey, 1996).

Flow cytometry is a sensitive analytical technique that can rapidly monitor physiological states of bacteria. This report outlines a method to optimize staining protocols and the flow cytometer instrument settings for the enumeration of VBNC and VC bacterial cells within 70 min (Khan 2010), using SYTO dyes with different fluorescent probes (SYTO 9, SYTO 13, SYTO 17, SYTO 40) for detection of total cells and PI for detection of dead cells.

Khan *et al.* (2010) reported a study using FCM methods to detect cells with intact and damaged membranes. They assumed that cells having intact membranes are live (VC) and those with damaged membranes are dead or theoretically dead (VBNC).

The main objective of this study was to establish the quickest, most accurate, and easiest ways to estimate the proportions of VBNC and VC states and dead cells, as indicated by membrane integrity of these four Gram-negative bacteria: *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, and *Salmonella enterica* serovar Typhimurium (Khan, 2010).

The FCM data were compared with those for specific standard nutrient agar to enumerate the number of cells in different states. By comparing results from cultures at late log phase, 1 to 64% of cells were nonculturable, 40 to 98% were culturable, and 0.7 to 4.5% had damaged cell membranes and were therefore theoretically dead. Data obtained using four different Gram-negative bacteria exposed to heat and stained with PI also illustrate the usefulness of the approach for the rapid and unbiased detection of dead versus live organisms.

Similar analysis was proposed by McHugh (2007) for detection of Gram positive and Gram negative vegetative bacteria (*Acinetobacter species*, *Burkholderia cepacia*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *Mycobacterium species*, and *Bacillus cereus*).

Another way in which FCM can achieve direct diagnosis is by use of different-sized fluorescent microspheres coated with antibodies against microbes. In this case is possible determine the absolute count of bacteria per unit of volume present in the sample analyses using following equation:

$$\frac{\text{\# of events in region containing cell population}}{\text{\# of events in bead population}} \times \frac{\text{\# of beads per test}}{\text{test volume}} \times \text{dilution factor} = \text{Concentration of bacterial population}$$

Flow cytometry (FCM) has potential as an alternative method for the quantification of fluorescently labeled bacterial cells in drinking water; it is fast, accurate, and quantitative.

Füchslin *et al.* (2010) have proposed an interesting method for the rapid and quantitative detection of *Legionella pneumophila* in water samples in according to ISO 11371. The method comprised concentrating by filtration and resuspension, immunostaining followed by immunomagnetic separation using labeling with paramagnetic MicroBeads (size 50 nm), separation on a high-gradient column, and finally flow cytometric detection. The individual steps of the procedure were separately validated under laboratory conditions, and the

results were compared with established standard methods such as cell enumeration with fluorescence microscopy and colony-forming units on selective agar plates. Furthermore, the whole method was tested with spiked tap water, and the detection limit was determined.

Use of fluorescent stains or fluorogenic substrates in combination with FCM allows the detection and discrimination of viable culturable, viable nonculturable, and nonviable organisms, can be used to microbial analysis of milk. *Gunasekera et al. (2000)* have demonstrated the potential application of flow cytometers in milk analyses developing a rapid method (less than 60 minutes) for detecting of total bacteria (*Gunasekera, 2000*). The authors have considered as potential contaminants of milk for represent gram-negative rods *Escherichia coli* and for gram-positive cocci *Staphylococcus aureus*.

Pure populations of *E. coli* and *S. aureus* were easily detected by FCM when they were suspended in phosphate-buffered saline (PBS), but when they were inoculated into ultra-heat-treated (UHT) milk, no distinct separation appeared. This is due to the presence of proteins and lipid globules that can bind nonspecifically to fluorescent stains and interfere with staining and detection of bacteria. Treatment of milk by centrifugation to remove lipids without also treating samples with proteases was insufficient to allow definition of bacteria. For these reason the authors have applied enzymatic treatment with protease K or savinase to remove or modify proteins and thereby enable distinction of bacteria by flow cytometry. The FCM procedure described estimates numbers of total bacteria in the processed sample, since SYTO BC binds to live culturable, live non-culturable, and dead cells.

This study demonstrates the ability of FCM to determine total bacterial numbers after clearing of milk and staining of bacteria with a readily available fluorescent stain (SSC vs green fluorescence). The sensitivity of the FCM procedure was  $\leq 10^4$  total bacteria ml of milk<sup>-1</sup>.

*Pianetti et al. (2005)* proposed a protocol for the determination of the viability of *Aeromonas hydrophila* in different types of water by flow cytometry and compared this results with classical methods as spectrophotometric and plate count techniques. Flow cytometric determination of viability was carried out using a dual-staining technique that enabled us to distinguish viable bacteria from damaged and membrane-compromised bacteria, using simultaneous permeable (SYBR Green dyes) and impermeable fluorescent probe (PI). The traditional methods showed that the bacterial content was variable and dependent on the type of water. The results obtained from the plate count analysis correlated with the absorbance data. In contrast, the flow cytometric analysis results did not correlate with the results obtained by traditional methods; in fact, this technique showed that there were viable cells even when the optical density was low or no longer detectable and there was no plate count value. Furthermore, it permits fast detection of bacteria that are in a viable but nonculturable state, which are not detectable by conventional methods.

FCM can be used to demonstrate multiplexed detection of bacteria and toxins using fluorescent coded microspheres.

