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Microscopy techniques: DEFT and flow cytometry

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9.1 Introduction

The food microbiologist has two questions when considering pathogen contamination of foods: is the pathogen present? and, if it is present, how much is there? There are many more methods for answering the first question than the second one. The reason is clear. Detection (is the pathogen present?) is often all that is needed in practice, because in most cases, the mere presence of the pathogen in a food is sufficient cause for concern and reason for action. Most of the rapid assay kits that have been commercialized for pathogen analysis in foods are detection methods, and the options available are many and varied. However, accurate enumeration (how much is there?) is needed for a variety of determinations: growth and survival of pathogens, their incidence in foods, human exposure levels and the efficiency of control methods. These quantitative determinations can also contribute to the development of accurate risk assessments for pathogens in foods. For enumeration, the technical options available to the food microbiologist are quite limited.

This chapter will focus on two specific approaches for the microscopic detection and enumeration of foodborne microbial pathogens: microscopic methods including the direct epifluorescent filter technique known as DEFT and flow cytometry (FC). These techniques share in common a utilization of optical technology, combined with specific or non-specific staining of the organisms in question with fluorescent dyes. There are numerous differences between the two methods, however, and each possesses unique strengths and weaknesses. DEFT recalls the basic foundation of microbiology in that it relies on the direct microscopic observation of organisms. FC is a technique that combines optical, fluidic and electronic technologies for the detection and characterization of

single cells or other small particles. Some flow cytometers are also capable of sorting, or physically isolating cells or particles with definable characteristics. FC technology was a commercially available technology by the 1970s (Hulett *et al.*, 1969; Van Dilla *et al.*, 1969) and evolved rapidly with the primary emphasis on lymphoid cell analysis. Microbiological analysis has developed more slowly. Although the potential use of FC in microbiology was recognized relatively early (Bailey *et al*., 1977), the technology has been slow to be adapted or widely accepted by many microbiologists. As is the case with most technologies, widespread adaptation depends on the development of at least one indispensable method uniquely dependent on the technique.

It is debatable whether or not FC is a true microscopic technique because enhancement of the apparent size of the organism and direct observation by the human eye are not necessarily involved in this technique. FC shares with DEFT the use of optics and fluorescent dyes to distinguish microorganisms. Both methods also offer the promise of direct detection and enumeration of foodborne pathogens. The aspect of direct detection is especially significant in that organisms need not be cultured in order to be enumerated, as is the case with traditional microbial colony counting. This not only eliminates the need for incubation of agar plates, thus contributing to rapidity of the methods, but also allows for detection of organisms that cannot be cultured such as stressed bacteria or protozoan pathogens such as *Cryptosporidium* spp. In order to detect specific pathogenic species or strains, both methods are dependent on the availability of high-quality antibody or nucleic acid probes. Analysis of samples by both methods requires adequate sample preparation that ensures the removal of potentially interfering particulates.

The problems encountered in trying to analyze foods for the presence of pathogens are well known. The pathogen usually exists in much smaller numbers than the indigenous microbial populations in the food, which makes detection difficult. Also, the complex food matrix is likely to interfere with detection. Most pathogen detection methods include a procedure to alleviate these problems, the most common being enrichment culture. Enrichment culture provides conditions that allow the pathogen to increase in number while inhibiting the growth of indigenous microorganisms. The food sample is added to an enrichment medium and incubated to provide optimal growth conditions for the pathogen. Enrichment culture, followed by colony isolation and identification on selective and differential agar media, has been the standard method for detection of pathogens in foods for many years. Commercial rapid assay kits generally include enrichment as a first step, which compromises the speed of the assay. Nevertheless, the ability to proceed directly from enrichment culture to detection result via a rapid assay, thereby circumventing lengthy colony isolation/identification procedures, greatly improves the speed of analysis. Enrichment, however, destroys the ability to enumerate the pathogen in the food, except when it is used in the most probable number procedure, a lengthy, tedious and laborious method for determining pathogen counts in foods

DEFT and other true microscopic methods are primarily manual and can be tedious. A skilled observer is also required. These factors offer both pros and cons: they tend to limit the number of samples that can be analyzed, however, few automated methods can equal the discriminating power of the trained eye. FC, as an automated technique, offers advantages in the area of speed, and potential automation of the process, thereby increasing sample throughput. Flow cytometers can analyze in excess of 1000 particles, or events, per second. Events can be tabulated at the time of analysis, or can be stored as archival data. FC data is also quantitative, involving the analysis of many thousands of individual particles, not possible manually. Problems can arise in relation to signal versus noise when attempting to detect rare cells amidst a high number of background cells or particles. This problem may be better addressed by direct observation of the cells (or bacteria) of interest by a trained observer. Because of the strengths of both methods, it is not surprising that some hybrid methods have been developed more recently. These methods combine the quantitative data collection and analysis features of FC with the slide or filter-based microscopic approach (Darzynkiewicz *et al*., 1999). The capability to visually observe cells of interest may also be a feature of these instruments.

9.2 Stains, fluorochromes and probes

Most microbial cells require some type of staining to provide sufficient contrast for visualization of the cells against the background medium. Some exceptions include pigmented microorganisms, e.g. the toxigenic dinoflagellates, and the pathogenic protozoan *Cyclospora,* which produces naturally autofluorescent oocysts. A huge variety of staining procedures have been described over the years.

9.2.1 Conventional stains

Categories of conventional stains include direct stains, differential stains and structural stains (Bier *et al*., 2001). Direct staining is useful for broadly classifying microbial groups (bacteria, yeasts, molds, protozoa) and for morphological descriptions (rods, cocci, spirals, hyphal segmentation). Identifications of certain molds can be made solely on the basis of morphologies of their spore-forming structures. Differential staining procedures allow distinctions between groups of microorganisms based on a particular property. In Gram staining, perhaps the most commonly used differential stain, bacteria may be broadly grouped as Gram positive or Gram negative, based on their reaction to the four-step staining procedure, a consequence of fundamental differences in cell wall structure. Structural staining procedures highlight a particular structure of the microbial cell, e.g. endospores, flagella, inclusion granules, and are helpful for identification purposes.

9.2.2 Fluorochromes

Some procedures such as DEFT and FC may require the use of fluorescent dyes to distinguish cells of interest (bacteria or protozoa) from other particulates. These fluorochromes can be used in two ways: by taking advantage of their chemical properties to interact directly with cellular targets such as DNA, RNA, lipids or proteins, or by using them as labels for molecular probes such as antibodies or oligonucleotides. All of the fluorochromes share the common characteristic of the ability to absorb and emit specific wavelengths of light. A detailed discussion of the physics of absorption and emission of light energy from fluorescent compounds is beyond the scope of this chapter. Briefly, fluorochromes absorb energy in the form of light, resulting in an excited state in which electrons are shifted to a higher energy level. This state is unstable and the electrons eventually return to their unexcited state. In this process, energy is released in the form of emitted light. Based on its molecular structure, each fluorochrome has unique light absorption and emission properties. Common to all, however, is the property that the energy released when electrons return to their ground state is at a lower level than the energy absorbed. The practical effect is that the light wavelength used to excite a fluorochrome is always shorter (higher energy) than the wavelength of the emitted light (lower energy). The difference between the two wavelengths is known as the Stokes shift, and is characteristic for each fluorochrome. These properties occur in the form of spectra with specific absorption and emission wavelength maxima for each. The broadness of the spectra and the magnitude of the Stokes shift can differ greatly between fluorochromes with important implications for their usefulness as probes or labels.

