

# Application of Flow Cytometry to the Detection of Pathogenic Bacteria

Deirdre Kennedy<sup>1</sup> and Martin G. Wilkinson<sup>2</sup>

<sup>1</sup>Food Chemistry and Technology Department  
Teagasc Food Research Centre, Moorepark,  
Fermoy, Ireland deirdre.kennedy@teagasc.ie

<sup>2</sup>Department of Life Sciences, University of  
Limerick, Limerick, Ireland  
martin.wilkinson@ul.ie

DOI: <https://dx.doi.org/10.21775/cimb.023.021>

## Abstract

Outbreaks of infections have emphasized the necessity for rapid and economic detection methods for pathogens in samples ranging from those of clinical origin to food products during production and retail storage, and increasingly, in environmental samples. Flow cytometry (FCM) allows the rapid acquisition of multi-parametric data regarding cell populations within fluidised samples. However, the application of FCM to pathogen detection depends on the availability of specific fluorescent probes such as antibodies and RNA probes capable of detecting and isolating pathogens from these diverse samples. A particular issue for FCM methodology is the ability to recover and discriminate bacteria from the sample matrix which may pose a major technical hurdle towards accurate and sensitive analysis. This review article focuses on detection of pathogens using FCM in samples originating from food, water, environmental and clinical sources and outlines the current state of the art and potential future applications.

## Microbial pathogenesis

Understanding the biology of microbial pathogens and the pathways to disease are of major importance to medical microbiology and form the cornerstone for the development of preventative or therapeutic approaches to such conditions Wilkinson (2016). Disease can result from the introduction of a single factor into the host such as a pathogenic agent e.g. bacteria or virus, or may require a series of complex interactions to occur

between the host and the pathogen e.g. requiring a specific mode of transmission or an altered status of the host immune system. The diversity of microbial end products e.g. toxins generated in contaminated food products may account for the spectrum of human diseases (Stein, 1998). In recent decades, infectious diseases have become a vital and resurgent field of research in which FCM is becoming a highly useful analytical technique. Traditional approaches used to study and detect bacterial, fungal or parasitic pathogens may involve a combination of culturing on non-selective and selective media, microscopic examination, followed by a range of biochemical, immunological, and genetic confirmatory tests. Infectious pathogens of interest for FCM analysis include viruses such as human papillomavirus (HPV) and rubella virus, bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and a range of spore formers including *Bacillus* and *Clostridia* species, fungi such as *Candida* and *Aspergillus*, protozoa such as *Plasmodium* spp., and multicellular parasites such as *Trichinella* and *Schistosoma*. Each of these agents presents a significantly different challenge related to their specific detection. Detection teams who wish to identify these potential pathogens will face analysis of complex samples including those originating from foods, human or animal tissue or clinical fluids. These samples may contain a mixture of endemic microorganisms, the target pathogen(s), spores, and/ or inorganic debris which complicate the identification procedure for the target pathogen. From such diverse materials, one needs to develop FCM based assays for rapid and specific detection of the harmful pathogens. Such FCM assays should be rapid, sensitive, credible by validation, suitable for simultaneous agent detection (multiplex analysis) and applicable to a variety of complex samples (Zahavy *et al.*, 2012). More recently, because of concerns regarding bioterrorism, accurate analytical techniques for pathogenic agents are needed which can be deployed at public transport hubs or scenes of terrorist incidents. Many

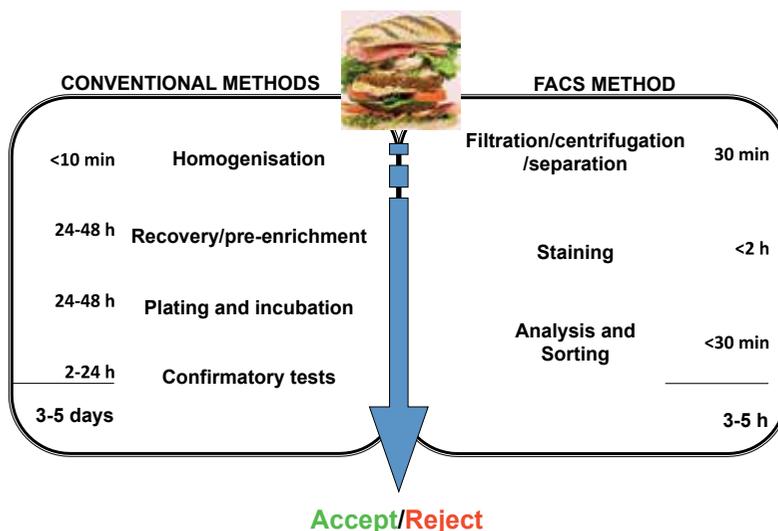
of the current detection kits depend on specific binding of antibodies to the surface antigenic components of pathogenic bacteria or viruses. Immunological detection mechanisms have both advantages and disadvantages; however, their combination with differential FCM staining and multi-parametric analysis may allow significant improvements in assay sensitivity, selectivity and data analysis (Robinson, 2001; Wilkinson, 2015).

### Advantages of flow cytometry for pathogen detection

From the point of view of a microbiologist, a key advantage of FCM methodology is the ability to simultaneously collect multiple data outputs for an individual cell (Kennedy and Wilkinson, 2015; Winson and Davey, 2000). This analysis typically generates data regarding cell viability, cell vitality, structural integrity, physiological status or stage of growth cycle. Flow cytometers offer the advantage of being able to analyse thousands of cells/events per second, depending on sample type and cell concentration in the sample. While some flow cytometers may require significant monetary investment with specialized training for operators, more cost-effective and user friendly machines are becoming increasingly available. It is clear from the increasing numbers of publications and other technical data that FCM is emerging as an alternative rapid method for microbial detection,

enumeration and population profiling. Indeed a number of studies have reported its use for the study of a range of pathogenic microorganisms (Allegra *et al.*, 2008; Berney *et al.*, 2006; Cronin and Wilkinson, 2007; Jarzembowski *et al.*, 2008; Nocker *et al.*, 2011; Ryumae *et al.*, 2010; Ueckert *et al.*, 1995). The techniques currently used to identify microbial pathogens rely upon well-established conventional clinical microbiology approaches but suffer from a number of limitations. While standard culture and susceptibility tests permit pathogen identification they are laborious, time-consuming, expensive, and may require the use of labile natural products such as egg yolk supplements and other ingredients with a short shelf life (see Figure 1). Detection and counting is carried out using optical methods, mainly by ocular inspection (Lazcka *et al.*, 2007) and detection of typical colonies is often followed by sub-culturing and use of a variety of tests for species identification (Entis *et al.*, 2001). For the modern medical or public health laboratory there is clearly a gap between these slower and well accepted traditional detection methods and the requirement to provide real time data to enable decisions to be made regarding medical treatment options, or implementation of risk management programmes (Kennedy and Wilkinson, 2015; Wilkinson, 2015).

