Detection and Quantification of Bacteria and Fungi Using Solid-Phase Cytometry

Lies ME Vanhee, Eva D'Haese, Ils Cools, Hans J Nelis, and Tom Coenye

Abstract Solid-phase cytometry (SPC) was developed to meet the demand for fast and sensitive microbial detection and quantification methods. By combining the principles of epifluorescence microscopy and flow cytometry, this technique allows accurate, fast and automated detection of single microbial cells. SPC analysis is a five-step procedure, including membrane filtration, fluorescent labelling of the retained cells, scanning of the membrane filter, data analysis by a computer and microscopic validation. The aim of this review is to present the basic principles of SPC, its advantages and disadvantages and to discuss the existing applications as well as some perspectives for future research.

Keywords Solid-phase cytometry • rapid detection • quantification

Introduction

Each year microbiologists analyse millions of clinical, water, food and beverage samples to determine total plate counts (total number of culturable cells) and to demonstrate the presence or absence of specific undesirable microorganisms.

L.M.E. Vanhee, E. D'Haese, I. Cools, H.J. Nelis, and T. Coenye
Laboratory of Pharmaceutical Microbiology, Ghent University,
Harelbekestraat 72, 9000 Ghent, Belgium

E. D'Haese
Health Care Department, National Institute for Health and Disablement Insurance,
Brussels, Belgium

I. Cools
GULLIMEX BV, Erpe-Mere, Belgium

T. Coenye (✉)
Laboratory of Pharmaceutical Microbiology, Ghent University,
Harelbekestraat 72, 9000 Ghent, Belgium
e-mail: TOM.COENYE@UGENT.BE

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However, traditional culture-based techniques are often labour-intensive and may take days to yield a result. Additionally, they often underestimate the number of microorganisms. First of all, only culturable microorganisms are detected, while the non-culturable ones, which can comprise a significant percentage of the total, escape detection. Secondly, because of differences in growth requirements, no single condition will allow growth of every microorganism. Finally, fast-growing microorganisms may overgrow slower ones.

In industrial, environmental and clinical settings, real-time microbial monitoring is often required for rapid decision making. Analytical microbiology has undergone great changes with the introduction of modern instrument-based technologies, each with a different performance and application range. The detection limit, accuracy, speed of analysis and type of sample to be analysed are important criteria for the selection of an instrumental technique.

Fluorescence-based microbial detection systems, including epifluorescence microscopy (EFM), flow cytometry (FC) and solid-phase cytometry (SPC), lend themselves to rapid, in-situ analysis of individual microorganisms, without the need for multiplication (Lemarchand et al. 2001; Lisle et al. 2004). This review will focus on the basic principles of SPC, its advantages, disadvantages and applications, and outline some future perspectives.

Basic Principles of SPC

In SPC, the main principles of EFM and FC, being fluorescent labelling of cells and laser detection, are combined (Lemarchand et al. 2001). The different steps in a SPC protocol are presented in Fig. 1.

First, samples are filtered over a black membrane filter (e.g. polyester or polycarbonate) with an appropriate pore size (i.e. 0.4 μm for bacteria and 0.8–2 μm for eukaryotic cells). These screen filters are used because of their low background fluorescence and high contrast, which facilitates validation using the epifluorescence microscope (see below) (Brailsford 1996). Secondly, the retained cells are fluorescently stained using one or more physiological or taxonomic probes (see Section Fluorescent Stains for SPC).

Next, the fluorescence emitted by the labelled cells is detected using a solid-phase cytometer (ChemScan C or RDI), which consists of an argon laser for fluorophore excitation and two to three photomultiplier tubes (PMTs) for signal detection. A laser beam from the argon laser (488 nm) is guided via a two-axis scanning device and a spot, 7 μm in diameter, is focused on the membrane surface. The beam scans the surface at a speed of 1 m/s in the X-direction. Along one scan line, PMTs with wavelength windows set for the green (500–530 nm) and amber (540–585 nm) regions collect the fluorescent light emitted at 0.5 μm intervals (samples). As each scan line is being offset in the Y direction by 3 μm from the previous one, the two-directional scanning is thus fully overlapping, ensuring that every point of the membrane is scanned at least twice by the laser beam. An entire