Antibodies specific for selected bacteria and toxins were conjugated to the coded microspheres to achieve sensitive and selective binding and detection. The respective limits of detection for bacteria and toxin are different (*Kim, 2009*). The microflow cytometer can detect for *Escherichia coli*, *Listeria*, and *Salmonella*  $10^3$ ,  $10^5$ , and  $10^4$  cfu/mL, respectively, while the limits of detection for the toxins as cholera toxin, staphylococcal enterotoxin B, and ricin were 1.6, 0.064, and 1.6 ng/mL respectively (*Kim, 2009*).

### 3.1.2 Fungi

The use of FCM to detect fungal pathogens was first described by *Libertin et al.* in 1984 for *Pneumocystis carinii* (now *Pneumocystis jirovecii*) and following was evaluated by *Lapinsky* in 1991.

*Pneumocystis jirovecii* is an opportunistic pathogen responsible for severe pneumonia in immune-compromised patients. Its diagnosis has been based upon direct microscopy either by classic staining or by epifluorescence microscopy (immunofluorescence staining, IFS), both of which are time-consuming and low on sensitivity. Its aim was to develop a flow cytometric (FC) protocol for the detection of *P. jirovecii* on respiratory samples. *Barbosa et al.* (2010) analyzed in parallel by IFS and FC, 420 respiratory samples and compared the results with clinical diagnosis to its resolution upon specific anti-Pneumocystis therapy. The optimum specific antibody concentration for FC analysis was determined to be 10 µg/ml, without any cross-reactions to bacteria or fungi. All positive cases detected by IFS were positive by FC; however, FC classified eight samples to be positive which were classified as negative by routine technique. These samples were obtained from patients with respiratory symptoms who responded favourably to Pneumocystis-specific therapy and were subsequently considered to be true-positives. Using clinical diagnosis as a reference method, FC showed 100% sensitivity and specificity, whereas IFS showed 90.9% sensitivity and 100% specificity. According to their results, a new diagnostic approach is now available to detect *P. jirovecii* in respiratory samples.

*Prigione et al.* (2004) proposed an alternative study to traditional methods for the enumeration of airborne fungi: the possibility to evaluate by FCM the assessment of exposure to the fungus aerosol. They compared FCM with epifluorescence microscopy direct counting (gold standard). Setting up of the method was achieved with pure suspensions of *Aspergillus fumigatus* and *Penicillium brevicompactum* conidia at different concentrations, and then analyses were extended to field samples collected by an impinger device. Detection and quantification of airborne fungi by FCM was obtained combining light scatter and propidium iodide red fluorescence parameters. Since inorganic debris are unstainable with propidium iodide, the biotic component could be recognized, whereas the preanalysis of pure conidia suspensions of some species allowed us to select the area corresponding to the expected fungal population. Moreover, data processing showed that FCM can be considered more precise and reliable at any of the tested concentrations, and suggest that FCM could also be used to detect and quantify airborne fungi in environments different, including agricultural environments (*Prigione, 2004*).

*Page et al.* (2005) have developed two assays utilizing two different methods capable of identifying clinically important ascomycetous yeast species in a single-well test. They identified different species of *Candida* (*C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*) using a direct hybridization method and allele-specific primer extension method. The amplicons are analyzed by FCM.

### 3.1.3 Parasites

FCM may also be applied to study parasites included analysis of the cell cycle, DNA quantification and analysis of membrane antigens. Specific clinical applications came later, when used associations of monoclonal antibodies, FCM, and immunofluorescence microscopy

for the direct identification of parasites, as *Naegleria fowleri* and *Acanthamoeba spp.*, in clinical specimens (Flores, 1990).

Other applications of FCM regarding malaria's detection. The diagnosis of malaria is primarily cell-based and involves visual detection of intraerythrocytic parasites by transmitted light microscopy in a peripheral blood smear stained with Giemsa's stain, a mixture of eosin and methylene azure dyes first described over a century ago. Identification of the various stages of parasites depends heavily on morphologic information, requiring observation at high power. Although it has been known for many years that methods based on fluorescence microscopy, using acridine orange and other dyes, compare in accuracy with light microscopy and may require less time and a less skilled observer, the required fluorescent microscope has, until recently, been too expensive for most laboratories in areas where malaria is most prevalent. If malaria were more common in affluent countries, we might expect that cytometry would, by now, have supplanted microscopy of Giemsa stained smears for malaria diagnosis, just as it has for differential leukocyte counting and reticulocyte counting.

In clinical diagnosis, it is important to distinguish between malaria caused by *Plasmodium falciparum* and malaria due to the other species (Shapiro, 2007), because microscopy is an imperfect "Gold Standard" diagnostic device (Makler, 1998).

Many recent publications on cytometry in malaria (Li, 2007) have used asymmetric cyanine nucleic acid dyes of the SYTO and YOYO series. These dyes, structurally related to thiazole orange (Makler, 1987), can be excited with blue or blue-green (488 nm) light and emit in the green or yellow spectral region. Unlike acridine orange, which quenches on binding to nucleic acids, the cyanines are not DNA-selective and enhance fluorescence substantially on binding, typically by a factor of 1,000 or more; this results in lower background fluorescence, which makes it easier to detect smaller (haploid) forms of the malaria parasite (Shapiro, 2007; Shapiro, 2010).