There is important interplay between the properties of fluorochromes and instrumentation. The characteristics of available fluorochromes often dictate the configuration of the instrumentation used in measurements. This is particularly true in the case of flow cytometers because the source of excitation is usually a laser. Unlike the illumination lamps of most fluorescent microscopes that emit a broad range of potential excitation wavelengths, lasers are typically limited to one or two wavelengths or laser lines of emission. The most common laser source, the argon laser, was originally chosen for use in early flow cytometers because its 488 nm emission was close to the absorption maximum of Fluorescein, a fluorochrome that was (and still is) widely used for labeling antibodies. The widespread use of the argon laser has, on the other hand, spawned the discovery and development of a number of fluorochromes with absorption spectra compatible with the 488 nm emission, but with distinct emission peaks that can be separated optically. This has led to the important capability of simultaneous analysis of multiple fluorochromes using a single excitation wavelength. Were it not for the early and widespread adaptation of the argon laser in flow cytometry, it is likely that none of these developments would have occurred because they would be unnecessary for fluorescence microscopy where mercury or xenon lamps can produce multiple excitation wavelengths. Thus, there are some fluorochromes, such as rhodamine, and some dyes that excite in the ultraviolet range that are used

almost exclusively in microscopy methods and rarely in FC. Fluorochromes are used either as direct probes for cellular components, or as reporters for the binding of some type of molecular probe. Examples of the former include nucleic acid binding dyes and viability stains. In many cases, nucleic acid binding dyes can function as viability stains. Comprehensive lists of fluorochromes, their spectral properties and uses can be found in most general references (Givan, 2001a; Haugland, 1996; Robinson, 1998).

9.2.3 Viability stains

The traditional determinant of microbial cell viability is colony formation. Not all viable cells can be grown in culture, however. Of practical consideration to food microbiologists are sublethally injured cells, which may not be able to grow on selective agar media (Ray, 1979). Microscopic methods may be used as an alternative tool for demonstrating cell viability. Various viability stains, based on different aspects of the nature of cellular viability, have been described and reviewed (Roszak and Colwell, 1987; McFeters *et al*., 1995; Porter *et al*., 1995; Haugland, 1996; Breeuwer and Abee, 2000; Takeuchi and Frank, 2001a).

Acridine orange is a fluorescent dye which has an affinity for nucleic acids. Binding to single-stranded RNA or double-stranded DNA results in red-orange or green fluorescence, respectively, and this differential binding was the basis for its use as a viability stain (Hobbie *et al*., 1977; McFeters *et al*., 1995). However, the color differentiation was found to depend on the medium in which the cells were suspended, and did not always display predictably in cells exposed to various processing treatments; thus the stain is no longer regarded as a reliable indicator of viability (Kepner and Pratt, 1994). It is, however, commonly used in DEFT for quantification of the total microbial population in a food sample.

Redox probes constitute another category of viability indicators, based on the presence of a functioning electron transport system (Haugland, 1996). Redox probes accept electrons from the electron transport system components in respiring cells and accumulate within the cell as insoluble chromogens or fluorescent derivatives. Fluorescent antibody staining has been coupled with the redox probe CTC (cyanoditolyl tetrazolium chloride) to specifically count viable cells of *E. coli* O157:H7 (Pyle *et al*., 1995).

Many viability stains are based on cell membrane integrity (Haugland, 1996). Exclusion of certain dyes by an intact membrane is an indication of viability, while permeation of the dye shows that the membrane has been damaged and the cell is not viable. For example, staining of cells by the fluorescent dye propidium iodide has been used to show their nonviable state. Staining of cells by fluorescein diacetate is based on two properties related to cell viability: enzyme activity and membrane integrity. This nonpolar, nonfluorescent compound can diffuse across an intact membrane. However, when esterase enzymes cleave the compound, the fluorescein released cannot pass the membrane and accumulates within the cell as a fluorescent indicator of viability.

Other nucleic acid binding dyes such as the SYTO series (Molecular Probes) and thiazol orange are permeant to intact cell membranes. Nucleic acid binding dyes in association with their viability indicator properties have been employed extensively in microbial methods of FC. The exploitation of these properties for analytical purposes will be discussed as they apply to specific methods.

9.2.4 Molecular probes

Fluorochromes are used as labels primarily for antibody or oligonucleotide probes. Unlike conventional stains, which have little discriminating power, molecular probes allow for specific identifications to be made by microscopy or FC. Microbial cells may be distinguished at various taxonomic levels, e.g. genus, species, strain, depending on the specificity of the probe. An antibody binds to an antigen specifically, through non-covalent molecular interactions as a result of conformational features in protein structure, in a way similar to the interaction between an enzyme and its substrate. This specificity allows antibodies to be used as probes for the presence of a particular antigen, e.g. a unique microbial cell component. Antibody-based immunofluorescence methods involve some form of stable chemical coupling of the fluorochrome to antibody molecules directed against a cellular or bacterial target protein. Fluorescent antibody staining is the basis of an official method for detection of *Salmonella* in foods (AOAC International, 1997b). Oligonucleotides are short polymers of nucleotides, the structural components of DNA and RNA. These short nucleotide chains can bind to specific nucleic acid sequences by complementary base pairing (adenine:thymine and cytosine:guanine), which involves formation of hydrogen bonds between the bases. Like antibodies, oligonucleotides may be labeled with a fluorochrome, and as such, can function as fluorescent probes for specific nucleic acid sequences. Oligonucleotides are less expensive and easier to produce than antibodies, because they can be synthesized chemically.

Identification of the molecular target for the probe that uniquely belongs to a particular microbial group (genus, species, strain, etc.) is the first consideration in designing the probe. Also to be considered is the relative abundance of the target in the cell. Antigens are usually present in the microbial cell in amounts sufficient for direct detection by microscopy; however biosynthesis of some antigens may be environmentally regulated, and they may not be expressed at all times by the cell. Nucleic acid sequences, on the other hand, are usually always present regardless of environmental conditions, but in amounts too low for direct microscopic detection. A notable exception is the relatively abundant level of ribosomal RNA present in actively growing cells. Fluorescent oligonucleotides specific for ribosomal RNA sequences have been applied as 'phylogenetic stains' for detection and identification of groups of microorganisms (DeLong *et al.*, 1989). Their use for detection and tracking of microbial populations by fluorescence *in situ* hybridization (FISH) has been demonstrated for a variety of natural samples, from drinking water to intestinal tissue (Manz *et al.*, 1993;

Poulsen *et al*., 1994). Methods have also been developed for use of fluorescent oligonucleotide probes in FC analysis of microorganisms (Amann *et al*., 1990; Fuchs *et al*., 1998; Porter and Pickup, 2000). In the area of flow cytometric methods applied to microbial pathogens in food, few, if any, such methods have been developed.

Fluorescent antibodies are easy to use, involving a simple process of adding them to a suspension of microbial cells, allowing them to react for a few minutes, then washing away the excess, unbound fluorescent antibodies before microscopic observation. The antibody probes are generally directed against some type of cell surface component and can bind immediately, therefore no preliminary treatment of the cells is required. The antibody-antigen reaction occurs at the cell surface. Use of oligonucleotide probes, on the other hand, usually requires some type of pre-treatment of the cells to allow the probe to pass across the cell wall to reach the nucleic acid component. In addition, heating and chemical treatment steps are necessary for hybridization of the oligonucleotide to the target nucleic acid sequence.

Gene sequences for proteins that fluoresce or luminesce can be spliced into the genome of a microorganism by recombinant DNA techniques or incorporated into the cell as a plasmid component. Unlike fluorescent antibodies or oligonucleotides, these molecular markers are not used to detect or identify naturally occurring microbial cells. Rather, they are helpful for determining microbial growth and survival, after inoculation of the marked strain into a food. Firefly luciferase and green fluorescent protein are two light-emitting markers that have been used commonly (Fratamico *et al*., 1997). The commercial availability of the gene sequences for these markers has contributed to the popularity of these types of studies. For example, strains marked with the green fluorescent protein have been used to study survival of *Salmonella* (Gandhi *et al*., 2001) and *E. coli* O157:H7 (Takeuchi and Frank, 2001b) in produce, and bioluminescent strains have been used to study sublethal injury in *Salmonella* (Ellison *et al*., 1991; Chen and Griffiths, 1996) and penetration of *Campylobacter* into egg shells (Allen and Griffiths, 2001).

