

SPECIAL GUEST EDITOR SECTION

Rapid Detection of Single Cell Bacteria as a Novel Approach in Food Microbiology

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Solid-phase cytometry (SPC) is a novel technique that allows rapid detection of bacteria at the single cell level, without the need for a growth phase. After filtration of the sample, the retained microorganisms are fluorescently labeled on the membrane filter and automatically counted by a laser scanning device. Each fluorescent spot can be visually inspected with an epifluorescence microscope connected to the ChemScan by a computer-driven moving stage. Depending on the fluorogenic labels used, information on the identity and the physiological status of the microorganisms can be obtained within a few hours. Although SPC was originally recommended for the determination of the total viable microbial count in water and other liquid samples, it may also be a promising technique for the detection and enumeration of bacteria in food samples, provided they can be isolated from the unfilterable matrix. The short detection time inherent in this approach is a considerable advantage over conventional plate counting, especially for slow-growing microorganisms. The basic principles of SPC are discussed as well as its potential for the detection of *Mycobacterium paratuberculosis*, a model example of a slow-growing bacterium in milk.

Pathogens, spoilage organisms, or indicator bacteria can be detected in food by various methodologies. Conventional culturing still remains the gold standard, as the demonstration or enumeration of target bacteria in a liquid broth or on solid agar media is unmistakable proof of their viability, defined here as their ability to multiply. Unfortunately, some microorganisms that are difficult to grow may require long incubation times or complex growth conditions. Immunological methods allow a more rapid detection of food pathogens, such as *S. aureus*, *E. coli*, *Salmonella*, *Listeria*, and *Campylobacter* in various matrixes (1, 2). However, these methods are based on the detection of bacterial surface antigens and do not provide information on the viability of the target organisms. Nucleic acid-based techniques, such as poly-

merase chain reaction (PCR) and hybridization assays, have also found widespread application in the field of food microbiology (3–9). Although they allow a highly specific detection of bacteria, they do not provide quantitative results or information on bacterial viability. Flow cytometry (FCM) and epifluorescence microscopy can also be used for detection of bacteria in food products, but they suffer from limitations, including rather high detection limits. Although these strategies are adequate for commonly recognized food pathogens, the detection of certain specific microorganisms remains difficult. Problems may arise when target bacteria grow very slowly, when the detection sensitivity of the method is too low, or when results should be obtained faster than achievable by the current methods.

This paper demonstrates that the recently developed technique of solid-phase cytometry (SPC) could be a promising alternative to meet some of these existing demands. It introduces the basic principles of SPC and outlines its potential for detection of the extremely slow-growing bacterium *Mycobacterium paratuberculosis* (MAP) in milk.

Direct Epifluorescent Filter Technique (DEFT) and Flow Cytometry in Food Microbiology

DEFT was originally developed for determination of the total viable count (TVC) of bacteria in raw milk (10–13), but has also been applied to other matrixes such as meat (14–17) and fish (18). In DEFT, an aliquot of the sample is filtered over a polycarbonate membrane filter; the retained bacteria are fluorescently stained and subsequently counted in a limited number of microscope fields with an epifluorescence microscope. The resulting count is multiplied by a magnification factor to calculate the number of cells in the original sample. This factor accounts for the number of microscope fields evaluated, the sample volume filtered, and the optical system used. Although determination of the TVC of a filterable sample takes <0.5 h to perform, the detection of specific bacteria in foods is far more complex. An extensive sample pretreatment is usually necessary to make solid food samples filterable. Enrichment, immunocapture, or preincubation is needed to enhance assay sensitivity when low numbers of specific target pathogens are to be detected (19, 20). In addition, given the nonspecificity of common fluorogenic dyes like 4',6-diamidino-2-phenylindol (DAPI) or acridine orange, a more specific labeling is needed. In the so-called anti-

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body-DEFT, target bacteria are selectively stained by fluorescent antibodies (21). More recently, specific nucleic acid techniques like fluorescence in situ hybridization (FISH) have been used in combination with DEFT (22). The use of a modified DEFT has been described for detection of *E. coli* O157:H7, *Salmonella*, and *Listeria* spp. (23–26).