## Conventional Detection vs. FACS



**Figure 1.** Time comparison of typical conventional microbial detection and enumeration methods with potential flow cytometric methods for detection of target bacteria.

Molecular biology and immunology has provided a broad array of materials available for labelling of specific pathogens including some commercially available antibodies and a more extensive sequence database of information for designing molecular probes. These two approaches have advanced the field with the development of commercial rapid methods for pathogen detection (Harkins and Harrigan, 2004). However if combined with FCM, developments in both molecular biology and immunology should potentially enable further advances such as on/at line detection of target cells present at low levels within mixed populations of non-target cells with almost instantaneous results. This is also of particular benefit to allied healthcare, pharmaceutical and food industries where product release can only be carried out after full testing results are obtained and interpreted (Wilkinson, 2015).

### Challenges in pathogen detection

Most detection and enumeration of pathogenic microbes is derived from cultures growing in pathogen-specific enrichment and/or selective media. However, this methodology requires that pathogenic bacteria are both viable and culturable in order to be detected which limits detection to a specific physiological state (Harkins and Harrigan, 2004). If a pathogen is not capable of growing under defined conditions these methods may lead to false-negative results or underestimate the population within a given sample. More importantly, tests that are routinely utilized for pathogen identification do not directly characterize virulence factors and do not provide the necessary information regarding the potential pathogenicity or virulence of the identified organisms (Vora *et al.*, 2004). In terms of laboratory work load and sample throughput, conventional microbiological techniques do not lend themselves well to analysis of large numbers of environmental or clinical samples. To quickly determine the presence of pathogen(s), microbiologists need reliable and accurate tools which can respond to the increasing pressures to find faster, more accurate analytical techniques for identifying infectious agents. Such efforts have led to the development of rapid FCM-based methods which can potentially rapidly detect low concentrations of pathogens in water, food and clinical samples (Hammes *et al.*, 2008; Lautenschlager *et al.*, 2013; Riyaz-UI-Hassan *et al.*, 2008). The main experimental difficulty in analysing bacteria using FCM is that many of their biological characteristics (including size, shape and DNA content) vary depending upon the growth conditions

used, or the sources from which the organism were obtained. For example, exponentially growing cells are larger than dormant or starved cells and may also contain higher levels of nucleic acids (Sincok and Robinson, 2001). Therefore, strict reproducibility of conditions is required in order to produce consistent data and all new methods require validation before widespread acceptance. In addition, the capital cost involved in flow cytometry analysis, especially where cell sorting is involved, is quite high which can restrict its widespread analytical usage (Ivnitski *et al.*, 1999). An area of potential concern to the laboratory analyst using cell sorting of biosafety level two pathogens is the generation of an aerosol of bacteria and some studies have reported the use of a killing step during sample preparation for this reason. Clearly this killing step is not practical for methods where cell sorting, viability testing or culturing is required. However, most flow cytometers capable of sorting have aerosol control such as filters but the biosafety levels required will vary with the type of sorting being carried out (Schmid *et al.*, 2001) especially where they are also routinely used for sorting of cells from infectious human blood samples (Raybourne and Tortorello, 2003). However, it is possible to house cell sorters within laminar flow hoods as is the case in the author's own laboratory for use in sorting of pathogens such as *E. coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (Kennedy *et al.*, 2011).

### Instrument set up

As bacteria are much smaller than mammalian cells, the detection and analysis of pathogenic microorganisms poses particular challenges for FCM. If the instrument is not specifically designed for microbial analysis one must first determine whether it is capable of such measurements and then optimize its performance (Harkins and Harrigan, 2004). Generally flow cytometers used for such applications are fitted with in-line filters to prevent contamination from microorganisms that may be present in sheath fluid or sheath fluid tanks. Therefore in the initial machine set up, the operator must validate the ability of the machine to detect and enumerate a specific pathogen(s). A range of fluorescent beads are commercially available for calibration and counting and are used as standards to provide a reference point allowing the operator to optimize instrument settings for detection of a particular pathogen. These beads allow detection at the size level required for analysis of small particles such as bacteria and are available in the range of 0.1  $\mu\text{m}$  upwards. Thereafter determination of

threshold values and photomultiplier tube (PMT) voltages are undertaken to enable optimization of a range of parameters for analysis of pathogens. Fluorescence and side-scatter or forward scatter ratio information allows the operator to anticipate where, for example, labelled pathogens are located on a two-parameter histogram. The concept of using FCM to analyse bacterial suspensions is not new, however the successful commercial development or modification of flow cytometers devoted entirely to the detection of a specific bacterial pathogen is still relatively uncommon.

A RAPID-B flow cytometer (LITMUS RAPID-B now Vivione BioSciences, AR, USA) described by Wilkes *et al.* (2012) is purported to have superior optical and physical characteristics for microbial applications including 130 nm resolution, a large cross-section flow cell channel, and syringe-controlled sample introduction for detecting and quantifying bacterial sized particles in complex matrices. The RAPID-B approach utilizes fluorescein-conjugated polyclonal antibodies that tag the target pathogen while a second detergent based reagent exposes bacterial cell surface epitopes. Thereafter the labelled pathogen is detected in a flow cytometer using the modified detection cell. Currently, this company claims to have developed rapid immuno-FCM tests for pathogens such as *E. coli* O157, and *Staphylococcus aureus*. Shortened enrichment times (4.5 hours) enables data to be generated rapidly with simultaneous information on total counts and viability based on Propidium Iodide (PI) staining. In total, sample preparation was reported as ~30 minutes, followed by enrichment of 4-6 hours with analysis times of 3-4 minutes. Hence the assay could deliver data within a typical manufacturing process cycle and was claimed to have a limit of detection (LOD) of 1 viable cell per 25 g of a contaminated spinach sample. Therefore the system may be used for process quality control and/or bio-mapping of a food production facility.