Several approaches have been developed in the last few years to detect intracellular parasites, such as *Plasmodium*; but these all rely on clinical suspicion and, consequently, an explicit clinical request. Such work took advantage of the absence of DNA in erythrocytes. Thus, if the parasite is inside the cell, its DNA can be stained with specific fluorochromes and detected by FCM. The multiparameter analysis permitted by FCM can be used to study other characteristics, such as parasite antigens expressed by the erythrocyte (which can be detected by antibodies conjugated with fluorochromes) or the viability state of the parasitised cell using fresh or fixed cells (Janse, 1994; Jouin, 1995).

Although some methods lend themselves to automation (e.g. flow cytometry), no technique can yet be used for routine clinical automated screening. Li et al. (2007) have recently proposed a new methodology to measure a parasitemia of *Plasmodium falciparum* using flow cytometry analysis, because the microscopic analysis of patient blood smears represent an imperfect "Gold Standard" diagnostic device (Makler, 1987). In fact, there was reported significant misdiagnosis with regard to false positives (7–36%), false negatives (5–18%), and false species (13–15%) and an high frequency of technical errors (e.g. wrong pH or a poor quality film).

Different dyes, such as Hoechst 33258 (Brown, 1980), acridine orange (Whaun, 1983), thiazole orange (Makler, 1987), or hydroethidine (Wyatt, 1991), have been considered for the determination of parasitemia in cultures of *P. falciparum* by FCM.



Jacobberger *et al.* (1984) used DiOC1, a membrane potential responsive dye and Hoechst 33342 to evaluate parasitemia levels in mice (Makler, 1987). YOYO-1, a dimeric cyanine nucleic acid dye, is among the highest sensitivity fluorescent probes available for nucleic acid staining and has been added to this list (Rye 1992; Barkan 2000). YOYO-1 has an extremely high affinity for DNA, and it can be excited at 488 nm, which is the excitation wavelength available from most lasers employed in FCM. Its bright fluorescence signal and low background make it ideal for flow cytometric analysis of stained malaria nucleic acids (Barkan, 2000; Jimenez-Diaz, 2005; Xie, 2007). The FCM analysis in cultured *P. falciparum* models of malaria is impeded by significant reduction of reticulocytes and normocytes containing detectable amounts of nucleic acids after blood treated. Therefore, the absence of reticulocytes and normocytes may reduce the interference in measurement of parasitemia (Hirons, 1994). Li *et al.* (2007) compared FCM method to traditional microscopic analysis of blood smears and the microdilution radioisotope method for the evaluation of parasitemia in parasite culture with *P. falciparum*. They report a dual-parameter procedure using autofluorescence to make a distinction of infected erythrocytes from uninfected erythrocytes and normocytes. This method is particularly well suited for measuring low and high parasitemias and significantly increased the sensitivity.

Several reports show YOYO-1 is better than Hoechst 33258 to easily differentiate between uninfected and infected RBC when parasitemia is low. The parasites in the reticulocytes population should exhibit the same YOYO-1 associated fluorescence intensity as the parasites in the normal RBC. Compensation of YOYO-1 emission in FL-2 is an essential step whose only objective is to set up accurately the region of infected cell events. The region must be empirically determined by comparison of blood samples from uninfected and malaria infected rats by increasing compensation of YOYO-1 emission in FL-2 until a defined region for infected cell events is obtained (Li, 2007).

Other important parasite for malaria is *Plasmodium vivax* which preferentially invades reticulocytes. It is therefore relevant for vaccine development purposes to identify and characterize *P. vivax* proteins that bind specifically to the surface of reticulocytes. Tran *et al.* (2005) have developed a two-color flow cytometric erythrocyte binding assay (F-EBA) using the *P. vivax* Duffy binding protein region II (PvDBP-RII) recombinant protein as a model. This protein binds to all erythrocytes that express the Duffy receptor (Fy) and discriminates binding between normocytes and reticulocytes. This technique have several advantages over traditional erythrocyte binding assays (T-EBAs) used in malaria research. Interesting is the Malaria's detection with haematology analysers, as diagnostic tool in the work-up of febrile patients. For more than a decade, flow cytometry-based automated haematology analysers have been studied for malaria diagnosis, and recently work for incorporate into modern analysers different "malaria alert" especially in scenarios with low pre-test probability for the disease. Ideally, a flag for malaria could be incorporated and used to guide microscopic evaluation of the patient's blood to establish the diagnosis and start treatment promptly. Automation of a "malaria alarm" is currently possible for some analysers as Cell-Dyn®, Coulter® GEN S and LH 750, and the Sysmex XE-2100®.

The Cell-Dyn instruments use a multiple-angle polarized scatter separation for WBC analysis to distinguish eosinophils from neutrophils based on the light depolarizing properties of their granules, but has also been found to detect haemozoin-containing monocytes and granulocytes. The malaria-related events are shown in a scatter-plot with 90°



side-scatter on the x-axis and 90° depolarized side-scatter on the y-axis. Coulter GEN S and LH 750 haematology analysers use Volume-Conductance-Scatter (VCS) technology to obtain positional parameters of all WBC by measuring impedance for cell volume; radiofrequency conductivity for internal structure and nuclear characteristics; and flow cytometry-based helium-neon laser light scatter analysis for cellular granularity, nuclear lobularity and cell surface structure.