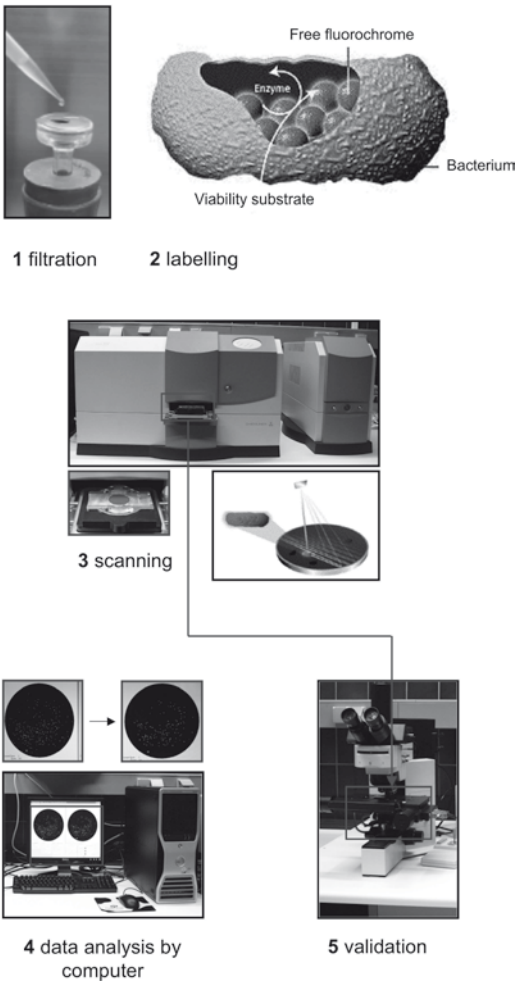

Fig. 1 Schematic overview of solid-phase cytometry. Steps include: membrane filtration, fluorescent labelling, scanning, data analysis by a computer and microscopic validation

membrane surface of 25 mm diameter can thus be scanned in 3 min. To this end, the membrane filter is placed in a stainless steel holder, which is automatically brought in focus and is cooled by Peltier elements.

Subsequently, the produced signals are processed by a computer to differentiate valid signals (labelled microorganisms) from fluorescent particles by evaluating data for several software parameters such as the size of the fluorescent spot, the specific intensity, the color ratio and the signal pattern (Fig. 2) (Brailsford 1997a, b; Guyomard 1997; Mignon-Godefroy et al. 1997; Rolland et al. 1999; Wallner et al. 1997). Upper and lower constraints can be placed on the number of scan lines and samples (reflecting the size) as well as on the fluorescence intensity admissible for a positive event.

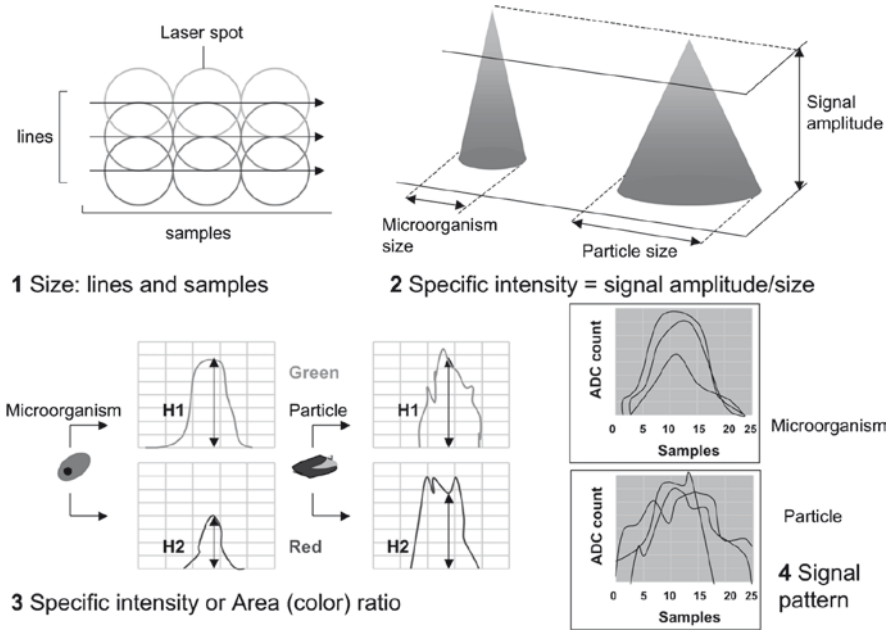

Fig. 2 Overview of the software parameters used by the computer to discriminate among microorganisms and autofluorescent particles. The four main discriminants are size, specific intensity, color ratio and signal pattern of the detected fluorescent spot

Additionally, the integration of the fluorescence by the computer further allows the calculation of a red to green ratio for each event. Finally, the degree of fitting of the fluorescence curves with the Gaussian interpolation is a reflection of the shape of the detected fluorescent spot, and can also be used as a parameter for discrimination. At the end of the analysis procedure, results are displayed as green spots on a membrane filter image in a primary and, after software elimination of background spots, in a secondary scan map (Mignon-Godefroy et al. 1997).