When developing methodology for microbial detection and enumeration by FCM it is important to first prepare and analyse pure cultures of the pathogen of interest for setting up the instrumentation and for initial validation of the labelling and enumeration method. This also sets the control profiles for the particular microbial species in terms of typical live, dead or damaged cells and where they may be expected to be located within a typical cytograph. It is important to note that species, sub-species and strain-related differences

in FCM profiles can be expected and the analyst, in many cases, will have to develop a database of these profiles for each pathogen of interest in order to recognize and differentiate target from non-target bacteria and to interpret data. Sample preparation protocols may be quite extensive in order to remove debris and to allow a sample meet the requirements of a reliable assay. Signal filtering techniques and instrument set up are important as are validation of counts as described by Subires *et al.* (2014). These workers used a polyclonal antibody conjugated to R-phycoerythrin (RPE) along with viability staining by PI and SYBR Green 1 for detection of *E. coli* O157:H7. This pathogen is the causative agent of human haemorrhagic enteritis which may lead to severe kidney failure in specific population cohorts. Signal filtering was based on evaluation of SSC events from 50-200, with optimal signal to noise ratio obtained with an SSC trigger at 150 events for threshold value. This allowed a lower limit of detection (LOD) to be established from  $10^3$ CFU/ml while a limit of quantification (LOQ) was established at  $10^4$ CFU/ml. Using signal triggering at 150 SSC the authors reported very good correlation between FCM data and viable agar plate counts. Some assay formats will require the exact enumeration of pathogens within a specific sample volume, e.g. a 1ml analyte volume derived from 25 g of sample in the case of pathogens in foods. These detection levels are significantly lower than those frequently presented in FCM research studies where optimal levels of  $10^6$  cells/ml are analysed as pure cultures obtained from dilute media without the presence of significant interfering substances. Therefore, sensitivity and clearly defined limits of detection need to be considered and optimized when setting up assays for low levels of target pathogens present in food, clinical or environmental samples.

### Sample preparation

Generally, most microbial testing is performed on the final product. Both the food and pharmaceutical industries have implemented a hazard analysis at critical control points (HACCP) system which also includes raw-material and in-process testing for pathogens. Therefore methods for rapid screening of a variety of sample types are of significant interest to these industries. Samples of various origins to be screened for pathogens often require a clean up/pretreatment step for successful analysis by FCM. This is necessary as substances within a sample matrix can affect the accuracy of flow cytometric counts (King, 2007), especially if particles are auto-fluorescent and are within the same size range as the bacteria of interest. These

issues cause high background fluorescence and interfere with light scatter profiles. Samples may also contain high background levels of non-pathogenic microflora which will require to be accounted for, most likely by selective labelling of target bacteria and/or discarding of other microorganisms. Finally, all bacteria to be analysed by FCM must be separated in a free form without the presence of adhering matrix particles. Sample preparation may involve homogenization of solid samples with a stomacher, with or without serial dilution, followed by separation and concentration steps such as centrifugation and filtration. Large particulates in samples can be removed via a series of filtration steps that may include initial filtration through a larger mesh size such as 2000µm and subsequently through a smaller filter (e.g. 3 µm) (Harkins and Harrigan, 2004). Centrifugation may be carried out in conjunction with filtration as it assists in sedimenting large particles leaving bacterial cells in suspension in the supernatant. Addition of special reagents may also be involved such as use of a clearing solution that removes micelles from milk or egg samples (McClelland and Pinder, 1994). Growth in enrichment media can increase the number of target organisms to detectable levels and allow recovery of stressed cells. This step may be necessary as the infectious dose of certain pathogens in samples may be lower than can be potentially detected by FCM. However, data gathered using an enrichment step is usually accepted as being qualitative rather than quantitative for the target microorganism.

Food or pharmaceutical samples are typically diluted into a defined volume of enrichment medium for a specific pathogen following clear guidelines (e.g. 65 g beef per 585 ml of pathogen specific enrichment medium for testing of *E. coli* O157:H7), followed by a culture period ranging from 18 to 72 hours depending on the target microorganism. For many samples, it may be necessary to both concentrate cells and remove matrices from the sample. Concentration may involve immunomagnetic separation (IMS) which is used as a pre-treatment and/ or pre-concentration step to capture and extract the target pathogen from a bacterial suspension using antibody-coated magnetic beads (Lazcka *et al.*, 2007). Many studies have shown IMS to be a useful concentration and isolation method for bacteria (Favrin *et al.*, 2003; Hibi *et al.*, 2006; Power *et al.*, 2003). Hibi *et al.*, (2006) showed that IMS combined with FCM could detect and enumerate *L. monocytogenes* within one minute in the range of  $10^2$ - $10^8$  CFU/ml after a 2 hour

preparation time with excellent agreement with standard plate counts ( $r = 0.97$ ). Without IMS, sensitivity decreased with high R values noted at cell concentrations of  $> 8 \times 10^4$  CFU/ml. Power *et al.* (2003) combined IMS with FCM for the sensitive detection of *Cryptosporidium* in faecal samples. The IMS/FCM technique was found to be approximately 50-fold more sensitive than formol-ether concentration, commonly used for *Cryptosporidium* epidemiological investigations. Tortorello *et al.* (1998) compared six rapid methods of enriching and detecting *E. coli* O157:H7 in apple juice samples. At the lowest inoculum level (0.1 CFU/ml), FCM required a 24-hour total enrichment and preparation time (and ranked third after ab-DEFT and IMS-PCR) but had the shortest analysis time of just 1 minute per sample.

Rymae *et al.* (2010) reported a study that used high gradient magnetic separation (HGMS) with flow cytometry to detect *Flavobacterium psychrophilum*. This technique uses a strengthened magnetic force by introducing a magnetic gradient between the magnetic filter and nearby column. This technique resulted in a much more sensitive assay with cell numbers determined by FCM correlating with those obtained using the colony counting method in the range of approximately  $10^1$ - $10^5$  CFU/ml.