The Sysmex XE-2100 automated haematology analyser uses combined impedance and radiofrequency conductance detection, semiconductor diode laser light 90° side-scatter (SSC) and 0° frontal-scatter (FSC) detection, and polymethyne fluorescence nucleic acid staining 90° side-fluorescence (SFL) detection (Campuzano-Zuluaga, 2010).

Other applications of FCM regarding the use of FCM in combination with immunofluorescence, conventional or immunofluorescence microscopy for detection of samples containing small number of cysts as *Giardia lamblia* (Dixon, 1997).

In recent years, flow cytometry has been gaining in popularity as a novel method of detecting and enumerating different parasites as *Giardia* cysts and *Cryptosporidium* oocysts present in environmental and fecal samples (Ferrari, 2003; Moss, 2001; Power, 2003). Many papers reported flow cytometry as a method more sensitive than either conventional or immunofluorescence microscopy for the detection of *Giardia* sp. cysts in fecal samples (Dixon, 1997; Dixon, 2002; Ferrari, 2003), in detection of *Cryptosporidium* in SCID mice (Arrowood, 1995), seeded horse feces (Cole, 1999), and seeded human stool specimens (Valdez, 1997). In addition to detection and enumeration, large-scale sorting could also be used in conjunction with flow cytometry to yield partially purified oocysts for research purposes, such as food-spiking and recovery experiments, viability determination, or molecular characterization (Dixon, 2005).

All *Cryptosporidium* and *Giardia* surface monoclonal antibodies (mAbs) isolated thus far are directed against the same immunodominant epitope, therefore independent noncompeting mAbs are not available. Multiparameter FCM analysis largely depends on the use of noncompeting mAbs to quantify phenotype percentages or cellular activation.

Ferrari *et al.* (2003) proposed an analysis for detection of *Giardia* who combining Immunomagnetic Separation (IMS) and Two-Color flow cytometry (Green fluorescence vs Orange fluorescence). In particular a two-color assay using competing surface mAbs has been developed for the detection of *Cryptosporidium* oocysts. Regions were defined around mAb-PE-stained cysts (R1), FITC-stained cysts (R2), and dual-stained cysts (R3); a gate was defined whereby any particle present within R1, R2, and R3 was positive and was sorted on membranes for microscopic confirmation.

In this assay the immunoglobulin G1 (IgG1) oocyst wall-specific mAb CRY104 was conjugated to phycoerythrin (PE) and fluorescein isothiocyanate (FITC). The greatest specificity in water was obtained with this combination over other *Giardia* cysts were spiked into a backwash water sample with and without prior hybridization to peptide nucleic acid (PNA) probes. Immunomagnetic separation (IMS) as a pre-enrichment step was compared with filtration of the water sample. Cysts were recovered with two-color FCM. Those cysts hybridized with PNA and fluorescein isothiocyanate (FITC) were dual stained with monoclonal antibody (mAb) conjugated to phycoerythrin (PE); those not hybridized to PNA were dual stained with mAb-FITC and mAb-PE. A fourfold increase in fluorescent signal

intensity was obtained when combining the mAb-PE and PNA probe compared with two-color antibody staining. When combined with IMS, *Giardia* was successfully identified by FCM, with no false positives detected. Analysis-only FCM detection of *Giardia* in water is feasible. Further method development incorporating PNA probe hybridization after IMS is necessary.

Moreover, the authors developed PNA probes directed to *Cryptosporidium* oocysts, so a dual *Cryptosporidium* and *Giardia* detection method is possible. This method described can be used in small cytometers with no cell sorting capabilities. Such a system would provide a rapid, online method for screening water samples. This method using PNA probes also was species specific; therefore, water utilities would gain important information on the potential public health risks of a contamination event. This research demonstrated that analysis-only FCM detection of *Giardia* is feasible. To achieve the sensitivity required, a combination of IMS and two-color immunofluorescence staining with independent probes (mAb and PNA probe) was necessary, followed by two-color FCM analysis. Although we could detect the cysts, the cyst seed used was hybridized to the PNA probes before spiking in water. Further method development is required for the hybridization of cysts to be carried out after IMS and before FCM.

*Cryptosporidium parvum* is transmitted through water and can cause severe diarrhea. The diagnosis is usually based upon observer-dependent microscopic detection of oocysts, with rather low sensitivity and specificity. *Barbosa et al. (2008)* recently proposed a study with an objective to optimize a FCM protocol to detect *Cryptosporidium parvum* oocysts in spiked human stools, using specific monoclonal antibodies. In particular, a specific monoclonal antibody conjugated with R-phycoerythrin was incubated with dead oocysts to determine the optimal antibody concentration, which was calculated in 3.0 mg/ml. Staining procedure was specific, as no cross-reactions were observed. This reliable and easy FC protocol allows the specific detection of *Cryptosporidium* oocysts, even at very low concentrations, which is important for public health and further studies of treatment efficacy.

Comparison of fluorescence signal intensities of *Cryptosporidium parvum* oocysts analyzed at FL2 showing autofluorescence of  $2 \times 10^5$  oocysts/ml and different concentrations of labeled oocysts with specific antibody (R-Phycoerythrin vs Counts) (*Barbosa, 2008*).