Finally, to further analyse their properties, the retained spots are visually inspected using an epifluorescence microscope equipped with a computer-driven moving stage. To that end, the sample holder is transferred to the motorized stage in exactly the same orientation as in the ChemScan. Highlighting of a green spot in the secondary scan map directs the microscope to the respective position on the membrane filter, allowing rapid and accurate visual discrimination between labelled cells and fluorescent particles (‘validation’).

Fluorescent Stains for SPC

Fluorescent stains used in EFM, FC and SPC include physiological and taxonomic probes (Joux and Lebaron 2000). An overview of their target sites is given in Fig. 3.

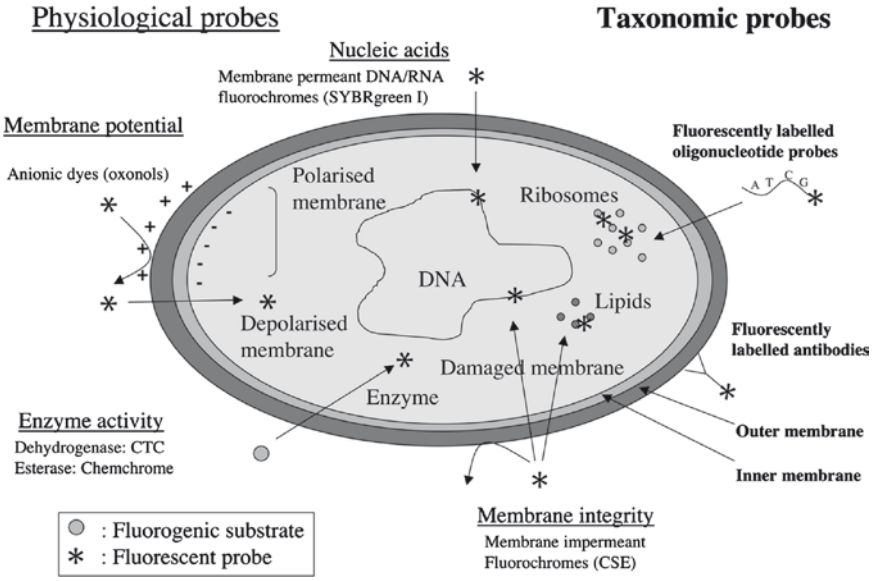

Fig. 3 Different cellular target sites for physiological and taxonomic fluorescent dyes (Based on Joux and Lebaron 2000)

Physiological probes directly bind to particular molecules in the cell or are markers of metabolic activities (such as enzyme activity and membrane potential). These probes are non-specific, i.e. their action is independent of the type and the identity of the cell. Depending on whether a stain is membrane permeant or impermeant it will label both viable and dead cells or dead cells only, respectively. ChemChrome V6 (formerly ChemChrome B or V3) contains carboxyfluorescein diacetate (Fig. 4), a nonfluorescent compound that is taken up by metabolically active cells and cleaved by intracellular esterases to yield an intensely green fluorescent product (carboxyfluorescein). When used in conjunction with an appropriate labelling buffer (ChemSol B12), the fluorescent carboxyfluorescein is only retained in cells with an intact cytoplasmatic membrane (Catala et al. 1999; Parthuisot et al. 2000). As the dye will rapidly leak from dead cells because of their damaged membranes, ChemChrome V6 functions as an activity and cell integrity probe that measures both enzymatic activity and cell-membrane integrity.

Little has been reported on the use of other fluorescent dyes for SPC. Broadaway et al. (2003) described the use of SYBR Green I in combination with ChemScan detection. Van Poucke and Nelis (2000a, b) reported the specific SPC detection of a target bacterium (*Escherichia coli*) using a physiological probe, i.e. fluorescein-ß-D-diglucuronide, a substrate for the marker enzyme ß-glucuronidase. Although some non-target bacteria occurring in water also contain this enzyme, specificity for *Escherichia coli* was derived from a quantitative difference in

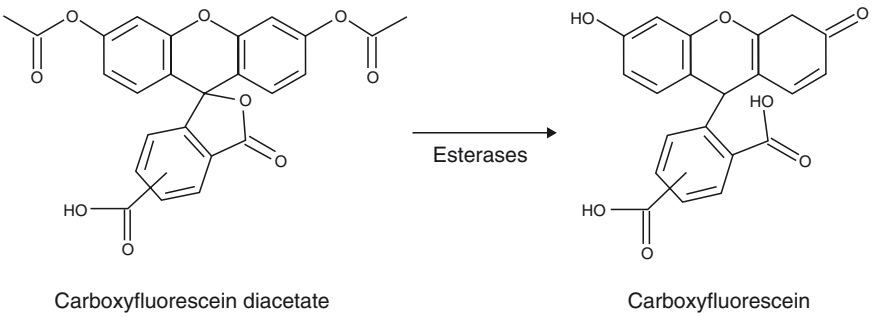

Fig. 4 Cleavage of ChemChrome V6 by esterases to yield the green fluorescent carboxyfluorescein

ß-glucuronidase activity resulting from the use of a proprietary cocktail of inducers and stabilizers.