In DEFT, only part of the membrane filter is covered, even along with imaging techniques. As a result, the heterogeneous distribution of target bacteria in the sample or on the membrane filter surface contributes to substantial counting errors. On the average, >300 cells must be counted to obtain reasonable accuracy, which results in rather high detection limits. This important method limitation is overcome in SPC, where the entire membrane filter surface is subjected to laser scanning and, hence, theoretically a single cell per membrane filter can be detected.

The other technique that laid the basis for SPC is FCM. Its application in the field of food microbiology is seriously hampered by the fact that a homogenous suspension free from interfering particles must be injected in the system. Therefore, FCM is limited to the enumeration of bacteria or yeasts in liquids such as milk and beverages (1, 27–31). Because detection limits are generally not below 10^3 – 10^4 colony-forming units (CFU)/mL, the assessment of low numbers of pathogens in complex solid foods may be problematic. In addition to an extensive sample pretreatment, a pre-enrichment or selective isolation step like immunomagnetic separation (IMS) must be included in the procedure. Specific target bacteria are usually stained with fluorescently labeled mono- or polyclonal antibodies. Some recent papers describe the use of immunofluorescence FCM for the detection of *E. coli* O157:H7 in ground beef, juices, and milk (32) and for *Salmonella* in dairy products (33–34).

Principles of SPC

SPC combines aspects of FCM and epifluorescence microscopy and allows the rapid enumeration of microorganisms as individual cells (cytometry) on a membrane filter (the solid phase), without a preceding growth step (35, 36). To this end, the microorganisms, mostly bacteria or fungi, are isolated from their matrix by membrane filtration, fluorescently labeled with an Argon (Ar) laser-excitable dye and automatically counted by a laser scanning device called ChemScan (Chemunex, Ivry-sur-Seine, France). During a 3 min scanning process, the entire membrane filter surface is scanned, yielding a theoretical detection limit of 1 cell per membrane filter and, hence, per filtered sample volume. Owing to the instrument's high dynamic range, accurate cell counts up to 10 000 cells per membrane filter can be obtained. During this scanning process, 2 photomultiplier tubes with wavelength windows set for the green (500–530 nm) and amber (540–585 nm) regions of the fluorescein emission spectrum detect the fluorescent light emitted by the labeled cells. The signals produced are computer-processed with software discriminants that allow instrumental differentiation between valid signals (target cells) and background noise (electronic noise and fluorescent particles). Scan results are displayed as

colored spots in a primary scan map (representing the membrane filter) and, after elimination of background by software discrimination, secondary scan map. The actual nature of each fluorescent spot can be further confirmed visually by an epifluorescence microscope (Olympus BX40, Olympus, Tokyo, Japan) connected to the ChemScan by a computer-driven moving stage (Prior Scientific, Fulbourn, UK). Highlighting of each spot in the secondary scan map directs the motorized stage to the corresponding position on the membrane filter for microscopic inspection of the appearance of each detected event.

Because the ChemScan was originally designed for determination of the TVC of bacteria, yeasts, molds, and spores in water and liquid samples, the standard labeling procedure using ChemChrome V3 as a substrate is nonspecific. This substrate, later replaced by its improved version, ChemChrome V6, is a nonfluorescent fluorescein derivative, which is taken up by metabolically active cells and cleaved by intracellular esterases to yield an intensely green fluorescent product (37–39) that can only be retained in cells with an intact cytoplasmic membrane. Hence, viability labeling is based on enzymatic activity as well as membrane integrity. Strictly speaking, the term “viability” is not appropriate in this context because labeled bacteria do not necessarily have the ability to multiply, and active, nonculturable (ANC) bacteria will also be detected (40).