*Dixon et al. (2005)* involves an evaluation of the effectiveness of flow cytometry for the detection and enumeration of *Cyclospora cayentanensis* oocysts in human fecal specimens. Using flow cytometry, oocysts could be separated according to their autofluorescence, size, and complexity, and a cluster representing *Cyclospora* oocysts could be clearly observed on the dot plots of positive samples (autofluorescence vs SSC, with gate region R1 for *Cyclospora* oocysts). *Dixon et al.* concluded that while the sample preparation time for flow cytometry may be similar to or even longer than that for microscopy, depending upon the concentration and staining procedures used, the time it takes to analyze a sample by flow cytometry is considerably shorter than the time it takes to analyze a sample by microscopy. Sample analysis took only minutes, whereas microscopic examination is often a very time-consuming procedure. As a result, a larger number of samples could be analyzed by flow cytometry in a relatively short period of time. More importantly, as the method is largely automated, the results are not influenced by an analyst's levels of fatigue and expertise, as they may be with microscopy. While stool specimens are generally not examined for *Cyclospora* oocysts unless specifically requested, the results of the present study suggest that

flow cytometry may be a useful alternative to microscopy in the screening of large numbers of fecal specimens for the presence of *Cyclospora* oocysts.

### 3.1.4 Virus

With FCM is possible to detect and quantify virus-infected cells present simultaneously (directly) in clinical samples, using antibodies that specifically recognize surface or internal antigens. We can investigate particular components of virus, as proteins or nucleic acids.

For example FCM could be applied to the detection of animal or human viruses in different clinical sample, such as the simultaneous detection of CMV, HSV, and HBV in organs destined for transplantation as well as in transplanted patients and co-infections in HIV-infected individuals.

It's possible discriminate stages of virus antigen expression (es. immediate-early, early, late) using monoclonal antibodies, detect viremia or FCM can be applied in a single device for the detection of HIV-1 viral load in association with other parameters to monitoring in the blood the success of antiretroviral therapy (HAART), as: CD4 Tcell count, CD4 percentage of lymphocytes (CD4%), and viral load (Greeve, 2009).

Greeve *et al.* (2009) proposed a new viral load test based on flow cytometric to detect HIV-1 viral load. They performed a FCS/SSC plot, and set a gate on each population, using PE-specific fluorescence and measure the sample in fluorescence 2 (FL2 with a 590 nm bandpass filter). In particular ten thousand events were collected, and the mean fluorescence intensity was calculated by setting a range over the whole measuring scale (0-1,000) in FL2 (log 4) separately for each microbead population.

Different methods have been routinely used to detect specific antibodies to viral antigens (ELISA, complement fixation, indirect immune-fluorescence microscopy, Western blotting), but the detection and quantification of antibodies to viral antigens can be carried out by FCM.

This technique has been used to detect and quantify antibodies to CMV, herpesvirus (HSV-1 and HSV-2), hepatitis C virus (HCV) and HIV-1 virus.

#### 3.1.4.1 Detection and quantification of viral antigens

FCM can detect viral antigens other on the surface and/or within infected cells. It can rapidly detect and quantify virus-infected cells using antibodies that specifically recognize surface or internal antigens; in the latter case, permeabilization of the cells is required.

Direct and indirect fluorescent-antibody methods are used. Direct detection involves the use of fluorescently labeled antibody (labeled with FITC or phycoerythrin). In the indirect fluorescent-antibody method, unlabeled antibody is bound to infected cells, which are then incubated with fluorescence-labeled anti-Ig that binds to the first viral antibody.

Based on the potential of FCM for multiparametric analysis, there are two key advantages to its use in studying viral infection: its ability to analyze several parameters in single infected cells at the same time and its ability not only to detect but also to quantify infected cells. These parameters may be related to particular components or events of the infected cell or components (proteins or nucleic acids) of the virus. For this reason, FCM has been a powerful

tool to characterize the mechanisms of viral pathogenesis. Furthermore, FCM allows simultaneous detection of several viruses in a sample by using antibodies to different viral antigens conjugated to different fluorochromes, or specific viral antibodies conjugated to latex particles of different sizes. As stated above, the presence of different viral antigens is detected by differences in the forward-scattered light as a consequence of the different sized particle used for each antibody. Different plant, animal or human viruses in any clinical sample is possible simultaneously detected.

#### 3.1.4.2 Detection and quantification of viral nucleic acids

The emergence of PCR and RT-PCR techniques has allowed the highly sensitive detection of specific viral nucleic acids (DNA or RNA) in virus-infected cells. These methods are indeed the most sensitive for the detection and characterization of viral genomes, especially in rare target viral sequences. However, the association between the viral nucleic acid and an individual cell is lost, and therefore no information about productively infected cell populations is obtained by this method. FCM analysis of fluorescent in situ hybridization in cell suspension overcomes this problem, since this assay can be coupled with simultaneous cell phenotyping (by using specific antibodies to different cell markers).

With FCM is possible to monitor EBV-infected cells in blood sample using in situ hybridization combined use with two or more fluorochromes.

### 3.2 Serological diagnosis

The diagnosis of acute hepatitis C virus (HCV) infection is based on the detection in serum or plasma of HCV RNA, anti-HCV IgG, and elevation of alanine aminotransferase levels. However, none of these markers alone or in combination can be used to identify acute infection, since they may also be detectable during the chronic phase of infection.