9.3 Microscopy

Microscopy is a powerful tool for analysis of microbial populations, because it allows us to see things at the microbial level, below the detection limit of the human eye, in a direct way that cannot be matched by other techniques. Two essential features of the microscope are its magnification and resolving power, which are inherent properties of its optical lens system. Magnification is the ability to make objects appear larger than they are. Resolving power is the ability to distinguish two closely-placed objects as distinct and separate. As the magnifying power of a lens system increases, resolving power generally decreases. The usefulness of a microscope obviously depends on the quality of the lens system with regard to both magnification and resolving power.

For food microbiological analysis, microscopy offers advantages, but also has disadvantages in comparison to other analytical methods. As a direct method of analysis, microscopy is difficult to match for its speed of detection; however, the food matrix is a major interfering factor. Because such a small sample size is analyzed in microscopy, its sensitivity is often less than that of other methods. However, microscopy may be combined with separation/concentration techniques to relieve food matrix interferences and to improve the sensitivity of detection. Specificity can be a problem, because microbial cells generally cannot be differentiated microscopically. However, staining techniques that employ molecular probes, such as antibodies and oligonucleotides, may be used specifically to identify microorganisms in the sample.

Microscopy provides one of the best technical options available for rapid enumeration of microorganisms. The other standard methods of enumeration, viable counts in agar media and the most probable number procedure, are more sensitive than microscopy but require one to several days to complete. Microscopic enumeration, by comparison, can be completed within an hour. The speed of analysis not only makes microscopy useful for research studies but also for practical applications in food analysis.

Often microscopy is used for identification procedures, after enrichment culture and isolation of the microorganism from the food. In these applications, direct analysis of the food is not a goal. Furthermore, microbial enumeration cannot be performed because the microbial population has been allowed, via enrichment culture, to increase in number beyond its original level. Nevertheless, such uses of microscopy provide unique capabilities, and, in many cases, may help to shorten the time required to identify the microorganism.

In summary, microscopy is a versatile technology, capable of allowing both rapid detection and enumeration of microorganisms in foods, or when used in identification procedures, improving the time of analysis. A number of procedures for the microbiological analysis of foods involve microscopy in various forms.

9.3.1 Bright field microscopy

Bright field microscopy is readily available in food testing laboratories and is useful for microbial identification procedures, such as Gram staining, endospore visualization, and fungal morphology characterization. Certain quantitative procedures also employ bright field microscopy. In the Howard Mold Count, for example, product quality determinations are based on the number of mold fragments in a sample deposited onto a special type of slide (AOAC International, 1997c). In milk grading, microbial counts are obtained from microscopic examination of the milk prepared as a stained, dried film on a microscope slide (Packard *et al.*, 1992). Bright field techniques usually involve fixation and staining of the sample, to provide sufficient contrast or visualization of the microbial cell against the background.

9.3.2 Fluorescence microscopy

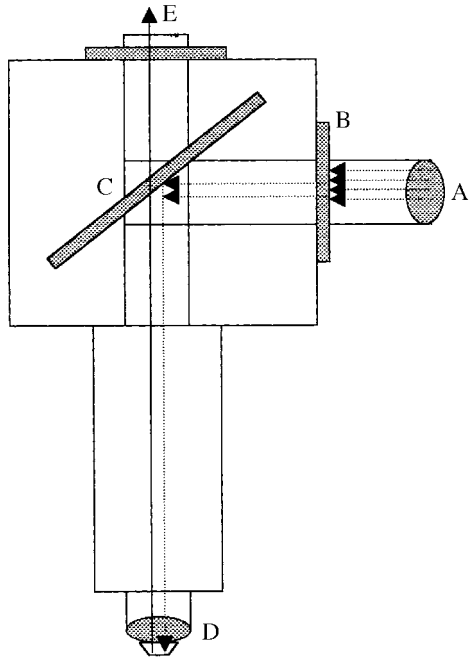
Fluorescence involves the excitation of a substance (i.e. the fluorochrome) by exposure to short wavelength light, causing emission of longer wavelength light by the fluorochrome. In fluorescence microscopy, various types of fluorochromes may be used as stains for visualization of microbial cells. The excitation and emission wavelengths are controlled and separated by appropriate filters positioned within the microscope. The design of a fluorescence microscope is based on either of two types of illumination, that is, transmitted or incident light. For food microbiological analysis, the most practical and widely used is the epifluorescence microscope, which features illumination of the sample by incident light (Fig. 9.1). There are two primary advantages afforded by epifluorescence or incident light illumination. First, it yields superior performance at the high magnifications needed for microbial cells. Second, because the light originates from above the sample, causing illumination from the sample surface, epifluorescence provides the ability to analyze the thick or opaque specimens that transmission fluorescence microscopy does not allow. The direct epifluorescent filter technique (DEFT, discussed below) takes advantage of these features of the epifluorescence microscope and is one of the most rapid methods known for enumerating microbial cells. The incorporation of molecular probes in the DEFT increases its utility, to allow rapid enumeration of specific microorganisms.

9.3.3 Confocal laser scanning microscopy

The capacity of the epifluorescence microscope for analysis of thick specimens, such as foods, is limited. In epifluorescence, the incident light illuminates the entire thickness of the specimen, causing out-of-focus fluorescent emissions to reach the eye and blur the image. In confocal laser scanning microscopy (CLSM), these out-of-focus emissions are removed, thus allowing sharp, high-contrast imaging of objects deep within a structure. The specimen is illuminated by rastering an intense beam of light across the plane of focus. A computer collects and stores the data, and when several focal planes are analyzed, high-contrast three-dimensional images may be created. Thus, CLSM allows analysis of thick specimens that would require sectioning and staining in other microscopic techniques. It provides the capability to study pathogens *in situ* in foods, without fixation or other sample preparation procedures that might give rise to artifacts. Biofilm work has been particularly advanced by CLSM. However, the cost and complex instrumentation of CLSM make it primarily a research technology, and not generally applicable for routine use in detection or enumeration of microorganisms in foods. For a review of CLSM applications in food microbiology, see Takeuchi and Frank (2001a).

9.3.4 Electron microscopy

Like CLSM, electron microscopy is basically a research tool, and not useful for routine analysis of microorganisms in foods. Two types of electron microscopy,



A, Light source, e.g. mercury, halogen or xenon lamp
B, Excitation filter
C, Dichroic mirror
D, Objective lens
E, Barrier filter

Fig. 9.1 Components of the epifluorescence microscope. In epifluorescence microscopy, light from (A) is passed through an excitation filter (B) which directs selected wavelengths to a dichroic mirror (C; also known as a dichromatic beam splitter), placed at a 45º angle to the vertical axis of the microscope. The dichroic mirror reflects (shorter) excitation wavelenghts and transmits (longer) fluorescence wavelengths. The reflected light from C is focused onto the specimen through the objective lens (D), which collects the fluorescent emissions for transmission back up to the eyepiece through C and the barrier filter (E). Only the fluorescent wavelengths of the fluorochrome in use are transmitted to the eyepiece. A filter set (B, C, E) is usually assembled for use with a particular fluorochrome and may be exchanged for another set within the microscope housing when use of a different fluorochrome is desired.

transmission (TEM) and scanning (SEM), have been of use in microbiological research. TEM has tremendous magnification and resolving power, and with detection limits in the nanometer range, it can provide details of subcellular structures of microorganisms. Although artifacts may be generated as a result of extensive sample preparation procedures required in sectioning and staining of the specimen, it has been a workhorse for studies of microbial cell ultrastructure. The mechanisms of cell inactivation of food processing treatments have been investigated using TEM (Calderon-Miranda *et al*., 1999; Dutreux *et al*., 2000). In SEM, the specimen is coated with a thin film of a heavy metal, and a beam of

electrons is scanned across it. The technique gives a three-dimensional image of the surface of the specimen and has been useful for studies of microorganisms in their natural environments, for example attachment of pathogens to foods and equipment surfaces (Fett, 2000; Woody *et al*., 2000; Han *et al*., 2000; Lindsay and von Holy, 1999).