### Immunolabelling

A combination of FCM with monoclonal antibodies against specific cellular antigens can enable FCM to identify specific cell populations within the background microflora of a sample matrix. Light scatter is first utilized to identify the cell population(s) of interest, while measurement of fluorescence intensity provides specific information about each individual cell or target labelled cells which will fluoresce in the channel of interest. Both polyclonal and monoclonal antibodies against a variety of different microbes can be sourced commercially from a range of suppliers. Polyclonal and monoclonal antibodies are becoming increasingly available against a variety of different microbes such as *Salmonella* Typhimurium, *Listeria monocytogenes*, *Escherichia coli* O157, *Staphylococcus aureus* (Guttikonda *et al.*, 2007; Jung *et al.*, 2003; McCarthy and Culloty, 2011; McClelland and Pinder, 1994). Polyclonal antibodies are generally noted as giving a more adequate and sufficient signal for FCM detection. Antibodies may be purchased in pure unconjugated form, as enzyme conjugates for ELISA assays, biotin tags, or fluorescent markers, depending upon the vendor and their intended use. For FCM analysis many

researchers use pure unconjugated primary antibodies to label target molecules. Thereafter, a range of commercially available secondary antibodies conjugated to a range of markers are used to reveal the labelled complex (primary antibody-antigen). Hence the fluorescence intensity of the target microbial population is generally measured by using a primary/secondary antibody labelling approach. For example, the use of 635-nm (Red) excitation will require the analyst to perform a conjugation step with either allophycocyanin (APC), or Alexa Fluor 647, or to employ an indirect labelling method using a commercially available secondary antibody conjugate (Harkins and Harrigan, 2004). The use of 488nm (blue) laser excitation allows the use of common fluorochromes such as Phycoerythrin (PE), fluorescein isothiocyanate (FITC), and peridinin chlorophyll protein (PerCP). Because many combinations of lasers, detectors, filters and fluorochromes are possible for multicolour analysis, precautions need to be taken (i.e. bandpass filters, dichroic mirrors, longpass filters, etc.) by the operator to ensure each fluorochrome is being detected by only one detector including selection and set up of bandpass filters, dichroic mirrors, longpass filters, etc. When bacteria are labelled using antibodies, a variety of factors can affect surface antigen density (growth condition, species, serovars, and strain variation).

Schellenberg *et al.* (2006) described an immunostaining procedure for *Staphylococcus aureus* in a mixed microbial population containing six various strains of lactic acid bacteria. Cells were first diluted in PBS, centrifuged, and the resultant pellet re-suspended in phosphate-buffered saline (PBS) and 1% Tween to prevent agglomeration. Samples were then heat fixed and cooled prior to staining using an *S. aureus*-specific fluorescently conjugated (FITC) antibody. These workers counterstained samples with PI for the purpose of analysis by FCM and oil immersion fluorescent microscopy. In this study, a combination of the specificity and sensitivity of immunofluorescent probes with cytometric analysis of thousands of bacterial cells was claimed to be a powerful analytical approach allowing a new insight into bacterial ecology. Significant correlation was found between estimated mean cell-unit concentrations as determined by the FCM and plate counting methods ( $r^2 = 0.732$ ,  $P = 0.0004$ ) (Schellenberg *et al.*, 2006). FCM analysis and sorting studies using monoclonal antibodies against defined surface markers on normal or neoplastic cell populations has created the basis for routine clinical diagnostic assays, for example leukaemia

classification or monitoring CD4 T-cell loss during HIV disease progression (Herzenberg *et al.*, 2000). These diagnostic applications of FCM are based on the availability of highly specific labelling antibodies. FCM detection of *Bacillus anthracis* spores (Dang *et al.*, 2001) demonstrated the utility of antibody-based labelling techniques for monitoring of environmental pathogens. Spores were treated with irradiation or by autoclaving and then analysed using a number of methods including FCM immunolabelling by three types of primary antibodies. The study illustrated the necessity to optimize and choose suitable primary antibodies for the spores of interest as some epitopes were destroyed after inactivation treatments and hence the staining ability of these antibodies was lost. Despite the obvious advantages in time saving that microbial immunofluorescence (FCM) can offer, antibodies are not always available for a particular application and overall few antibodies are commercially available which target whole cells of pathogenic bacteria or other specific bacterial groups of interest.

#### **Fluorescence *in situ* hybridisation (FISH)**

Historically, FISH and other *in situ* hybridization data played a primary role in mapping genes on human chromosomes. In microbial analysis, FISH is used to label specific nucleic acid sequences inside intact viable cells and to enable identification of bacterial species present in various samples that may contain mixed microbial populations. The first step in the procedure involves preparation of short sequences of single-stranded DNA probes that hybridize to a portion of the target gene. The next step is to label these probes by attaching one of a number of colours of fluorescent dye. Depending on the desired level of specificity, the oligonucleotides complementary to these sequences may function as group or species-specific probes for microbial cells in a sample. As the probe is fluorescent it is possible to detect the site of hybridization directly. In general, the small subunit ribosomal RNA (rRNA) provides an abundant intracellular target that is commonly used as a phylogenetic marker of bacterial classification. This abundance allows generation of a signal strong enough for detection. FISH with rRNA-targeted oligonucleotide probes has been shown to be a rapid method for identification of human pathogenic bacteria and yeast in samples such as blood cultures (Banerjee *et al.*, 2002; Kempf *et al.*, 2000; Moter and Göbel, 2000). Most FISH detection and analysis is carried out using fluorescence microscopy. However, a combination of FISH and FCM (FC-FISH or FLOW-FISH) has been shown in certain cases to be both a rapid and

reliable technique for bacterial identification (Rigottier-Gois *et al.*, 2003). These workers successfully identified *Clostridium coccooides*, *Fusobacterium prausnitzii*, *Bifidobacterium* spp. and Enterobacteria in human faecal samples with data obtained not statistically different from standard counting methods. Probe retention/ binding can be quantified by measuring median fluorescence in a population of cells, using a fluorescence channel of interest on the cytometer. The primary advantage of this technique is that it eliminates the time required for preparation and subsequent analysis of slides. One of the most intriguing potential applications of FCM is the possibility for direct extraction of specific subpopulations from samples, omitting a cultivation step, followed by genetic or possibly genomic characterization (Kalyuzhnaya *et al.*, 2006).

FISH procedures for human pathogen detection using peptide nucleic-acid (PNA) probes in place of nucleic-acid probes have also been reported (Kenny *et al.*, 2008; Stender *et al.*, 2002). PNA is a DNA mimic, where the entire negatively charged sugar phosphate backbone has been replaced with a neutral one consisting of repeated N-(2-aminoethyl) glycine units linked by peptide bonds and which is stable both chemically and biologically. Peptide bonds can enable PNA to be readily conjugated with peptides, fluorescent dyes, and other useful biomolecules. Because PNA probes have a neutral backbone, the kinetics of binding and the stability of the hybridization are significantly better than that obtained using standard nucleic-acid probes (Harkins and Harrigan, 2004). These PNA probes can also be used in conjunction with FCM as the same principles apply if they are conjugated to a fluorescent marker. Hartmann *et al.* (2005) used a combination of PNA FISH probes in conjunction with FCM to rapidly identify *Staphylococcus aureus* in blood cultures. Results obtained with PNA FISH using FCM did not reveal any discrepancies in sensitivity or specificity compared to standard laboratory methods and offered a much quicker alternative to standard methods as results were obtained within 4 hours. The detection of growing pathogens (e.g. *Stenotrophomonas maltophilia*) in blood cultures was achieved more rapidly by FCFISH compared with standard agar plate detection methods (Kempf *et al.*, 2005). The target organisms grown on agar plates subsequently had to be identified with standard laboratory methods including: cytochrome oxidase test, API 20E strip, API 20NE strip, ID32 C, Vitek II system. Using the FCFISH method required a one hour hybridization followed by washing and analysis on a FACS