*Araujo et al. (2011)* developed a multiplexed, flow-cytometric microsphere immunoassay, to measure simultaneously anti-HCV-IgG responses to multiple structural (E1, E2, core) and nonstructural (NS3, NS4, NS5) HCV recombinant proteins. Furthermore this assay has the potential to discriminate between the acute and chronic phases by testing of single specimens (*Araujo, 2011*).

The detection of specific antibody to HCV is an important assay in the identification of individuals infected with HCV. Routinely screening (enzyme immunoassay or EIA) and confirmation analysis (recombinant immunoblot assay or RIBA) of blood sample is well addressed using the commercially available assays. *McHugh et al. (2005)* considered that the increased rate of false-positive antibody test results coupled with the data indicating that the concentration of HCV specific antibody can help to indicate the likelihood of antibody positivity proposed a semi-quantitative assay to improvement the resolution of low levels of specific HCV antibody using microsphere assay.

*Bhaduri-McIntosh et al. (2007)* propose flow cytometry-based assay to investigated IgA antibodies are a marker for primary Epstein-Barr virus (EBV) infection, and compared this assay to presence of IgM antibodies to viral capsid antigen and the absence of antibodies to EB nuclear antigen (EBNAs). The authors compare the occurrence of IgA serologic responses to EBV total antigens and early antigen (EAs) during primary EBV infection and



in healthy individuals persistently infected with EBV, for differentiation between individuals with primary EBV infection and healthy EBV-seropositive individuals, using flow cytometry-based assay.

The use of flow cytometry to measure the number of infected cells has been demonstrated previously for other types of viruses (McSharry, 2000). These methods were aimed at the measurement to quantify virus infectivity in a sample previously infected with a virus suspension and relied on the assumption that only one round of infection had occurred and that virus adhesion is quantitative and synchronous. These has been described for various enveloped and non-enveloped viruses.

These studies are based on direct enumeration of infected cells in the flow cytometer and discrimination from noninfected cells by immunostaining using monoclonal antibodies specific for viral antigens.

Recently Gates *et al.* (2009) introduced a semi-automated flow cytometry protocol to quantitative measurement of Varicella-Zoster Virus Infection. They describe an alternate infectivity assay for the attenuated VZV strain, based on the enumeration of infected cells 24 to 72 h post-infection by semi-automated capillary flow cytometry. The discrimination of infected cells from non infected cells is performed by indirect immunofluorescence to detect the expression of viral glycoproteins on the surface of infected cells. The new assay provides a rapid, higher-throughput alternative to the classical plaque assay. It was used a semi permeable vitality dye (7-AAD) to identify and quantify live-infected cells.

Measurement of VZV infection using flow cytometry was made considering FSC ( $x$  axis) and red fluorescence intensity channel (RED;  $y$  axis) correlation dot plot showing selective gating on 7-AAD-negative events (live cells and non nucleated debris) and tested monoclonal antibodies which are directed at both immediate early/early genes (IE62) and late, structural proteins (VZV gE, gI, gB, and gH and MCP) and were titrated to measure mean fluorescence intensities at saturation (Gates, 2009).

Lemos *et al.* (2007) developed a flow cytometry-based methodology to detect anti-*Leishmania* (*Leishmania*) *chagasi* immunoglobulin G as a reliable serological approach to monitor post-therapeutical cure in patients affected by Visceral Leishmaniasis. They have demonstrated that although conventional serology (indirect immunofluorescence and enzyme-linked immunosorbent assay) remained positive after treatment, the antimembrane-specific antibodies detected by flow cytometry were present only during active disease and not detected after successful chemotherapy.

Similar work was proposed by Rocha *et al.* (2006) for clinical value of anti-live *Leishmania* (*Viannia*) *braziliensis* immunoglobulin G subclasses for diagnosing active localized cutaneous leishmaniasis.

### 3.3 Antimicrobial effects and susceptibility testing by flow cytometry

In the 1990s, there were interesting advances in this field from microbiology laboratories, and the number of scientific articles addressing the antimicrobial responses of bacteria (including mycobacteria), fungi, and parasites to antimicrobial agents increased considerably (Gant, 1993).



FCM has proved to be very useful for studying the physiological effects of antimicrobial agents (bactericidal or bacteriostatic effect) on bacterial cells due to their effect on particular metabolic parameters (membrane potential, cell size, and amount of DNA).

Pina-Vaz *et al.* (2005) described a flow cytometric assay, simple, fast, safe and accurate, to assess the susceptibility of *Mycobacterium tuberculosis* to the antimicrobial susceptibility (streptomycin, isoniazid, rifampicin, ethambutol) and compared it with standard laboratory procedure (BACTEC MGIT 960). The described assay is a quick, safe and accurate method, as heat-inactivated mycobacteria cells are analysed following staining with SYTO 16, a nucleic acid stain, which distinguishes them from debris. The time needed to obtain susceptibility results of *Mycobacterium tuberculosis* using classical methodologies is still too long (two months), and flow cytometry is a promising technique in the setting of the clinical laboratory, giving fast results. A safe, reliable and rapid method to study susceptibility to streptomycin, isoniazide, rifampicin and ethambutol is described. Isolates of mycobacteria, grown for 72 h in the absence or presence of antimycobacterial drugs in the mycobacteria growth indicator tube (MGIT), were heat-killed, stained with SYTO 16 (a nucleic acid fluorescent stain that only penetrates cells with severe lesion of the membrane) and then analysed by flow cytometry. Comparing the intensity of fluorescence of *Mycobacterium* cells incubated with antimycobacterial drugs with that of drug-free cells, after staining with SYTO 16, it was possible to distinguish between sensitive, intermediate and resistant phenotypes. Bacterial cells respond to different antimicrobial agents by decreasing or increasing their membrane potential. Antimicrobial susceptibility shows a decrease in green fluorescence (live cells) and an increase in red fluorescence (dead cells).