Taxonomic probes, including antibodies and nucleic acid or peptide nucleic acid (PNA) probes selectively stain particular target cells by association with antigens or DNA/RNA. The corresponding approaches are designated as immunofluorescence (IF) and fluorescence in situ hybridisation (FISH), respectively. The major limitation of these labelling procedures is the low fluorescence intensity, which results in poorly labelled cells that escape detection.

Signal intensity can be increased by double antibody labelling, a procedure in which both the primary and secondary antibody are tagged with fluorescein isothiocyanate (FITC). As these two fluorescent signals are additive, the signal intensity will be markedly increased (Aurell et al. 2004).

In tyramide signal amplification (TSA), the target cells are labelled with an antibody or a nucleic acid probe, followed by secondary detection with a horseradish peroxidase (HRP) labelled antibody. HRP activates multiple copies of fluorescently labelled tyramide derivatives, yielding fluorescent tyramide radicals that are deposited in the vicinity of the HRP-target interaction site (Fig. 5).

The implementation of a direct viable count (DVC) approach may also lead to an increase of the fluorescence intensity. This procedure is based on the activation of the cellular metabolism in the presence of a nutritive source and a DNA gyrase inhibitor (e. g. nalidixic acid), which stops cell division, increases the intracellular rRNA content and causes elongation of sensitive cells. This leads to a higher fluorescence intensity as there are more rRNA targets available for subsequent FISH labelling (Baudart et al. 2002, 2005).

Finally, a double labelling with antibodies and a viability substrate can be performed. De Vos and Nelis (2003, 2006) combined ChemChrome V6 with tetramethyl rhodamin isothiocyanate (TRITC) labelled antibodies for the detection of *Aspergillus fumigatus*. In these approaches, the ChemChrome reagent, yielding green fluorescence, ensures the primary detection by the ChemScan, whereas the TRITC label results in red fluorescence, to be observed microscopically.

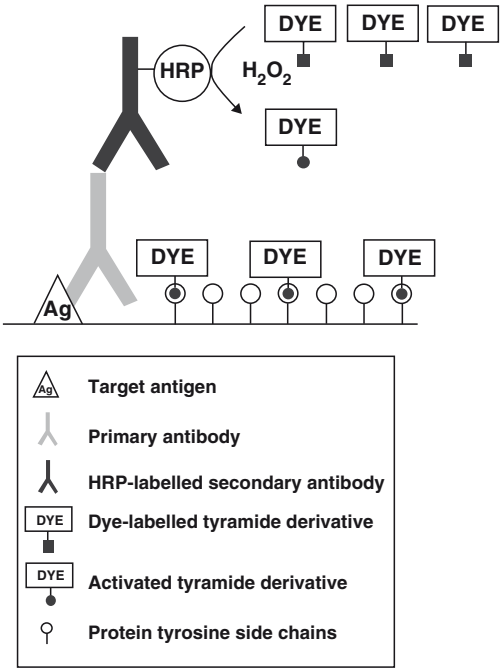

Fig. 5 Schematic presentation of tyramide signal amplification (TSA)

Applications of SPC

The fact that pathogenic microorganisms can be present in low numbers often hampers their detection. Conventional culturing may take several days and underestimate the microbial load. Therefore, more rapid and sensitive microbial detection methods would be useful for many applications to complement or replace these traditional culture methods. As SPC is a fast and sensitive tool to detect low numbers of microorganisms, it has been used for the detection and quantification of several important species.

Applications of SPC (summarized in Table 1) can be divided into three main categories. The first one concerns SPC methods (using physiological probes for fluorescent labelling) used to determine a total (viable) microbial count for quality assessment of water and air samples (Section Determination of the Total (Viable) Count of Water and Air Samples). A second group (Section Specific Detection of Target Organisms) consists of methods for the detection of specific target organisms in water, air, food and clinical samples. This type of SPC analysis requires taxonomic probes for selective labelling, sometimes in conjunction with a physiological probe to assess the viability of the cells. In the third category of applications (Section Studies on the Physiological State of Microorganisms), SPC is used as a tool for studies on the physiological state of microorganisms, e.g. the formation of

Table 1 SPC detection of different types of cells, the matrix in which detection was performed, as well as the fluorescent label used