9.3.5 Microscopic enumeration of microorganisms

One of the most powerful applications of the microscope in food microbiology is its potential for counting microorganisms directly and rapidly. In fact, the examination of a food film dried onto a microscope slide is among the oldest techniques described in food microbiology. Originally developed and used for the grading of milk (Breed, 1911; Packard *et al*., 1992), microscopic enumeration of microorganisms in thin films has also been applied to other foods (Bryce and Poelma, 1995; AOAC International, 1997a). A known quantity of the sample is applied to the slide, and the number of microbial cells counted per microscope field is converted into numbers per gram based on the area of the field, as described below.

The microscope slide used for dried film analysis has a $1\,cm^2$ circular area imprinted on it, which is used to contain the sample in a defined area. A 0.01 ml quantity of the sample is spread within the circle, dried, fixed and stained. The number of microbial cells is counted in 10–100 fields, and the average count per field is calculated. To obtain the concentration of cells in the food, the Microscope Factor (MF), which is unique for every lens, must be determined. The MF is the number of microscope fields in the $1\,cm^2$ slide area, divided by the quantity of sample (0.01 ml) applied. The formula for the area of a circle (πr^2) is used to determine the area of the microscope field for a particular lens; r is determined by measuring the field diameter using a stage micrometer and dividing by two. The MF is obtained by dividing the slide sample area ($1\,cm^2$) by the field area to obtain the number of microscope fields in the slide area; and then dividing this quantity by 0.01 ml. Finally, the concentration of microorganisms in the food is calculated by multiplying the average count per field by the MF.

9.4 The direct epifluorescent filter technique (DEFT)

The dried film technique can be used for enumeration only when microbial cell densities are quite large, because of the small sample size examined. Greater sensitivity (i.e. lower detection limit) can be achieved by including a sample concentration step, as in the direct epifluorescent filter technique (DEFT). In the DEFT, membrane filtration of the sample improves sensitivity by several orders of magnitude. When the sample is passed through the filter, the microbial cells are concentrated and collected on the filter, which provides the surface on which the microscopic analysis is performed. Incident light illumination (epi-fluorescence) is used to examine the filter surface.

The DEFT had its origins in microbiological studies of natural aquatic systems (Collins, 1957; Francisco *et al*., 1973). Well-acquainted with the failings of conventional culture media to recover microorganisms from environmental samples, microbial ecologists looked to microscopy for direct analysis of the systems under study. Use of the acridine orange stain, with its affinity for nucleic acids, allowed microscopists to distinguish biological (i.e. cellular) matter from detritus. Considering all things that might be present and visible in a microscopic sample, this was a significant technological advance. For sample concentration, black membrane filters were used, which provided contrast between the fluorescent cells and background. Later, the development of polycarbonate membranes of uniform pore size provided improved microbial counts compared to cellulosic filters, due to greater capture of cells on the filter surface, as opposed to within the depths of the filter.

Food microbiologists explored the method's use originally for counting microorganisms in milk, as a more sensitive alternative to the dried film method (Cousins *et al*., 1979; Pettipher *et al*., 1980). To effect filtration through the 0.6 μm pore filter, the milk was pre-treated with protease, surfactant and mild heat. Homogenized foods, including meat, fish, vegetables, and spices, were next, and the term 'direct epifluorescent filter technique', or DEFT, was coined (Pettipher and Rodrigues, 1982a). For these samples, a preliminary step of pre-filtration through a 5 μm pore nylon filter was added to the procedure to facilitate passage of the food through the analytical filter. A digital camera attached to the microscope with linkage to an image analysis system showed the potential to automate the technique (Pettipher and Rodrigues, 1982b). The speed of the DEFT for direct enumeration of microorganisms was unmatched, and it compared favorably with other techniques with respect to sensitivity and precision (Pettipher, 1986). Evaluations were performed to support its use as a standard method for the microbiological analysis of meats (Ovist and Jakobsen, 1985; Shaw *et al*., 1987; Boisen *et al*., 1992).

The following procedure has been recommended for the microbiological analysis of raw milk by the DEFT (Pettipher *et al*., 1980; Bier *et al*., 2001). All reagents, buffers and solutions for use in the DEFT must be passed through 0.22 μm pore sterilization filters to remove contaminating microorganisms that would be visible microscopically and influence counting results. The milk sample (2 ml) is mixed together with 0.5 ml trypsin and 2 ml Triton X-100 (each at 0.5% w/v), and incubated at 50ºC for 10 min. The somatic cells and lipid micelles in the milk are lysed by this procedure, facilitating filtration. The lysed mixture is then added to a previously warmed filter assembly holding a 25 mm diameter, 0.6 μm pore black polycarbonate membrane filter (shiny side up). A vacuum is applied to the filter assembly, which is then rinsed with 5 ml Triton X-100 (0.1% w/v). The vacuum is disconnected, and the membrane filter is overlayed with 2 ml acridine orange stain (available commercially, or prepared as a 0.025% w/v solution in 0.1 M citrate-NaOH buffer, pH 6.6). After staining for 2 min, the membrane filter is rinsed under vacuum with 2.5 ml 0.1 M citrate-NaOH buffer, pH 3, followed by 2.5 ml 95% ethyl alcohol. The membrane filter

is air-dried, then mounted on a glass microscope slide in a drop of nonfluorescent immersion oil. Finally, a cover slip is applied for examination of the membrane filter by epifluorescence microscopy, using a 450–490 nm wide-bandpass filter appropriate for acridine orange fluorescence.

It is best to choose the microscope objective lens that provides the widest field of view for scanning the filter for the presence of fluorescent cells. For detecting and counting bacteria, the 100X oil immersion lens, which is the lens with the smallest field of view, is generally necessary. However, other microorganisms, e.g. yeasts or protozoa, may be detectable using lower power lenses which provide larger fields of view. During a preliminary scan of the membrane, a rough estimate of the number of cells per field is made; if there are typically more than 100 cells per field, the sample should be diluted before counting is attempted. Fluorescent cells in randomly selected fields across the membrane filter are then counted. The number of fields that should be analyzed depends on the microbial cell density, recommended as follows: if there are 0–10 cells per field, 15 fields are analyzed; for 11–25 cells per field, 10 fields are analyzed; for 26–50 cells, 6 fields; for 51–75 cells, 3 fields; for 76–100 cells, 2 fields. Calculation of the number of cells per ml of milk is done as for the dried film procedure (above), by multiplying the average number of cells per field by the MF; however, in the DEFT the area of the membrane filter is factored (membrane filter microscope factor, MFMF). The MFMF is the area of the membrane filter divided by the area of the microscope field. The concentration of microbial cells in the sample is calculated by multiplying the average number of cells per field by the MFMF, then dividing by the volume of material filtered. Any dilution of the original sample should be factored into the calculation.

Adaptations of the DEFT have been made to extend its functionality. Determination of viable cells has been performed by incubation of the membrane filters for several hours on agar media after sample filtration, followed by staining and examination of the microcolonies formed by epifluorescence microscopy (Rodrigues and Kroll, 1988, 1989). The microcolony technique was further modified to include the use of fluorescent antibodies as staining reagents, allowing for specific identification of viable cells. The potential for rapid detection of viable cells of *Salmonella* (Rodrigues and Kroll, 1990) and *Listeria* (Sheridan *et al*., 1991) in raw meats was demonstrated by the microcolony-fluorescent antibody method.