Calibur cytometer. Rapid identification of pathogens was achieved using specific FISH probes, Ckru-1453: specific for *C. krusei*, Sma633: specific for *S. maltophilia*. Hybridization probes with FCM identified *C. albicans* and Enterobacteriaceae directly from blood culture bottles. *C. albicans* hybridized with the universal yeast probe PF-2 (green) and with a *C. albicans* specific probe. Enterobacteriaceae, hybridized with the universal bacterial probe and also with a Enterobacteriaceae specific probe. Results were then confirmed with microscopy and species identified further with standard microbiology tests. For example, blood cultures were artificially spiked with approximately 10-50 CFU/ml *S. maltophilia*. BacTec9240® blood culture system indicated bacterial growth 31 hours after inoculation, where using FC-FISH, bacteria were detectable after 23 hours.

### Green fluorescent protein (GFP)

Several bioluminescent coelenterates use a secondary fluorescent protein, green fluorescent protein (GFP), in an energy transfer reaction to generate green light. The most studied of these proteins have been GFPs from the jellyfish *Aequorea victoria* and the sea pansy *Renilla reniformis*. Proteins from these organisms have a chromophore, which is derived from the primary amino acid sequence of GFP (Chalfie, 1995). The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509nm, which is in the lower green portion of the visible spectrum. GFP from *Renilla reniformis* has a single major excitation peak at 498 nm. The gene for GFP has been isolated and has become a widely used and useful tool for generating chimeric proteins of GFP where it is linked to other proteins and functions as a fluorescent tag. As a non-invasive fluorescent marker in living cells, it has a wide range of applications as a cell lineage tracer, a reporter of gene expression, or as an indicator of protein-protein interactions (Mocz, 2012). Variants with more intense fluorescence or alterations in the excitation and emission spectra have been produced and FCM has proven to be a useful method with which to study infectivity and host-pathogen interactions.

*Ornithogalum dubium* (commonly known as the Star of Bethlehem) is a flowering plant and is a natural host of the soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc). Golan *et al.* (2010) developed a quantitative assay for Pcc expressing a GFP reporter gene, using fluorescent

activated cell sorting to quantify bacterial cell proliferation in *O. dubium* plantlets inoculated with Pcc. The cell sorting approach for quantification of Pcc was found to be accurate, reproducible and reduced analytical time. Douesnard-Malo and Daigle (2011) used FCM to demonstrate that *Salmonella* Typhi can persist for extended periods within environments containing a suitable host organism. This study investigated interactions between *Salmonella* Typhi and *Acanthamoeba castellanii* (a host Amoeba) by using co-cultures. Growth of both organisms was estimated by cell count, viable count, FCM and fluorescence microscopy. These workers tracked the growth and infectivity of *Salmonella* Typhi by monitoring auto fluorescence from the Amoebae and green fluorescent protein signal from the salmonellae. Cell population analyses by FCM confirmed a decline in the bacterial population, clearly seen by the loss of fluorescence over time when bacteria were grown in pure culture. It was only in the presence of amoebae that fluorescence was observed after 14 days. Results confirmed what earlier co-cultures have shown by viable plate counts, i.e. survival of *Salmonella* Typhi for 14 days. This FCM method could provide a means to monitor the interaction between a pathogen and its host, allowing a better understanding of pathogen transmissibility.

### Quantum dot labelling

Commonly used cell sorting systems are based upon single or dual excitation as excitation source both for light scatter parameters and for several fluorescence detectors. Hence, for multi-labelling detection, there is a requirement for fluorophores having a broad excitation wavelength and sharp emission bands. Such fluorophores should have high fluorescence efficiency, stability, and be available for conjugation with other bio-molecules. Quantum dots (QDs) provide practical features which meet many of these criteria (Zahavy *et al.*, 2012) as they are inorganic crystalline nanoparticles made of semiconducting materials. QDs used for biological applications typically are composed of three different layers starting with a core, normally cadmium selenide (CdSe) coated with a semiconductor outer shell of zinc sulphide (ZnS). The core-shell ranges in size from 3 to 10 nm and the core size defines the fluorescence emission of the QDs. A third layer is composed of a polymer which solubilizes the QDs while incorporating specific functional groups such as proteins or chemical compounds which allow specific binding to the desired target. QDs are currently being used as a novel fluorophores as their physical and chemical

properties confer significant advantages over traditional dyes, including brighter fluorescence and resistance to photobleaching (Ibanez-Peral *et al.*, 2008). In comparison to organic fluorophores, a single excitation source can simultaneously excite several QDs with distinctive emission bands which can be easily separated by optical filters (Zahavy *et al.*, 2012). QDs have applications for bacterial and pathogen detection in combination with FCM as well as for bacteriophages (Edgar *et al.*, 2006). A QD method has combined *in vitro* biotinylation of engineered host specific bacteriophage and conjugation of the phage to streptavidin-coated QDs. This phage-based assay reduces the amplification to a shorter time (20-45 minutes from infection to lysis) because each infected bacterium can result in a release of 10-1000 phage particles that can be readily detected by QDs. Ibanez-Peral *et al.* (2008) investigated the potential of QDs for FCM based assays by labelling beads in various ways. These workers indicated that the minimum fluorophore concentration required for detection of QDs above an autofluorescent background was 100-fold less than for FITC. The fluorescent signals of Dynabeads®, magnetic separation beads from Invitrogen, labelled with both fluorophores (FITC and QD655) were analysed by FCM and the MFI values collected. When QDs were used as fluorophores to label beads, they still exhibited lower detection limits in FCM in comparison to FITC. The effects of coupling an oligonucleotide labelled with FITC and QDs modified with biotin to paramagnetic beads (Dynabeads®) as probes targeting specific DNA sequences from microorganisms may be of interest. Dynabeads are of a larger size than bacteria and can be distinguished readily by FCM. However, there are few reports of specific nucleotide probes being used for microbial identification using FCM. Most reports have described QDs conjugated to specific antibodies for this purpose where they have also been used in immuno-phenotyping applications of human cells (Chattopadhyay *et al.*, 2006; Summers *et al.*, 2010). Conjugates need to maintain the immunospecific properties of the antibodies with the fluorescence properties of the QDs. However, QDs have been found to be unsuitable in certain applications such as environmental sampling for detection of *Cryptosporidium* (Ferrari and Bergquist, 2007). These workers noted the superior spectral characteristics of QDs; however, they noted that oocysts stained with QD-conjugates exhibited significantly lower fluorescence intensity than organic conjugates. Additionally, the level of non-specific binding by QD-conjugates to other particles

present in the water concentrate was significantly higher than that of the organic conjugates. Overall, it is important when developing accurate FCM methods for water analysis to recognize that there may be high levels of auto-fluorescent and background particles in the samples. Water analysis and FCM is dealt with extensively in another review (Egli and Köttsch, 2015).