Application	Matrix	Fluorescent label	Reference
Total counts			
	Water	SYBR Green I	Broadaway et al. 2003
Total viable counts			
	Natural waters	ChemChrome B, ChemChrome V6	Parthuisot et al. 2000
	Pharmaceutical water	ChemChrome V3	Guyomard 1997
	Pharmaceutical water	ChemChrome V3	Brailsford 1996
	Pharmaceutical water	ChemChrome V3	Jones et al. 1999
	Pharmaceutical water	ChemChrome (not specified)	Wallner et al. 1997
	Pharmaceutical water	ChemChrome (not specified)	Wallner et al. 1999
	Potable water	ChemChrome B	Reynolds et al. 1997
	Potable water	ChemChrome B	Reynolds and Fricker 1999
	Tap water, seawater, purified water	ChemChrome V6	Catala et al. 1999
	Water	ChemChrome V3	Lisle et al. 2004
	Air	ChemChrome V6	Vanhee et al., 2008, Vanhee et al., 2009a
Total viable fungal count			
	Hospital waters	ChemChrome V6 + TRITC-concanavalin A	De Vos and Nelis 2006
Specific target organisms			
Water samples			
Enterobacteriaceae	Freshwater, drinking water	Nucleic acid probe + DVC + TSA	Baudart et al., 2002, 2005
Escherichia coli	Drinking water	Nucleic acid probe + TSA	Lepeuple et al. 2003
Escherichia coli	Tap water	PNA probe + TSA	Prescott and Fricker 1999
Escherichia coli	Tap water, well water, surface water	Fluorescein-ß-D-glucuronide	Van Poucke and Nelis 2000a, b
Escherichia coli O157:H7	Tap water, seawater	FITC-Ab	Lemarchand et al. 2001
Escherichia coli O157:H7	Water	FITC-Ab + CTC	Pyle et al. 1999
Legionella pneumophila	Hot water systems	FITC-Ab (prim. & sec.)	Aurell et al. 2004
Cryptosporidium parvum	Raw and potable water	FITC-Ab	de Roubin et al. 2002; Reynolds et al. 1999; Rushton et al. 2000
Naegleria fowleri	Surface waters	Biotin-Ab + steptavidin-RPE-Cy5 HRP-Ab + TSA	Pougnard et al. 2002

(continued)

Table 1 (continued)

Application	Matrix	Fluorescent label	Reference
Prymnesium parvum	Seawater	Ab + FITC-Ab	West et al. 2006
Prymnesium parvum	Seawater	Nucleic acid probe + TSA	Töbe et al. 2006
Air samples			
Aspergillus fumigatus	Air	Ab + TSA	Vanhee et al. 2009b
Food samples			
Escherichia coli O157:H7	Meat	FITC-Ab + CTC	Pyle et al. 1999
Mycobacterium paratuberculosis	Milk	ChemChrome V6	D'Haese et al. 2005
Clinical samples			
Aspergillus fumigatus	BAL, sputum	ChemChrome V6 + TRITC-Ab	De Vos and Nelis 2003, 2006
Cryptococcus neoformans	CSF, serum	FITC-Ab, ChemChrome V3	Bauters et al. 2003
Human Papilloma Virus	Solid biopsy material	Biotin-nucleic acid probe + FITC-avidin	Butor et al. 1997
Physiological state applications			
Campylobacter jejuni	Pure cultures	ChemChrome V6	Cools et al. 2005
Total viable count	Spiramycin	ChemChrome V6	Ramond et al. 2000
Colistin	Milk	ChemChrome V6	D'Haese and Nelis 2000
Total viable count	Pharmaceutical oils	ChemChrome V6	De Prijck et al. 2008
Other applications			
Anaerobic bacteria and spores	Pure cultures	ChemChrome V6	Vermis et al. 2002
Fetal cells	Maternal blood	FITC-Nucleic acid probe + FastRed-Ab	Serradell et al. 2000
Somatic cells	Milk	ChemChrome V6	D'Haese et al. 2001
Filament count	*Candida albicans* cultures	ChemChrome V6	Nailis et al. 2009

TRITC: tetramethyl rhodamin isothiocyanate
DVC: direct viable count
TSA: tyramide signal amplification
PNA: peptide nucleic acid
FITC: fluorescein isothiocyanate
Ab: antibody
CTC: cyanoditolyl tetrazolium chloride
RPE-Cy5: R-phycoerythrin conjugated with Cy5
HRP: horseradish peroxidase
BAL: bronchoalveolar lavage fluid
CSF: cerebrospinal fluid

viable but non-culturable (VBNC) forms. Finally, SPC has proven its usefulness in various less common research applications (Section Other Applications).