Fluorescent antibodies were also applied directly in the DEFT without microcolony formation, as an alternative to acridine orange staining, for rapid specific detection and enumeration of microorganisms. The antibody-direct epifluorescent filter technique (Ab-DEFT) allowed specific enumeration of *E. coli* O157:H7 in milk and juice samples within one hour (Tortorello and Gendel, 1993). The sensitivity of the Ab-DEFT for detection of *E. coli* O157:H7 in beef compared favorably with a conventional enrichment culture technique, and it was shown to be useful for measurement of growth of the pathogen in beef (Tortorello and Stewart, 1994). The Ab-DEFT was evaluated as an alternative to

the most probable number method for enumeration of *Listeria* in vegetables (Tortorello *et al*., 1997).

In a variation of fluorescence *in situ* hybridization (FISH), application of fluorescent oligonucleotides in the DEFT has been demonstrated, but their use has been reported more frequently by microbial ecologists than by food microbiologists. The usual target of oligonucleotide probing is ribosomal RNA, because its abundance in the cell allows for a fluorescent signal sufficiently large to be detectable by microscopic observation. Depending on the level of specificity, oligonucleotides complementary to ribosomal RNA sequences may function as group- or species-specific probes for microbial cells in a sample. Direct enumeration of *E. coli* in water, various beverages and vegetable sprouts was demonstrated by using a fluorescent oligonucleotide in the DEFT (Tortorello and Reineke, 2000).

The detection limit of the DEFT is in the order of 10^3 cells/ml (Pettipher, 1986). Although the detection limit of the Ab-DEFT for *E. coli* O157:H7 in beef was reported as approximately 16 cfu/g, it was achieved by passing up to 100 ml of homogenate through the filter and by scanning hundreds of microscope fields (Tortorello and Stewart, 1994). In comparison to the recommended DEFT parameters for sample volume and number of fields analyzed (discussed above), the Ab-DEFT report represented a significantly greater effort. Furthermore, the excellent quality of the fluorescent antibody, which provided an intense fluorescent signal, was an important factor which contributed to the sensitivity of detection, for it allowed visualization of the fluorescent cells within a dense, complex background. These conditions may not be feasible or available for the routine application of the DEFT.

Modifications to the DEFT have been explored to improve its sensitivity for pathogen detection. A short period of sample enrichment prior to the Ab-DEFT, for example, substantially reduced microscope scanning time by allowing cells to multiply to readily detectable levels, thereby improving sensitivity (Restaino *et al*., 1996). This modification resulted in a 10 h screening assay, which, although it no longer allowed enumeration, resulted in a detection limit (0.1 cfu/g) comparable to that of standard cultural methods. The same report demonstrated the use of immunomagnetic separation (IMS) for confirmation of Ab-DEFT-positive samples within 24 h (Restaino *et al*., 1996). The ease and speed of the combined screening and confirmation protocol represented a substantial saving in time and effort for screening of beef for the presence of *E. coli* O157:H7. In comparison to other rapid methods (e.g., IMS-polymerase chain reaction and flow cytometry), the Ab-DEFT provided the quickest detection of *E. coli* O157:H7 in enrichments of apple juice inoculated at 0.1 cfu/ml (Tortorello *et al.*, 1998a).

9.5 Flow cytometry

Numerous excellent books and extensive review articles have been written on the technical aspects of FC including microbial analysis by FC (Davey and Kell,

1996; Givan, 2001a; Shapiro, 2000; Steen, 2000). The following is intended as a brief overview for those unfamiliar with the technology of flow cytometry with particular emphasis on the problems associated with analysis of microbial organisms.

9.5.1 Basic principles and instrumentation

This section will focus on FC as an off-the-shelf technology and not deal with custom, one of a kind instruments, or with proprietary instrumentation such as those designed specifically for the food industry. In a typical commercially available FC instrument, particles (cells, bacteria, nuclei, beads) move in an aqueous stream that intersects a focused, stable, high intensity beam of light, usually produced by a laser (Fig. 9.2). Some instruments may utilize two or more lasers. The primary laser is usually an argon laser, producing a 488 nm excitation beam. In order to achieve uniform light exposure of the cells, they must be stabilized at a uniform distance in relation to the focused excitation beam. This is accomplished by a laminar flow technique in which a narrow sample stream, or sample core, containing the cells or other particles is stabilized within a larger stream known as the sheath. By maintaining a precise pressure differential between the sample stream and the sheath, cells move through the laser beam in a single file line fixed at the focal point of the beam. This process is known as hydrodynamic focusing. Depending on the instrument, the intersection of laser and stream may occur in air (stream in air), within an optical quartz flow cell (stream in quartz), or on the surface of a cover slip (stream on surface). Each of these configurations offers certain strengths and

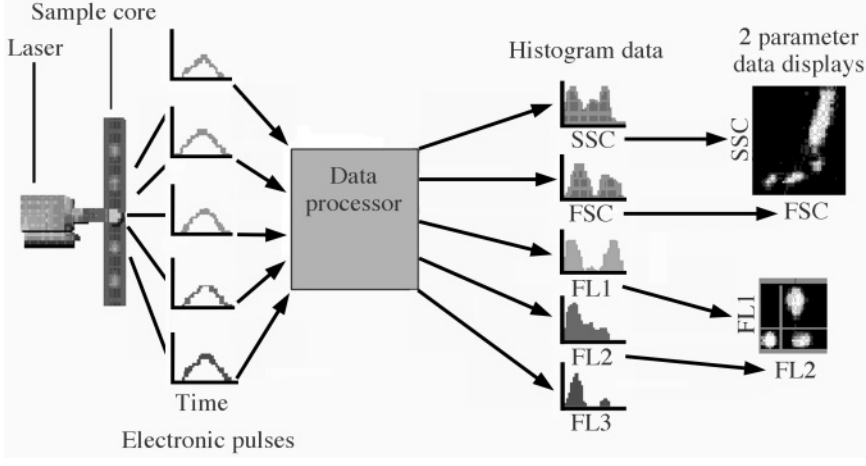


Fig. 9.2 Overview of signal and data pathways in flow cytometry. Scattered and emitted fluorescence light signals from cells passing the laser are converted to electronic pulses by PMTs. Pulses are converted to digital values, processed by the computer, and used to produce data displays. (Adapted from Becton Dickinson Immunocytometry Systems.)

weaknesses to be discussed later. When the cells pass through the laser, the light that is scattered is collected by detectors configured to detect light at a low angle in the direction of the laser beam path (forward light scatter) or light that is emitted at approximately 90º to the beam path (side scatter, or 90º light scatter). Forward light scatter (FSC) is correlated roughly with cell size, while side scatter (SSC) correlates with internal structure such as granularity, or with surface roughness. This relationship between physical properties of cells, including microorganisms, and light scatter provides an additional determinant for characterizing cells, exclusive of fluorescent probes. FSC and SSC as well as light emitted by fluorescent probes bound to the cells are collected by a series of focusing lenses and distributed to detectors along an optical pathway that includes appropriate wavelength transmitting or blocking filters. This results in each detector being specific for one light signal or parameter. The detectors are photomultiplier tubes (PMTs) for SSC and fluorescence emissions, and usually a photodiode for FSC. Both types of detectors convert photons into an electrical current. The current from the PMTs are then passed through linear and/or logarithmic amplifiers. Linear amplification is useful for analyzing particles of similar size or fluorescence (such as beads), whereas logarithmic amplification is suited to analysis of particles that are more broadly distributed with respect to a particular parameter. In general, where a high level of discrimination may be needed, such as light scatter, linear amplification is used. The amplified pulses are then processed and converted from analog to a digital number, typically from 1–1000 mV. These numbers are then used to generate a variety of data plots (Fig. 9.2).