Theoretically, every Ar laser-excitable dye could be used in SPC, but little has been reported on the use of reagents other than ChemChrome V3 or V6. However, in their method for the detection of *E. coli* O157:H7 in ground beef, Pyle et al. (41) describe the combined use of green fluorescent antibodies and the red fluorescent 5-cyano-3-ditolyl tetrazoliumchloride (CTC), a compound which is reduced by viable bacteria only. A detection limit of approximately 10 CFU *E. coli*/g ground beef was reported, with a total assay time of 5–7 h. This dual labeling combines detection specificity with information on bacterial viability. After the green fluorescent antibody labeled cells are detected by the ChemScan, the spots are microscopically inspected with a filter for collecting emitted red light only. Conversely, a green fluorescent viability label could be combined with a red fluorescent antibody.

Applications of SPC and Its Potential in Food Microbiology

Given the need for sample filterability, SPC has been used almost exclusively for the microbiological control of water. Applications include determination of the TVC of pharmaceutical waters (42–44) and the detection of specific microorganisms like *Cryptosporidium* or *E. coli* in drinking water (45–50). In these applications, speed is of paramount importance. Indeed, even for bacteria growing at a normal speed, culturing can still take too long before the result is known and remedial actions can be taken. We believe that the above-cited paper by Pyle et al. (41) describes the only application of SPC in food microbiology, to date. To selectively capture *E. coli* O157:H7 from beef, an IMS with supraparamagnetic beads coated with anti-O157 rabbit anti-

Table 1. Comparison of cell counts obtained with ChemScan and DEFT for different cultures of MAP

Culture No.	Mixing procedure	DEFT counts, cells/mL	SPC counts, cells/mL
1	Gentle mixing	8.0×10^6	1.9×10^7
	Syringe (3 times)	1.1×10^7	4.8×10^7
2	Vortex mixing	3.7×10^6	3.7×10^6
3	Vortex mixing	1.3×10^9	8.0×10^8
4	Vortex mixing	6.3×10^5	9.8×10^6
	Syringe (3 times)	6.3×10^6	1.3×10^8
	Syringe (6 times)	2.0×10^6	2.5×10^7
	Gentle mixing	6.5×10^6	9.6×10^6

serum was used. SPC was used after dual labeling, as described above. We previously reported the use of SPC for milk quality control, notably the enumeration of somatic cells and semiquantitative detection of certain antibiotic residues (51, 52). No other reports on the application of SPC in food analysis have yet appeared.

This paper highlights the potential of SPC for detection of MAP in milk. The elimination of a growth step makes this technique suitable for detection of this extremely slow-growing microorganism.

Detection of MAP in Milk: Background

MAP is the etiologic agent of Johne's disease, a chronic inflammatory bowel disease that mainly affects ruminants. This progressive disease is characterized by a sequence of different asymptomatic and symptomatic stages, and finally results in the death of the animal (53, 54). Milk destined for human consumption can be contaminated by direct shedding, even by asymptomatic carrier animals, or by fecal contamination (54). MAP can be transmitted to humans through raw milk and probably also through pasteurized milk (55). The potential health risk of MAP is that it may play a role in human Crohn's disease (56–63). Its survival of pasteurization is probably due to the extremely hydrophobic nature of the bacterium, which forms tight aggregates. The inner cells of these clumps are probably shielded from the detrimental effect of heat and, as a result, remain viable (55).

The hypothesis of MAP as a potential human health hazard, however, is subject to controversy, as no adequate method for the rapid detection of viable MAP is available to prove their survival of pasteurization (64). Existing methods for detection of MAP suffer from various drawbacks. It can take several months to grow MAP cells in culture, making them susceptible to contamination or overgrowth by other microorganisms. PCR, based on the detection of the specific IS900 sequence, offers a rapid (1–2 days) and specific identification, but does not discriminate between dead and viable cells (64).

Obviously, information on viability is of paramount importance for the evaluation of pasteurization efficiency. Immunological procedures like the enzyme-linked immunosorbent assay (ELISA) suffer from a too-low detection sensitivity and are not suitable for studies on pasteurization (65).

We believe SPC could be a potential alternative detection strategy, given its ability to rapidly quantitate viable cells without the need for a growth phase. The challenge of such a method is dual: an extensive sample pretreatment should selectively isolate MAP from a complex and unfilterable matrix, and a selective labeling procedure should ensure specific detection.