Determination of the Total (Viable) Count in Water and Air Samples

Analysis of Water Samples

Multiple studies have applied SPC for the determination of the total viable count (TVC) of mainly pharmaceutical and potable waters. For this purpose, viability stains such as ChemChrome B (Parthuisot et al. 2000; Reynolds et al. 1997; Reynolds and Fricker 1999a), ChemChrome V3 (Brailsford 1996; Guyomard 1997; Jones et al. 1999; Lisle et al. 2004) and ChemChrome V6 (Catala et al. 1999; Parthuisot et al. 2000) were used. ChemChrome V6 resulted in superior labelling when compared to the now obsolete ChemChrome B or V3 (Parthuisot et al. 2000). Although SPC counts were often higher than plate counts using R2A agar, a very good correlation was found between the results obtained with both methods (Brailsford 1996; Jones et al. 1999; Wallner et al. 1997, 1999). Only one study (Broadaway et al. 2003), however, determined the total count (viable and non viable cells) by using the nucleic acid dye SYBR Green I in addition to the TVC obtained with ChemChrome V6.

De Vos and Nelis (2006b) applied a double labelling for the selective detection of fungi in hospital waters (dialysis fluid and rinse water for endoscopic equipment), combining ChemChrome V6 for viability assessment and TRITC-concanavalin A for selective labelling of fungal cells.

Analysis of Air Samples

Recently, we have developed a novel approach for the rapid enumeration of airborne bacteria and fungi based on SPC. Air samples are collected by impaction on a water soluble polymer that is subsequently dissolved. For labelling of the airborne microorganisms, the viability stain ChemChrome V6 was used (Vanhee et al. 2008, 2009a).

Specific Detection of Target Organisms

Analysis of Water Samples

For the specific detection of target organisms taxonomic probes have to be used. In spite of the practical problems with the fluorescence intensity when these taxonomic probes are used (see Section Fluorescent Stains for SPC), several target

microorganisms have been detected in water samples by means of SPC, though with variable success.

The enumeration of *Enterobacteriaceae* and *Escherichia coli* in water using SPC has been the subject of several studies. These microorganisms serve as indicators of faecal contamination as part of the monitoring of the quality of raw and partially purified waters. They are also used to demonstrate the compliance of a final product with legal standards. For the selective detection of viable *Enterobacteriaceae*, Baudart et al. (2002, 2005) used a nucleic acid probe targetting the 16S rRNA. In order to enumerate the viable cells and to increase the fluorescence intensity, FISH was combined with a DVC procedure and TSA. Using this approach, as little as one fluorescent target cell could be demonstrated in the presence of 10^7–10^8 non-fluorescent other cells.

Several studies have focused on the specific detection of *Escherichia coli* with SPC, using different labelling procedures. While Lepeuple et al. (2003) used a 16S rRNA directed nucleic acid probe, Prescott and Fricker (1999) used a PNA probe. In both methods TSA was included to increase the fluorescence intensity. Van Poucke and Nelis (2000a, b) on the other hand used an enzyme substrate (fluorescein-ß-D-diglucuronide) for the demonstration of ß-glucuronidase activity in *Escherichia coli*. The quantitative difference in enzyme activity between target and non-target bacterial cells allowed for the specific detection of *Escherichia coli.*

Real-time monitoring of the quality of water is also important to prevent outbreaks caused by pathogenic microorganisms. Therefore, rapid and sensitive SPC detection methods have also been developed for selected pathogens including *Escherichia coli* O157:H7, *Legionella pneumophila*, *Cryptosporidium parvum*, *Naegleria fowleri* and *Prymnesium parvum* (Table 1). Lemarchand et al. (2001) used FITC-labelled antibodies without amplification to detect *Escherichia coli* O157:H7 in water. Pyle et al. (1999), on the other hand, used an antibody both for capturing (immunomagnetic separation, IMS) and labelling (FITC conjugated). Additionally, an incubation step with cyanoditolyl tetrazolium chloride (CTC) to determine cellular respiratory activity was incorporated. The resulting red fluorescence was observed microscopically during the validation of the scan results.

Aurell et al. (2004) were able to demonstrate *Legionella pneumophila* cells in water using a double antibody technique with specific monoclonal antibodies conjugated to FITC. A very simple amplification technique, using a secondary, FITC conjugated, antibody was used to increase the fluorescence intensity.

Cryptosporidium oocysts were selectively extracted (antibodies coupled to magnetic beads) from water concentrates using IMS. These oocysts were subsequently visualized using FITC conjugated monoclonal antibodies. However, dissociation from the immunomagnetic beads before labelling proved to be necessary (de Roubin et al. 2002; Reynolds et al. 1999; Rushton et al. 2000).