### FCM analysis of food samples

The extent of growth, survival and biochemical activity of microorganisms in foods is the result of stress reactions. These reactions are a response to the changing physical and chemical conditions within the food microenvironment, e.g. pH, oxygen, water activity, salt, and temperature and the presence of other microorganisms within foods. The above factors greatly influence the ability of bacteria to colonize the food matrix and to grow and subsequently be detected using traditional plating on solid selective media (Fleet, 1999). Legislation pertaining to food safety is particularly stringent, as failure to detect infection/intoxication in food products may have serious consequences for the consumer including illness and/or death. Hence, accurate and early detection of pathogenic bacteria is key to prevention and identification of problems related to food and public health. Despite the obvious need for obtaining analytical results in the shortest possible time, the continued widespread use of traditional and standard bacterial enumeration and/or identification methods can take up to 7 or 8 days to yield useful data. This timescale is clearly insufficient for products with a short shelf life including chilled ready meals. Therefore, researchers have focused their efforts towards the development of FCM-based rapid methods to replace or augment these methodologies (Lazcka *et al.*, 2007). This is quite technically challenging for FCM methodology, as certain pathogens have a low infectious dose and hence low limits of detection (LOD) and limit of quantification (LOQ) are required. The infectious dose of *E. coli* 0157:H7 is of the order of 10-100 cells and so a highly sensitive detection system is necessary for its detection in foods (Subires *et al.*, 2014; Sunwoo *et al.*, 2006). The ingestion of low numbers (< 10) of pathogens such as *Salmonella* Typhimurium may lead to food poisoning; thus, preparation methods are required to concentrate low levels of the target microorganisms (Rotariu *et al.*, 2005). Compared with chemical and physical hazards the occurrence or emergence of microbiological hazards depends on a variety of factors which may occur during food production, processing and storage. Therefore

implementation of well-structured hygiene and food safety management systems are essential (Untermann, 1998).

FCM is a commonly used technique for quantifying bacteria in milk (Gunasekera *et al.*, 2003; Holm *et al.*, 2004), milk powder (Flint *et al.*, 2006) and to monitor dairy starters during cheese ripening (Bunthof and Abee, 2002). Quantification of bacteria in these matrices are undertaken by several commercial instruments such as the BactoCount (Bentley Instruments Inc., Chaska, USA). A range of FCM based technologies are also available from under the Chemunex® brand, (Biomérieux, France) including the BactiFlow®, BactiFlowALS® and D-Count® for rapid viable cell analysis of bacteria, yeast and moulds in food processing environments. In addition, FCM-based methods have also been used for Gram-typing bacteria in bulk milk by labelling with various fluorochromes (Holm and Jespersen, 2003; Langerhuus *et al.*, 2013). The same principle could potentially be used for Gram-typing of mastitis-associated bacteria. Langerhuus *et al.* (2013) proposed an assay which allowed rapid screening of milk samples for mastitis-causing bacteria. Results indicated that it was possible to detect and determine the Gram-type of pathogenic bacteria in mastitis affected milk samples based on bacterial biotin-conjugated wheat germ agglutinin and Acridine Orange (AO). Wheat germ agglutinin binds to N-acetyl-glucosamine in the peptidoglycan layer, which is prevented in Gram-negative bacteria, as wheat germ agglutinin cannot penetrate the outer lipopolysaccharide layer. In addition, Gram-negative bacteria also had less uptake of AO. The authors admitted that the method cannot compete with the traditional culturing techniques or PCR technology with respect to bacterial identification. However, they claim it could potentially provide the dairy farmer or the attending veterinarian with on-site information on bacterial detection and Gram-type in cases of clinical mastitis (Langerhuus *et al.*, 2013).

### FCM analysis of clinical samples

Life-threatening and harmful infections that require prompt antimicrobial therapy need rapid and accurate diagnostic tests in order to be effective in treating such diseases caused by specific pathogens. FCM is a promising and emerging technique with enormous potential for clinical microbiology applications. Alvarez-Barrientos *et al.* (2000) published an excellent review entitled the 'Applications of flow cytometry to clinical microbiology' outlining the advances made using FCM. This section will now outline some of the more

recent advances in this area. FCM has been shown to detect viral antigens either on the surface of, or within, infected cells using antibodies that specifically recognize surface or internal antigens. FCM analysis of virus-infected cells is best suited to samples of blood, bronchoalveolar lavage fluid, and urine. However, it is also possible to analyse cells from tissues that have previously been pretreated for other clinical tests (Alvarez-Barrientos *et al.*, 2000).

As far back as 1990, McSharry *et al.* (1990) developed a method to detect and quantify human immunodeficiency virus (HIV)-infected peripheral blood mononuclear cells obtained from HIV-seropositive patients. Intracellular HIV antigens were detected by indirect immunofluorescence using monoclonal antibodies against HIV antigens as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G F(ab')<sub>2</sub> antibody as the secondary antibody. After overnight amplification of clinical samples suspected to contain HIV, FCM enabled detection of the virus 1 to 3 days prior to cytopathic effects were evident in cell culture. Honda *et al.* (1997) used FCM to detect cytomegalovirus (CMV)-infected cell populations in peripheral blood lymphocytes from CMV-infected patients. Using monoclonal antibodies directed against immediate-early CMV antigen or against several cell membrane markers to phenotype-infected peripheral blood cells from bone marrow transplant recipients; these authors developed a rapid and quantitative FCM method for the detection of immediate-early CMV antigen.