Preliminary Results and Future Strategies

The potential of SPC for enumeration of MAP cells in pure cultures using nonspecific viability labeling with ChemChrome V3 was demonstrated. The growth from a slant of Harrold's Egg Yolk Medium supplemented with mycobactin J (Synbiotics, Lyon, France) was harvested, suspended in Maximum Recovery Diluent (Oxoid, London, UK), and decimal dilutions were made in the same buffer. A 1 mL aliquot of each dilution was filtered over a Cycloblack-coated polyester membrane filter (0.4 μ m pore size, 25 mm diameter; Chemunex). The membrane filter was subsequently transferred to a sterile Petri dish containing a cellulose pad (25 mm diameter, Millipore, Bedford, MA) impregnated with a 1:100 dilution of ChemChrome V3 in ChemSol B1 labeling buffer (both from Chemunex), and subsequently incubated at 30°C for 30 min. The number of green fluorescent, hence, viable cells was determined by SPC and followed by microscopic confirmation. The 10-fold dilution rate was reflected in the 10-fold decrease of the cell counts (66). To minimize cell clumping, which is likely to affect accurate enumerations, different mixing protocols were tried. When 500 μ L aliquots of a pure MAP culture (3 dilutions of 10^{-3} , 10^{-4} , and 10^{-5}) were subjected to SPC, the following counts were obtained: 5080, 352, 34 (gentle mixing); 7154, 518, 40 (aspirating and expelling 3 times through a 5 mL disposable syringe with 0.8 $\times$ 40 mm needle); and 5383, 384, 36 (aspirating and expelling 6 times through a needle). These numbers corresponded to mean cell counts of 8×10^6 , 1.1×10^7 , and 8.6×10^6 /mL for the 3 different treatments, respectively. In another experiment, a comparison was made between the MAP cell counts obtained with conventional DEFT after acridine orange staining and SPC (Table 1). In view of the cell clumping phenomenon, different mixing procedures were also compared. The potential of SPC as an alternative cell counting technique for MAP was hereby demonstrated.

As part of the specific detection of MAP in milk, an extensive pretreatment protocol for raw and pasteurized milk, developed in close collaboration with Lieve Herman and co-workers (DVK-CLO Ghent, Melle, Belgium), is being investigated for its compatibility with ChemScan analysis. The procedure involves chemical and enzymatic milk destruction, centrifugation, hydrophobic interaction chromatography, and IMS (unpublished data). Although depending on the initial

cell concentration, a good recovery could be obtained with *M. smegmatis* as a model strain for MAP from artificially spiked sterile milk samples, as evaluated by plate counting on brain heart infusion agar (Difco, Sparks, MD). In repetitive experiments (3–6 replicates), recoveries between 40 and 100% were obtained for inocula of 10^2 CFU/mL; these numbers decreased to 11–22 and 1–13% for inocula of 10^5 and 10^7 /mL, respectively. However, the recovery of low numbers of target bacteria among an excess of background flora still needs to be evaluated. Moreover, problems of milk filterability occurred and fluorescent particles interfered with the ChemScan analysis, with their numbers depending on the type of milk tested. For example, in 15 mL ultrahigh temperature milk, only 10 particles were found. The same volumes of pasteurized and raw milk yielded more than 500 and 4000 particles, respectively. It was not feasible to determine whether these were real particles or cells belonging to the excess background flora. Therefore, to obtain a selective isolation of MAP from background flora, a selective decontamination step should be included in the protocol (64, 66). Unfortunately, all procedures tested thus far were either too drastic, killing mycobacterial cells (i.e., provoking loss of esterase activity and/or membrane integrity), or insufficiently effective, leaving a considerable part of the background flora intact (unpublished results). The selectivity of the assay should be further enhanced by selective labeling to ensure specific detection. A dual labeling study is being conducted, in which the use of green fluorescent antibodies is combined with that of the red fluorescent viability label CTC.

Conclusions

The above preliminary results show that SPC may be a promising tool for the rapid detection of microorganisms that are difficult to grow, even in complex food matrixes. Bottlenecks, however, remain the selective isolation of the target organism and the development of specific labeling. Our ongoing research on MAP focuses on these 2 aspects.

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