Naegleria fowleri (Pougnard et al. 2002) has been detected by SPC in surface water. A monoclonal antibody was conjugated with biotin or HRP and revealed by streptavidin conjugated to RPE-Cy5 (R-phycoerythrin conjugated with the cyanine dye Cy5) or FITC-conjugated tyramide, respectively. The RPE-Cy5 protocol was the most efficient and allowed the detection of both trophozoite and cyst forms in water.

Finally, two studies used SPC for the rapid quantification of the toxic alga *Prymnesium parvum* in seawater. West et al. (2006) used a specific monoclonal antibody and a FITC-conjugated secondary antibody, while Töbe et al. (2006) detected the algal cells with a new oligonucleotide probe conjugated to HRP and TSA.

Analysis of Air Samples

A novel approach for the quantification of *Aspergillus fumigatus*, based on SPC and immunofluorescent labelling, has recently been developed. Air samples were collected by impaction as described previously (Section Analysis of Air Samples). This was followed by labelling with a monoclonal anti-*Aspergillus* antibody and TSA to detect the cells. Additionally, a growth step at 47°C was included to improve the specificity (Vanhee et al. 2009b).

Analysis of Food Samples

Given the unfilterability of foods, reports on the application of SPC in this field are scarce (D'Haese and Nelis 2002). However, the few experimental methods that were developed may serve as a proof of concept.

Pyle et al. (1999) used SPC for the rapid detection of *Escherichia coli* O157:H7 in meat. IMS allowed the isolation of target cells from the food matrix and was followed by a double labelling, using green fluorescent FITC-conjugated antibodies and the red fluorescent viability substrate CTC.

D'Haese et al. (2005) studied the potential of SPC for the rapid enumeration of the extremely slow-growing *Mycobacterium paratuberculosis* in milk. However, as the viability stain ChemChrome V6 was used for labelling, no specific detection of the bacteria was obtained and only spiked samples were analysed.

Analysis of Clinical Samples

SPC has not yet found its way to clinical microbiology, although several studies have demonstrated its potential applicability. De Vos and Nelis (2003, 2006) describe the specific detection of *Aspergillus fumigatus* in bronchoalveolar lavage fluid (BAL) and sputum by means of a double labelling using ChemChrome V6 and TRITC-conjugated antibodies.

Bauters et al. (2003) demonstrated *Cryptococcus neoformans* in cerebrospinal fluid (CSF) and serum. Their 30-min procedure was based on the non-specific labelling with ChemChrome V3 in combination with a second analysis using immunofluorescence. To that end, cells were labelled with a specific primary antibody against a capsular polysaccharide and a secondary antibody conjugated with FITC.

Butor et al. (1997) were able to detect and map Human Papilloma Virus (HPV) infected cells labelled by means of FISH in cervical condyloma biopsies using a cDNA probe conjugated with biotin and subsequent detection with FITC conjugated avidin.

Studies on the Physiological State of Microorganisms

Cools et al. (2005) used SPC with ChemChrome V6 labelling to demonstrate the existence of a viable but non-culturable (VBNC) form of *Campylobacter jejuni* in water. For freshly cultured viable cells, an excellent correspondence was noticed between culture and SPC. Therefore, if discrepancies between the two results occur in older cultures, they can be attributed to the transition of culturable *Campylobacter jejuni* cells into the VBNC form.

Addition of pure cultures of four microorganisms to the antibiotic spiramycin (Ramond et al. 2000) did not compromise their recovery (irrespective of their susceptibility) using a SPC procedure with ChemChrome V6 labelling. In contrast, on a conventional plate, only spiramycin resistant strains were completely recovered.

Another study tested to what extent antibiotics affect the membrane integrity of *Escherichia coli* and hence inhibit the ChemChrome V6 labelling. Inhibition of the fluorescent staining was only observed for membrane permeabilizing antibiotics, even at concentrations below the MIC but not for antibiotics with other mechanisms of action e. g. ß-lactams. As an application, colistin could be determined in milk by measuring the decrease in the number of labelled *Escherichia coli* cells relative to the initial number that had been added to the milk (D'Haese and Nelis 2000).

For the evaluation of the survival of different strains of bacteria in pharmaceutical oils, SPC was recently compared to the plate method. In agreement with previous studies, differences in recovery between the two methods were indicative of the formation of VBNC cells (De Prijck et al. 2008).

Other Applications

The applicability of SPC has also been evaluated in several other areas. Vermis et al. (2002) were able to label (ChemChrome V6) and enumerate vegetative cells and spores of eight strains of anaerobic bacteria under aerobic conditions. For vegetative cells a labelling time of 3 h (as compared to the 30 min labelling needed for aerobic bacteria) was necessary, whereas spores required an anaerobic activation of 3 h followed by a 1 h labelling.