It is necessary to be able to distinguish cells of interest from debris and electronic noise. In order to accomplish this, a threshold level is established for one of the signals. Typically, light scatter signals are used for this purpose, because all cells, even in a sample that may contain non-fluorescently labeled cells, will produce a scatter signal. If the signal from the cell or particle is above this level, then the instrument is triggered to collect all other signals derived from the same cell or particle. This is the basis of a most important aspect of FC: multi-parameter data is generated on individual cells. FS, SSC, and fluorescence data are stored in the computer as correlated data for each cell (listmode data). This allows for the generation of data plots based on single or multiple parameters. Plotting the data in this way gives rise to populations of cells based on the accumulated single cell data. These populations can be included within graphically represented regions that can function as regions and gates. Data regarding other parameters linked to the cells that fall within the gate can then be derived. Multiple gates can be used to further define cell populations. Data saved on the computer as listmode can be re-analyzed as necessary to generate new data plots and additional information. The same gates can also be used to define populations of cells for sorting if the cytometer has that capability. Sorted cells can then be subjected to further analysis.

9.5.2 Flow cytometry of microorganisms

If the world of FC is large, the world of FC of microorganisms is fairly small, but growing. Since its inception, the potential usefulness of FC for the study of microorganisms has been considered; however, microbiologists have been relatively slow to adopt the technology for the study of bacterial populations. Studies using FC instruments to analyze bacteria were first published in the late 1970s (Bailey *et al.*, 1977). The clear advantage of FC for bacterial analysis lies in the ability to collect correlated data on single cells as described above and in other comprehensive articles (Davey and Kell, 1996; Shapiro, 2001). This capability represents a potential conceptual breakthrough from viewing bacterial populations as monolithic entities. Questions regarding variations in the antigenic, reproductive and physiologic state of individual cells in a large population can be addressed. Nevertheless, as pointed out by Harald Steen, one of the pioneers in the field of microbial analysis: 'No one seemed to realize that they had hit a scientific goldmine … even now, the majority of bacteriologists are not aware of the possibilities that flow cytometry offers for acquiring knowledge about bacteria which is difficult, if not impossible, to obtain by other methods' (Steen, 2000). The list of potential parameters that could be measured in microorganisms is lengthy (Shapiro, 2000). The reasons for this relatively underdeveloped state of knowledge may lie both in the fact that most microbiologists are unfamiliar with FC and that no area of information about bacterial populations has arisen from multiparameter FC that is considered to be unique and indispensable to microbiologists. In other words, there is no 'killer application' that makes FC universally essential to microbiologists. By way of analogy, the development of multiparameter lymphocyte phenotyping quickly made FC an integral part of almost every immunology and hospital clinical laboratory. A second handicap faced by microbiologists has been a lack of readily available reagents for specific identification of microorganisms. To extend the analogy with lymphocyte phenotyping, the widespread use of FC in immunology is both driven by and supports the development of a huge variety of commercially available FC reagents. This problem is particularly acute in the area of pathogen detection, where identification may depend on specific antibody or DNA probes. Within the past few years, some commercially available reagent kits for FC of bacteria have become available, such as the BacLight™ kit (Molecular Probes) for distinguishing live and dead bacteria (as in Raybourne, 1997).

The most basic information on microbial analysis by FC comes from research on organisms grown in culture in the laboratory. Applied applications have focused in the areas of clinical, food and primarily environmental microbiology. Most of the information gained from analysis of bacteria in pure culture or the environment is also relevant to detection of microbial pathogens in foods. Indeed, there is a direct relationship between environmental microbiology and food especially in the area of pathogens that may be generally considered waterborne, but can also be transmitted due to exposure of food to contaminated water. Of special interest in this area are FC methods related to the protozoan

Cryptosporidium spp. (Vesey *et al*., 1994; Valdez *et al*., 1997). While the list of foodborne microbial pathogens includes this and other protozoans, the focus here will be on bacteria.

The basic FC principles outlined above apply generally to analysis of microorganisms such as bacteria. However, these organisms do present some special problems. One such problem is due to the relatively small size of bacteria. Most commercially available flow cytometers are designed for analysis of particles in the size range of eukaryotic cells, for example lymphocytes, which are roughly ten times the diameter (or 1000 times the volume) of most bacteria. This problem is most critical in the detection of FSC and SSC signals. In many commercial flow cytometers FSC of bacteria may be difficult to separate from noise. The noise may come from electronic, optical and particulate sources (Hoffman, 1997). The latter is due to small particulates in the sheath fluid or the sample medium and can be dealt with by careful filtration of the sheath and other solutions involved in the experiment. Electronic noise becomes a problem when increasing amounts of voltage and/or amplification are applied to the FSC and SSC detectors. Optical noise is detected when signals derived from the laser excitation source are present in the absence of sample particles. The optical and electronic problem is particularly acute with FSC, since many commercial instruments use a photodiode, which is less sensitive (but adequate for eukaryotic cells) as the FSC detector. As with any FC analysis, the objective is to achieve optimal separation between the signals of interest and the threshold, below which noise occurs. Because, as previously mentioned, FSC is commonly used as the threshold or triggering signal, lack of separation between signal and noise can result in spurious accumulation of noise in all other parameters. Some degree of FSC noise is always present due to dust, particulates, stray laser light, and even room light, usually making it necessary to maintain some threshold at all times. As an example, this might be a threshold level of 50 channels out of 1000. The FSC amplification necessary to resolve lymphocytes, for example, would result in almost no noise above the threshold of 50.

As one attempts to resolve smaller and smaller particles, it becomes necessary to increase the degree of amplification of the FSC signal. Eventually this results in significant noise appearing above the threshold of 50. Of course, one could increase the threshold, but this strategy will only be successful if the signal of interest is not overlapping the threshold to a significant degree. Thus, analyzing bacteria using FSC as the threshold requires careful optimizing of signal to noise. Some factors that can help in this are careful optical alignment of the instrument, cleaning of optical filters and lenses and reduction of ambient light. Some cytometer optical configurations achieve better FSC resolution and sensitivity than others. Of the most widely distributed commercial instruments, those with stream in quartz configurations are superior to stream in air (Shapiro, 2001; Harkins, 1999). This is due to increased scattering of light from the sheath fluid when the laser interacts with the stream in air. Steen and colleagues (Steen, 2000) developed the stream on open surface configuration in which FSC is

collected using microscope optics. In this system, the FSC detector is a photomultiplier tube rather than a photodiode. This unique system configuration achieves resolution down to 0.2 μM. This type of instrument has been used to produce unique light scatter profiles as a means of rapidly characterizing bacterial populations. This characterization was further improved with the addition of DNA content as a parameter (Allman *et al*., 1992). Using this instrument, in conjunction with a specific antibody probe, it could be demonstrated that *E. coli* O157:H7 had enhanced FSC over background microbial flora in a beef enrichment culture (Raybourne, 1997).

Additional strategies have also been developed to resolve bacteria on mass marketed flow cytometers lacking sensitive, high resolution light scatter detection. One approach is the use of SSC as the threshold or triggering parameter (Harkins, 1999; Alsharif and Godfrey, 2001). The SSC detector is typically a PMT which gives this parameter increased sensitivity over the photodiode detector typically used for FSC. In addition, this PMT is not in the direct light path of the laser as in the case of the FSC detector, reducing the tendency for optical noise. Additionally, log amplification of the SSC and/or FSC results in increased sensitivity. The emphasis on FSC and SSC as threshold parameters can be avoided entirely in favor of a fluorescence parameter by the use of a fluorochrome labeled probe or other fluorochrome to distinguish particles of interest from particulate noise (Nebe-von-Caron *et al*., 2000; Pinder *et al*., 1990; Porter, 1999). This strategy is based on the assumption that all particles of interest, i.e. bacteria or other cells, will take up the dye and become more fluorescent than particulate debris. Dyes that bind to cellular structures such as nucleic acids (Davey and Kell, 1996) are commonly used for this purpose. Nucleic acid binding dyes that increase their fluorescence upon binding to DNA are particularly useful. Success also depends on the dye penetrating all the cells. To achieve this may require the use of a dye that is cell membrane permeant such as thiazole orange (Givan, 2001b), acridine orange, or Syto 9 (Molecular Probes, Eugene OR, USA). Even though these dyes are permeant in eukaryotic cells, they may have difficulty being taken up by bacteria. Treatment of the cells with EDTA to deplete the Lipopolysaccharide (LPS) can facilitate bacterial labeling (Alsharif and Godfrey, 2001). If there is no need to retain viability of the cells, treatment such as fixation by heat or other toxic method can be used to render the cells permeable to normally impermeable nucleic acid binding dyes, such as propidium iodide or ethidium bromide. This increases the number of possible fluorochromes that can be used for this purpose (Pinder *et al*., 1990; Davey and Kell, 1996).