Piuri *et al.* (2009) describe a virus-based assay in which fluoro-mycobacteriophages are used to deliver a GFP or ZsYellow fluorescent marker gene to *M. tuberculosis*, which can then be monitored by fluorescent detection approaches including fluorescent microscopy and flow cytometry. Pre-clinical evaluations show that addition of either Rifampicin or Streptomycin at the time of phage addition obliterates fluorescence in susceptible cells but not in isogenic resistant bacteria enabling drug sensitivity determination in less than 24 hours. Detection requires no substrate addition, fewer than 100 cells can be identified, and resistant bacteria can be detected within mixed populations. Fluorescence withstands fixation by paraformaldehyde providing enhanced biosafety for testing MDR-TB and XDR-TB infections (Piuri, 2009).

### 3.4 Monitoring of infections and antimicrobial therapy

FCM can be used to monitor patient's responses to antimicrobial treatments during the infections' treatment with antimicrobial therapy.

Rudensky *et al.* (2005) develop a rapid flow-cytometric antifungal susceptibility test for determining susceptibility of different species of *Candida* to fluconazole and echinocandin, and compare results with the standard methods (MIC determined by macrodilution and/or Etest according to National Committee for Clinical Laboratory Standard, now Clinical Laboratory and Standard Institute).

They used reference and laboratory strains of *Candida* (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*) who tested for susceptibility to fluconazole and echinocandin by

fluorescent flow cytometry using Acridine Orange (AO) as indicator of viability (AO fluorescence versus SSC). The flow method produced results in 5 h or less, and give excellent sensitivity and specificity to distinguish between sensitive, susceptible dose-dependent and resistant strains. The advantages of this method is to produce daily results and assist clinicians in the selection of appropriate antifungal therapy. The method is easy, reproducible, permit to measure the percentage of damaged yeasts in relation with drug concentration, and can be implemented in any laboratory with access to a flow cytometer.

In the same period, *Ramani et al.* (2003) test with FCM antifungal susceptibility of *Aspergillus fumigatus* against three important antifungal (voriconazole, amphotericin B and itraconazole) used in therapy of aspergillosis. The results obtained within 3 to 4 h proved to be a reliable indicator of a drug's antifungal activity against *A. fumigatus* isolates, and indicate a good correlation with the drug MICs obtained by the CLSI broth microdilution method.

Flow cytometry can be used to monitoring anti-leishmanial drugs susceptibility (pentavalent antimonial, pentamidine, amphotericin B, sodium stibogluconate). In particular, *Singh and Dube* (2004) proposed a flow cytometry assay based on green fluorescent protein a marker for *Leishmania* causing kala-azar (visceral leishmaniasis) and for transgenic *L. donovani* promastigotes that constitutively express GFP in their cytoplasm.

### 3.5 Other application of flow cytometry

Several strategies to optimize the detection of bacterial contamination in platelet preparations (PLTs) have been examined in the past. In fact, bacterial contamination is the major infectious hazard associated with transfusion of PLTs.

Screening of PLTs for bacterial contamination by prospective culture testing has been implemented as part of the quality assurance program in several blood services. Despite screening of PLTs for bacterial contamination by culture, it has been demonstrated that there is still a substantial infection risk associated with transfusing PLTs, and septic complications have been observed in recipients (*Dreier, 2009*).

Routine testing for bacterial contamination in PLTs has become common, but transfusion-transmitted bacterial sepsis has not been eliminated. *Dreier et al.* (2009) describe a new flow cytometry-based method for point-of-issue screening of PLTs for bacterial contamination. They used flow cytometry to detect and count bacteria based on esterase activity in viable cells, and compared the flow-cytometric assay to incubation culture system and rapid nucleic acid-based or immunoassay (reverse transcription PCR) methods.

Flow cytometry is rapidly becoming an essential tool in the field of aquatic microbiology. *Wang et al.* (2010) proposed an interesting application of FCM in aquatic microbiology, in the development range from straightforward total cell counts to community structure analysis, and further extend to physiological analysis at a single-cell level (*Wang, 2010*).

*Hammes and Egli* (2010) describe as the rapid detection of microbial cells is a challenge in microbiology, particularly when complex indigenous communities or subpopulations varying in viability, activity and physiological state are investigated. Numerous FCM applications have emerged 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. They focused the information that

can be gained from the analysis of bacteria in water, highlighting some of the main advantages, pitfalls and applications (Hammes, 2010).