A preliminary study showed that fetal cells can be detected in maternal blood using SPC and an immuno-FISH labelling protocol (Serradell et al. 2000). This could be promising for the prenatal diagnosis of chromosomal abnormalities.

SPC was also used for the rapid enumeration of somatic cells in milk (D'Haese et al. 2001). However, comparison with the routinely used fluoro-opto-electronic method revealed a poor comparability. Furthermore, problems of milk filterability and the interference of fluorescent particles hamper this application of SPC.

Finally, SPC has also been used to determine the fraction of *Candida albicans* filaments in a culture using labelling with ChemChrome V6 and a microscopic discrimination between yeast cells and filaments (Nailis et al. 2009).

Advantages and Disadvantages of SPC

One of the prominent advantages of SPC is its speed. As this method does not rely on culturing the microorganisms, quantitative results can be obtained within a few hours. Additionally, the membrane filter is scanned by the laser in only 3 min. Therefore, SPC can be used for real-time monitoring to provide an early warning and a rapid implementation of corrective measures.

The use of viability stains results in the quantification of not only the culturable cells but all viable cells. As recently stated by Newby (2007), the detection of VBNC microorganisms is crucial as pathogenic organisms may retain pathogenicity during the VBNC state. In general, SPC yields higher counts than plate methods because the VBNC microorganisms are also enumerated. Consequently, current warning and action limits based on plate counts need to be redefined.

SPC has a theoretical detection limit of one cell per filtered volume, but can also be used to determine the microbial load of highly contaminated samples because of its high dynamic range with an upper limit of approximately 10,000 cells per membrane filter. Unlike EFM and FC, SPC has the potential of detecting rare events, i.e. of finding low numbers of target cells among an excess of non-target cells (Mignon-Godefroy et al. 1997; Lemarchand et al. 2001; Lisle et al. 2004).

Microscopic inspection after the scan, to determine if a recorded fluorescent event represents a microorganism or a particle, is possible. Furthermore, SPC counts all microorganisms on the membrane filter, so that errors associated with counting microorganisms in a limited number of microscope fields and subsequent extrapolation of the counts to the total number of cells are minimized (Lemarchand et al. 2001).

SPC also has its limitations. A first one is the limited availability of compatible stains. Since the instrument is equipped with a single Ar laser source, the staining is restricted to the range of probes and dyes excitable at approximately 488 nm and emitting light in the range of 500–530 nm.

The most fundamental limitation of SPC is the requirement of filterable samples. Only clear, aqueous solutions can be used unless samples are more or less extensively pre-treated. For example, such pre-treatment proved necessary to obtain a modest improvement in the ability to filter BAL and sputum samples (De Vos et al. 2006). Alternatively, microorganisms can be isolated from complex matrices by e. g. IMS (de Roubin et al. 2002; Pyle et al. 1999; Reynolds et al. 1999; Rushton et al. 2000)

Furthermore, the possibility to culture and identify the microorganisms on the membrane filter would present a significant improvement.

In some samples, the occurrence of fluorescent particles may lead to an aborted scan or to a cumbersome validation when numerous spots are present in the secondary scan map. By adding a counterstaining step (Catala et al. 1999) and/or using a filter with a larger pore size, this problem can sometimes be overcome. Additionally, the fluorescence intensity of cells labelled using FISH or immunofluorescence is often low, making signal amplification necessary (see Section Fluorescent Stains for SPC).

The final disadvantage of SPC is its high cost and low throughput. A ChemScan is an expensive piece of laboratory equipment (approximately €170,000) and the cost for a traditional analysis with ChemChrome V6 is also relatively high (approximately €7.5 per sample).

Conclusions and Perspectives

The different applications of SPC clearly illustrate its usefulness in microbiology. However, some modifications could broaden the applicability of this method. The modification of SPC to a high throughput system (e. g. microtiter plate based) would enhance its use in industrial settings.

The introduction of other dyes for fluorescent labelling might provide information about other physiological cellular states and the development of new labelling procedures using taxonomic probes might enable the detection of other cells. Microorganisms genetically modified to express the green fluorescent protein are also likely to be detectable with SPC, although there are at present no reports in this regard.

Due to the use of a single light source, the choice of probes is rather limited. When two probes are combined, the technique becomes cumbersome and loses its advantage of speed. Therefore, extension of the instrument with a second or even a third light source would open the possibility for a whole range of new applications. For example, if a viability probe and a probe for the detection of a specific species could be combined, detailed studies on the physiological behaviour of bacteria in a complex community would be facilitated.

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