Dixon *et al.* (2005) evaluated an FCM method for the detection and enumeration of *Cyclospora* oocysts in human faecal specimens which had previously been associated with food-borne outbreaks of cyclosporiasis in Ontario, Canada. In the absence of commercially available anti-*Cyclospora* monoclonal antibodies, the FCM method was based on the autofluorescence of *Cyclospora* oocysts. FCM data were generally very comparable to the original microscopy results for these specimens, both in terms of the presence or absence of oocysts in samples and their relative concentrations.

Rapid and accurate methods to quantify bacterial adhesion and invasion to epithelial and various other types of cells has been evaluated using FCM (Grootaert *et al.*, 2011; Pils *et al.*, 2006; Trouillet *et al.*, 2011). Variants of *Neisseria gonorrhoeae*

expressing distinct colony opacity-associated (Opa) proteins bind to receptors of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family to direct their uptake by human cells. Pils *et al.* (2006) suggested that quantitative analysis of bacterial invasion *in vitro* may be key to understanding the molecular basis of the various internalization processes. The analytical approach was based on direct fluorescein labelling of bacteria prior to infection followed by FCM based detection of cell-associated fluorescence as an estimate of the number of internalized bacteria. FCM analysis not only determined the percentage of infected cells, but also provided data on the relative amount of internalized bacteria based on the strength of the fluorescence signal. These workers demonstrated that this protocol could correctly quantify intracellular bacteria when compared with a conventional antibiotic protection assay or microscopic techniques.

Flow cytometers are routinely used to measure specific cell subpopulations in blood, bone marrow aspirates, body fluids and tissues. Septicaemia or blood poisoning is one of the leading causes of death in hospital patients and in the USA is the tenth leading cause of death (Melamed and Sorvillo, 2009). Rapid identification of sepsis-causing pathogens is crucial as appropriate antimicrobial agents can be selected earlier, avoiding unnecessary/ineffective treatments with a potential for improved patient prognosis. Definitive identification of pathogens grown from blood cultures by traditional methods involves sub-culturing and biochemical analysis requiring at least 24 hours for data generation after the first indication of growth (Kempf *et al.*, 2005). As described earlier, the rapid identification of bacterial pathogens by FC-FISH provides a potential method to overcome this problem. Schellenberg *et al.* (2008) used FCM as a tool to quantify total bacterial cells in vaginal specimens from adolescents. Typically a reduction in *Lactobacillus* populations and an overgrowth by anaerobic and Gram-negative species defines a frequently asymptomatic clinical condition known as bacterial vaginosis. FCM was used by these workers as a simple method to quantify total bacterial cells in vaginal swab samples. Bacterial cells could easily be distinguished from eukaryotic cells on the basis of size and shape and the study provided data regarding patients over a six week period of sampling. Kadhoda *et al.* (2011) identified the requirement for a rapid, easy and reliable screening method for urinary tract infections (UTI), which would obviate the requirement to culture

samples that do not contain pathogens. These workers used a UF-1000i™ flow cytometer which stains cell components with a fluorescent dye and rapidly measures cells in the urine such as leucocytes, bacteria, and erythrocytes. The method rapidly determines both the white blood cell and bacterial counts providing useful information regarding which samples may or may not need to be forwarded for traditional culture methods. Hussein *et al.* (2002) used FCM and immunolabelling to detect the presence of *E. coli* 0157:H7 in bovine rumen fluid and faeces. Concentrations of  $10^4$  CFU per ml were accurately identified and enumerated, however lower concentrations required enrichment.

FCM has found applications in other medical fields such as the study of bacteria which may cause periodontal disease. Spirochetes such as *Treponema denticola* are highly fastidious organisms and are extremely difficult to culture and enumerate on solid agar media. The current method can take between 7 and 14 days to yield results. Orth *et al.* (2009) described an FCM based approach using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen). A standard curve was first prepared using known dilutions of cells and cell count estimates were then derived from this. The percentage of bacteria that were SYTO 9 and PI positive, i.e. dead cells, were excluded from analysis as viable counts were compared with a standard method. In enumerating cells, a known concentration of beads was added to each sample which could be easily identified from bacterial whole cells and cell debris by SYTO 9 staining and forward scatter. The actual viable cell numbers in the sample could be determined based on the ratio of the number of viable bacteria and the number of events in the gate containing the fluorescent counting beads. Using FCM methods *T. denticola* could be detected and enumerated by comparison with a known number of counting beads within a positive linear correlation coefficient ( $R^2$ ) of 0.9874.

### Physiology of microbial pathogens and FCM analysis

The ability to distinguish different physiological states is important in order to assess the survival and growth of pathogenic microorganisms in various environments such as aquatic environments (Allegra *et al.*, 2008; Berney *et al.*, 2007) or to assess the effects of processing treatments and potential antibacterial treatments on pathogen response (Cronin and Wilkinson, 2008; Kennedy *et al.*, 2011).

Bacterial responses and physiological states are of particular interest to the food industry as bacteria in various physiological states can be incorporated into food matrices where they are exposed to rapid and dynamic changes in environmental conditions which can allow recovery and outgrowth (Cronin and Wilkinson, 2009; Doherty *et al.*, 2010). Pianetti *et al.* (2008) used FCM to assess the survival and viability of a pathogenic strain of *Aeromonas hydrophilla* at various salt concentrations and temperatures over an extended period (188 days). SYBR Green and PI were used to stain the cells prior to FCM analysis for total cell count and viability studies. Membrane effects were assessed by adding 3,3'-diethyloxadicarbocyanine (DiOC<sub>2</sub>). Data obtained by FCM were generally comparable to those obtained by the plate count method. However, in the presence of 6% NaCl differences were noted between the numbers of viable cells detected by FCM and by agar plate counts. These workers also noted that trends resulting in populations with high membrane potential values, indicative of membrane integrity, were strongly correlated with the viable cells. This suggested that DiOC<sub>2</sub> may be a useful fluorochrome for detecting changes in the membrane potential of *Aeromonas* cells and changes in their viability.