In fact Comas-Riu and Rius (2009) proposed a mini-review to give an overview of the principles of flow cytometry and examples of the application of this technique in the food industry. By analysing large numbers of cells individually using light-scattering and fluorescence measurements, this technique reveals both cellular characteristics and the levels of cellular components. Flow cytometry has been developed to rapidly enumerate microorganisms; to distinguish between viable, metabolically active and dead cells, which is of great importance in food development and food spoilage; and to detect specific pathogenic microorganisms by conjugating antibodies with fluorochromes, which is of great use in the food industry. In addition, high-speed multiparametric data acquisition, analysis and cell sorting, which allow other characteristics of individual cells to be studied, have increased the interest of food microbiologists in this technique (Comas-Riu and Rius, 2009).

Penders *et al.* (2004) proposed an automated flow cytometry analysis of peritoneal dialysis fluid. Peritonitis is the major frequent complication of peritoneal dialysis fluid. The diagnosis and effective treatment of peritonitis depends on clinical evaluation and correlation with laboratory examination of the dialysate. Various techniques have been used to facilitate the recovery of microorganisms from dialysate, among them the use of selected broth media, processing of large volumes of dialysis effluent by concentration techniques or total volume culture. Nevertheless, microorganisms are not always recovered from dialysate during peritonitis. The authors have evaluated the possibilities to apply flow cytometry in the analysis of peritoneal dialysis fluid. In particular they have analyzed 135 samples with automated instrument Sysmex UF-100 and compared the obtained data with those of counting chamber techniques, biochemical analysis and bacterial culture (Penders, 2004). They concluded that flow cytometric analysis can be an useful additional tool for peritoneal dialysis fluid examination, especially in the emergency setting for detect leukocytes, bacteria and/or yeast cells.

Another application of flow cytometry regards screening of urine sample (Jolkkonen, 2010; Pieretti, 2011).

Urinary tract infection (UTI) is a widespread disease, and thus, the most common samples tested in diagnostic microbiology laboratories are urine samples. The “gold standard” for diagnosis is still bacterial culture, but a large proportion of samples are negative. Unnecessary culture can be reduced by an effective screening test. Pieretti *et al.* (2011) have evaluated the performance of a new urine cytometer, the Sysmex UF-1000i (Dasit), on 703 urine samples submitted to our laboratory for culture. They have compared bacteria and leukocyte (WBC) counts performed with the Sysmex UF-1000i to CFU-per-milliliter quantification on CPS agar to assess the best cutoff values. Different cutoff values of bacteria/ml and WBC/ml were compared to give the best discrimination. On the basis of the results obtained in this study, we suggest that when the Sysmex UF-1000i analyzer is used as a screening test for UTI the cutoff values should be 65 bacteria/ml and 100 WBC/ml. Diagnostic performance in terms of sensitivity (98.2%), specificity (62.1%), negative predictive value (98.7%), positive predictive value (53.7%), and diagnostic accuracy (73.3%) were satisfactory. The authors concluded that the screening with the Sysmex UF-1000i is acceptable and applicable for routine use because it has reduced the number of bacterial cultures by 43% and decreased the inappropriate use of antibiotics.

Similar study was proposed by Jolkkonen *et al.* (2010).

Li *et al.* (2010) have demonstrated as FCM represent a rapid and quantitative method to detect infectious Adenoviruses in environmental water and clearly distinguish them from inactivated viruses. This method has the potential for application in detection of infectious Adenoviruses in environments and evaluation of viral stability and inactivation during the water treatment and disinfection's process.

Cantera *et al.* (2010) described an alternative approach at classical serological and viral nucleic acid detection, that utilizes engineered cells expressing fluorescent proteins undergoing fluorescence resonance energy transfer (FRET) upon cleavage by the viral 2A protease (2Apro) as an indication of infection. Quantification of the infectious-virus titers was resolved by using flow cytometry, and utility was demonstrated for the detection of poliovirus 1 (PV1) infection.

Those methods, however, are time-consuming and labor-intensive, as the procedures included cell fixation, permeabilization, labeling, and washing steps prior to flow cytometry. Viral titers determined by FC were comparable to titers obtained by the plaque assay.

#### 4. Concluding remarks and future perspectives

In this chapter we remark the different applications of flow cytometry in clinical microbiology.

It's a wide field for many aspects still to be explored, and for future we think that it's very important introduce automation in routine of clinical microbiology laboratories the flow cytometric assay, but is necessary optimize the cost-benefit ratio of FCM.

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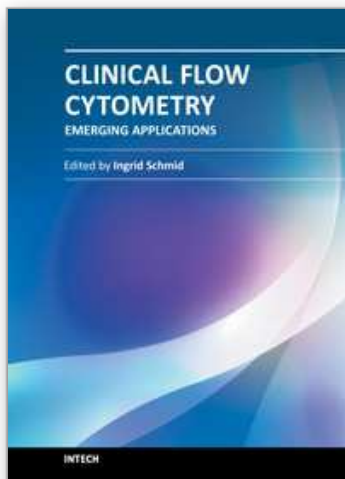
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"Clinical Flow Cytometry - Emerging Applications" contains a collection of reviews and original papers that illustrate the relevance of flow cytometry for the study of specific diseases and clinical evaluations. The chapters have been contributed by authors from a wide variety of countries showing the broad application and importance of this technology in medicine. Examples include chapters on autoimmune disease, cancer, and the evaluation of new drugs. The book is intended to give newcomers a helpful introduction, but also to provide experienced flow cytometrists with novel insights and a better understanding of clinical cytometry.

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