9.5.3 Microbial pathogens in food

Detection of microbial pathogens such as bacteria in foods presents some additional challenges to those outlined above. These problems relate primarily to the complexity of the food matrix and the need to identify bacteria with some specificity. Many foods are fairly complex and require some processing to

release bacteria into a suitable medium for further analysis. The processes for doing this (grinding, etc.) often result in the presence of particulates that can be extremely troublesome for FC analysis. Even liquid foods may require some processing. Foods are also generally not sterile and usually contain a presence of background bacterial flora or spoilage organisms that are not pathogens and are of little interest for regulatory purposes. Thus, the challenge becomes specific identification of pathogenic strains.

Under ideal circumstances, measurement of some of the markers listed above (DNA staining, FSC, SSC) may help identify certain bacteria, but it is likely that a specific marker such as an antibody or nucleic acid probe will be needed. The availability and standardization of antibodies of appropriate specificity can then become a limitation. In addition, the pathogens of concern in foods often have very low presumptive infectious doses making it necessary to be able to detect as few as 10–100 cells of the organism of interest per gram of food, in the presence of a large number of bacteria of no regulatory interest. In spite of these potential difficulties, there is a significant potential payoff in the use of FC in food microbiology in the areas of rapid detection and high throughput, and in the ability to identify organisms of interest through multi-parameter single cell analysis. In terms of rapid detection, FC produces real-time and archival data that may be particularly important for critical control point monitoring (Wang and Slavik, 1999). Flow cytometers equipped with cell sorting capability can also be used to isolate bacteria of potential interest for further culture and analysis. FC can also detect non-viable as well as so-called viable non-culturable bacteria (Nebe-von-Caron *et al*., 2000) that can be of regulatory significance.

A number of studies that have focused on detection of potentially pathogenic bacteria in food are summarized in Table 9.1. Excluded from Table 9.1 are studies that exclusively involve detection of specific pathogens derived from pure broth cultures (Goodridge *et al*., 1999; Clarke and Pinder, 1998; Kusunoki *et al*., 1998; McClelland and Pinder, 1994a), although the use of pure cultures is a necessary step in the development of methods applicable to a food matrix. There are several common elements to all of the studies listed in Table 9.1. These elements will be considered below. Experimental details can usually be found in the references cited. A more detailed protocol is also available (Raybourne, 1999) for studies with *E coli* O157:H7 in food.

9.5.4 Sample processing and enumeration of bacteria

Comparison with conventional agar plate counts is generally used as the standard for counting bacteria by FC. As a starting point for method development, target organisms can be diluted in a filtered diluent such as PBS. Aliquots can then be taken for FC counting and plating. These simple suspensions are useful for instrument standardization. Many investigators then proceed to spiking of a food extract with dilutions of bacteria followed by parallel FC counting and plating. Because such food extracts often contain large

Table 9.1 Some studies utilizing flow cytometry for detection of microbial pathogens in food

Organism	Food matrix-microbiological methods	FC methods	Reference
Listeria monocytogenes	Milk (selective enrichment from)	Cytofluorograf™: DNA (PI) Immunofluoresence Light Scatter (SSC)	Donnelly and Baigent (1996)
Salmonella typhimurium *Salmonella montevideo*	Milk (direct detection after clearing and non-selective enrichment)	Custom-built cytometer: Nucleic acids (Ebr) immunifluorescence (FITC & R = PE) Light Scatter (FSC)	McClelland and Pinder (1994a)
Salmonella typhimurium	Eggs and milk (direct detection after clearing and non-selective enrichment)	Custom-built cytometer: Nucleic acids (Ebr) Immunofluorescence (FITC) Light Scatter (FSC)	McClelland and Pinder (1994b)
Escherichia coli O157:H7	Ground beef (non-selective enrichment) FC vs. Ab-DEFT comparison	EPICS Elite™ and Bryte HS™Immunofluorescence (FITC) Light Scatter (FSC) Sorting	Tortorello *et al*. (1998b)
Escherichia coli O157:H7	Apple juice (non-selective enrichment) Comparison of six methods vs. time	Bryte HS™Immunofluorescence (FITC) Light Scatter (FSC)	Tortorello *et al*. (1998a)
Salmonella typhimurium	Washes from processed poultry (direct detection and selective enrichment) Immunomagnetic separation	FACSort™Immunofluorescence (FITC) Light Scatter (FSC)	Wang and Slavik (1999)
Total bacteria (*Escherichia coli* and *Staphylococcus aureus*)	Milk (direct detection after clearing)	FACScan™ and FACSCalibur™: Nucleic acid (SYTO BC) Light Scatter (FSC and SSC) Viability (PI) Sorting	Gunasekara *et al.* (2000)

amounts of particulate matter, some type of filtration step is necessary to avoid clogging of the flow cytometer. Filtration of the extract or enrichment culture through a 5.0 μm porosity filter has proven to be adequate to remove harmful large particulates (Tortorello *et al*., 1998b). If immunofluorescence is being used to identify the pathogen of interest, the samples must be stained before FC analysis. This is accomplished by addition of a predetermined optimal concentration of fluorescent antibody probe to the samples, followed by incubation and washing (Tortorello *et al*., 1998b). Some studies (Tortorello *et al*., 1998a; McClelland and Pinder, 1994a; Pinder and McClelland, 1994) use a killing step, which can be important as a safety consideration when working with biosafety 2 level pathogens because FC can generate aerosols. This would obviously not be practical in studies in which sorting and subsequent culture are planned. However, most flow cytometers capable of cell sorting have provisions for aerosol control due to the common use of these instruments to sort cells from infectious human samples (e.g. HIV infected blood). One advantage of a killing step is that it can facilitate the use of some nucleic acid staining dyes that are not normally membrane permeable. This can be used as a means to distinguish the bacterial cells in a sample from non-cellular debris and can thus aid in the identification of the entire bacterial population. In multiparameter analysis nucleic acid stains have been combined with fluorochrome-labeled antibodies to identify specific pathogens of interest in mixed bacterial populations (Donnelly and Baigent, 1986; McClelland and Pinder, 1994b; McClelland and Pinder, 1994a). Identification of the organisms of interest amongst a high background population is a significant practical consideration and can be addressed experimentally by spiking the food matrix with mixtures of the concerned pathogen and an irrelevant bacterial strain at various ratios (Pinder and McClelland, 1994), or by using similar mixtures in PBS (Tortorello *et al*., 1998b).

One approach to the problem of pathogen enumeration by FC involves determination of the volume of sample that has passed through the detection point of the instrument. If the instrument uses a microsyringe with controlled flow, the volume of sample, the number of events (bacteria) in the region of interest, and hence the concentration of bacteria, can be determined directly. In the studies listed, only the custom built cytometer and the Bryte HS have this capability. In most commercially-available instruments, it is necessary to estimate the sample volume based on flow rate and time (Gunasekera *et al*., 2000; Wang and Slavik, 1999). Another approach is to include in the sample fluorescent microspheres (beads) at a known concentration. These beads have light scatter and fluorescence properties that are distinct from the bacteria. The concentration of the bacteria of interest (e.g., FITC-stained *E. coli* O157:H7) can then be calculated as a function of the ratio of beads detected by FC vs. the known concentration. When investigated in parallel, the microsyringe and ratiometric methods produced equivalent results (Tortorello *et al*., 1998b). It is interesting to observe that, despite all of the numerous variables between these studies, all of them achieved a consistent lower limit

of detection of the pathogen of approximately 10^4 ml in various foods and in the presence of high levels of background flora compared to the 'gold standard' of viable counts.

The level of sensitivity and resolution for detection by FC seems unlikely to be improved to a level sufficient to detect directly pathogens that may be of regulatory concern at concentrations of less that 100 organisms per 25 g of food. It is for this reason that great emphasis has been placed on the use of enrichment procedures for detection of low numbers of pathogens. The simple idea of enrichment is to culture an extract of the contaminated food in microbial growth medium to a point where the pathogen can be detected directly. This can be done using a medium that selectively promotes the growth of the pathogen (selective enrichment) or a permissive medium (non-selective enrichment). The experimental procedure involves spiking of food with known numbers of the pathogen down to very low levels of about 1 organism per ml or less. At this point, one approach is to add a competing strain of bacteria at various ratios to the strain of interest in order to simulate background flora in some foods (milk and eggs; McClelland and Pinder, 1994b). Alternatively, if the food has a high inherent background (e.g., ground beef or chicken washes) a substantial natural background bacterial flora will grow during the course of enrichment (Tortorello *et al.*, 1998b; Wang and Slavik, 1999). Enrichment cultures can than be analyzed by both FC and plating at a fixed time point, or a series of enrichment cultures can be analyzed at various time points. This allows for the determination of the minimum enrichment time required to detect a given starting level of pathogen (Tortorello *et al*., 1998a; Wang and Slavik, 1999), an important consideration in a rapid method. With enrichments, enumeration by FC is relatively unimportant. The analysis is simply a positive or negative result with the detection limit extrapolated back to the original number of organisms added prior to enrichment.

9.5.5 Applications in food processing

In addition to pathogen detection, FC methods have been developed for use in food processing and quality control. Multiparameter FC analysis of dairy starter and probiotic products revealed the presence of populations of culturable, metabolically active but not culturable and permeabilized (non-viable) lactic acid bacteria (Bunthof and Abee, 2002). This approach serves to better characterize the microbial properties of such products than culture alone. FC studies were also conducted on *L. monocytogenes* subjected to high hydrostatic pressure as a potential food preservation technique (Ritz *et al*., 2001). Analysis of metabolic activity based on fluorescein diacetate, membrane permeability based on propidium iodide and light scatter were used to assess the morphological and physiological state of treated cells.

9.6 Comparing detection techniques and future trends

Reliance on enrichments to detect low numbers of pathogens is not unique to FC, and is commonly used in other rapid methods. We compared FC, Ab-DEFT, direct plating, and immunomagnetic separation combined with direct plating or polymerase chain reaction (IMS-PCR) for their ability to detect several strains of *E. coli* O157:H7 in apple juice (Tortorello *et al*., 1998a). The standard for comparison was the enrichment time required for detection of the slowest growing strain at various levels of contamination. At the lowest level (0.1 cfu/ml), Ab-DEFT proved to be the most rapid (8 h), followed by IMS-PCR (16 h), FC (24 h), IMS-direct plating, (32 h), and direct plating alone (48 h). In this direct comparison, FC and Ab-DEFT compared favorably with IMS-PCR, without the additional step of IMS. If the time required for each analysis were included it is likely that FC would be capable of analyzing many more samples per hour than other methods, since the approximate time for analysis of each FC sample is about 1 min.

9.6.1 Dedicated instruments

One major drawback to the use of FC as a detection method is the relatively high cost of the instrumentation that has kept the technology primarily in the realm of a research tool. Commercially available research flow cytometers minimally cost approximately $100,000 and require one skilled key operator to maintain the instrument. The capabilities of these instruments may be beyond what is needed for analysis in the arena of food microbiology. These factors have led to efforts to develop dedicated instrumentation, based on FC technology, designed for specific applications that may be less expensive and less complex to operate. One such instrument is the Bactoscan® which is essentially a flow cytometer with limited measurement parameters. This instrument has been used in conjunction with acridine orange staining to measure the number of microorganisms in raw milk (Rapposch *et al*., 2000). It has not been shown to be effective for probe-based detection of specific pathogens.

9.6.2 Laser scanning cytometry

Laser scanning cytometry (LSC)® is a technology related to FC and to confocal laser scanning microscopy in which the laser beam rasters across a sample which is stationary on a microscope stage (similar to confocal laser scanning). As in FC, the signals emitted from particles in the sample are transmitted to PMTs, converted to electrical pulses, and digitized for computer analysis (Darzynkiewicz *et al*., 1999). The result is an output of single and multiple parameter histograms and dot plots similar to FC. One unique feature of this technology is the ability to re-locate particles (e.g. potential pathogens) of interest on the microscope stage for visual confirmation. As with FC, this technology is a relatively sophisticated tool with many applications in research. A dedicated instrument based on similar

technology (ChemScanRDI™, Chemunex) has been developed and used in combination with IMS to detect small numbers of *E. coli* O157:H7 in ground beef and water (Pyle *et al*., 1999). In this study, liquid samples were concentrated by filtration using a polycarbonate nuclear etched membrane filter. This approach has the potential advantage of allowing for concentration of large volumes containing relatively small numbers of bacteria. In this way, it is somewhat similar to DEFT and has the potential to greatly increase sensitivity over conventional FC.

9.6.3 Application to counter-terrorism

An unfortunate reality of today's world is that incidents of contamination of food or water might not be accidental. The possible use of food as a vehicle for bio-terrorism might involve classical pathogens such as *Salmonella* or *E. coli* O157:H7, or the use of more exotic pathogens such as *Bacillus anthracis* or *Francisella tularensis*. In either case, the high throughput potential of FC might be of value in the event of such an incident, where numerous potentially contaminated samples might have to be analyzed rapidly. As outlined above, methods exist for FC detection of *Salmonella* and *E. coli* O157:H7 in foods. Methods have also been described for FC-based detection of *B. anthracis* spores (Stopa, 2000) using FITC-labeled antibodies. Some problems with cross-reactivity to related species were reported, but could probably be overcome with more specific antibody probes. A method has also been reported for FC detection of *F. tularensis* (Grunow *et al*., 1998). It seems likely that these methods could be adapted for use in food analysis.

9.7 Sources of further information and advice

Current Protocols in Cytometry, edited by J.P. Robinson, published by John Wiley and Sons, New York in 1998. This is a compendium of detailed methods for flow cytometry and image analysis. It is in loose-leaf form and is updated regularly.

Flow Cytometry, First Principles, second edition, by A.L. Givan, published by Wiley Liss, New York in 2001. An introduction to flow cytometry.

'Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single cell analyses', Davey, H.M. and Kell D.B. (1996) *Microbiological Reviews* **60**: 641–96. A comprehensive review of flow cytometry of micro-organisms.

International Society for Analytical Cytology (ISAC). www.isac.org A professional organization for scientists interested in flow cytometry and related technologies

Cytometry: Official publication of ISAC

Handbook of Fluorescent Probes and Research Chemicals, by R.P. Haugland, published by Molecular Probes, Inc., www.molecularprobes.com. Part reference manual, part catalogue.

Molecular Expressions Microscopy Primer. Michael W. Davidson, Mortimer Abramowitz, Olympus America, Inc. and The Florida State University. http://www.microscopy.fsu.edu/primer/index.html. An educational website devoted to

all aspects of microscopy. Includes interactive tutorials, virtual microscopes, photo gallery, references and links to web resources, both educational and commercial.

Introduction to Light Microscopy, S. Bradbury and B. Bracegirdle. The Royal Microscopy Society, Microscopy Handbook Series, Volume 42. (1997) Springer, New York.

The Royal Microscopical Society. International society of professional microscopists, publishes *Journal of Microscopy* and a series of technical handbooks providing practical instruction in microscopical techniques.

Fluorescence Microscopy, by F.W.D. Rost, published by Cambridge University Press, Cambridge (1992 and 1995), two-volume set.

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