The application of staining probes for physiological evaluation of the target microorganism for certain samples also requires a detailed knowledge of the effect of the dye itself on the cell itself as well as the physiology and structure of the microorganism (Kennedy *et al.*, 2011; Shi *et al.*, 2007). Bacterial endospores of *Bacillus* and *Paenibacillus* were characterized into live, dead, and germinating, based on a number of physiological properties monitored by staining with cytometric dyes (Comas-Riu and Vives-Rego, 2002; Cronin and Wilkinson, 2007). Using simultaneous staining protocols with different fluorochromes, FCM can reveal previously unknown levels of heterogeneity within a culture arising as a result of various degrees of cell injury and differing physiological states (Berney *et al.*, 2007; Davey, 2011; Want *et al.*, 2011). These studies tracked physiological states of bacteria subjected to different environments or growth limiting factors using stains such as PI and SYTO9, 3,3'-dihexylocarbocyanine iodide-DiOC<sub>6</sub>(3). An increasing range of functional probes exist, mainly for assessing microbial membrane potential, intracellular pH and metabolic activity within bacterial cells. Viability is typically classified as the ability of cells to form a colony on an agar plate (Davey, 2011; Davey and Kell, 1996). However, the

use of FCM as a microbiological tool casts new light on this definition. Using FCM, it is not necessary to culture an organism to determine its viability, at least in terms of metabolic state. Plate counts, although usually considered to be the 'gold standard' of viability measurement indicates only how many of the cells can replicate under the specific conditions provided for growth. FCM viability staining meanwhile provides data on how many of the cells can exclude, become permeable to, or utilize a particular stain or combinations of stains (Davey, 2011). Excellent descriptions of the range of dyes that are available to the microbiologist interested in FCM are published in a number of reviews (Alvarez-Barrientos *et al.*, 2000; Joux and Lebaron, 2000; Nebe-von-Caron *et al.*, 2000; Shapiro, 2003; Sincock and Robinson, 2001; Tracy *et al.*, 2010).

Much work has been reported regarding the suitability of various probes and combinations of probes for different assays, generating varied outcomes and recommendations. FCM methodology has also allowed development of quantitative procedures to assess antimicrobial susceptibility and drug cytotoxicity using rapid, accurate, and highly reproducible assays (Gauthier *et al.*, 2002; Mason *et al.*, 1994; Pore, 1994). The importance of using a number of viability indicators to characterize the physiological state of stressed bacteria has been emphasized by Berney *et al.* (2006) Hewitt and Nebe-von-Caron (2004) and Nocker (2011) as each parameter reflects differing levels of cellular integrity and functionality. Therefore the sum of multiple parameters (including culturability) may be employed to generate a wider perspective on the overall physiological state of bacteria. The use of cell sorting to evaluate culturability has also led to advances in understanding and interpretation of physiological states and staining methods. (Kennedy *et al.*, 2011; Nebe-von-Caron *et al.*, 2000; Want *et al.*, 2011). In these cases it has been used to establish the relationship between the culturable state of the bacteria on agar that had been classified by FCM as permeabilized/injured or dead.

In summary, the presence of a range of cellular features are considered as prerequisites for a 'living' cell and many of these can be rapidly and simultaneously measured by FCM. The viability-related parameters measurable by FCM includes: enzyme activity such as intracellular enzymes responsible for key reactions in metabolic pathways, membrane polarization reflecting the maintenance of the cellular energy status, membrane integrity to

indicate the presence of an intact functional barrier between the extracellular environment and the cytoplasm. These parameters which are easily measurable by FCM are of great relevance when studying the physiological state of pathogens originating from clinical, food or environmental samples.

### **Cell separation/cell sorting**

Sorting of bacteria by fluorescence activated cell sorting (FACS) has tremendous potential for sensitive and selective analysis and subsequent study of particular bacterial subpopulations in samples. This ability to detect, identify, and enumerate cells in a single device based on phenotype has proven to be invaluable in the fields of medicine, food science, military applications, and basic and clinical research (Pappas and Wang, 2007). However, while FACS is well established as a reliable method for sorting cells, it can be expensive, voluminous, and requires dedicated highly trained staff to operate the equipment (Pappas and Wang, 2007). Using cell sorters, cells with a specific characteristic or indeed a combination of characteristics can be separated from the sample for further analysis or growth (Winson and Davey, 2000). Many cell separation methods use immunofluorescence, previously described in this article, as the triggering signal for detecting and isolating pathogens of interest (Lazcka *et al.*, 2007; Pappas and Wang, 2007). The use of cell sorting has many novel applications in the analysis and study of pathogens. FACS has been used as a novel enrichment technique to isolate herpes simplex viruses (HSV) for further study (Kolb and Brandt, 2004). FCM and FACS have been employed to study host-pathogen interactions. For example, FACS methods have been used to compare the infectivity and other properties of host cells, e.g. weakly or highly *Salmonella*-infected cells (Thöne *et al.*, 2007). Spleen cells that differed in *Salmonella* infectivity were sorted and further analysed for the presence of cell surface markers to better understand the relevance of different host cell populations in *Salmonella* infections. Martinon *et al.* (2011) demonstrated that sorting of single cells using FACS was useful to generate defined population standards for quantification of *S. aureus* by PCR. This allowed improved standard curves for PCR quantification of *S. aureus* to be generated based on the fact that single cells could be sorted to give highly defined populations. FCM has been highlighted as potentially useful in studying antibiotic resistance of pathogenic microorganisms ( Jarzembowski *et al.*,

2008). In this study, the heterogeneity of populations treated with antibiotics was revealed using fluorescent penicillin. Isolated strains of *Enterococcus faecalis* and *S. aureus* were compared with known penicillin-resistant and penicillin-sensitive strains. Fluorescent intensities were lower in penicillin resistant and suspected penicillin resistant strains. The presence of dormant endospores in certain foods is a likely cause of food-borne poisoning. Bacterial endospores of *Bacillus* and *Paenibacillus* have been characterized into live, dead, and germinating, based on a number of physiological properties achieved by staining with cytometric dyes (Comas-Riu and Vives-Rego, 2002; Cronin and Wilkinson, 2007).

### Conclusion

This review has focused on the application and potential of FCM as a reliable technique for use in pathogen detection in a range of different sample types. It could be argued that if such methods were to become automated they would remove costs associated with highly trained microbiologists and continuous on-site operation would speed up detection. However, there is a huge challenge for microbiologists in standardizing FCM assays particularly from complex samples from various food/clinical/environmental origins. It also remains to be seen how quickly regulatory authorities will assess and allow FCM based methods to become part of the methodology for routine analysis of pathogens. Overall, it is clear that FCM offers a variety of new possibilities for rapid species and strain-specific labelling of target pathogens.

### References

- Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B., and Riffard, S. (2008). Use of flow cytometry to monitor *Legionella* Viability. *Appl. Environ. Microbiol.* **74**, 7813-7816.
- Alvarez-Barrientos, A., Arroyo, J., Canton, R., Nombela, C., and Sanchez-Perez, M. (2000). Applications of flow cytometry to clinical microbiology. *Clin. Microbiol. Rev.* **13**, 167-195.
- Banerjee, A., Yasserli, M., and Munson, M. (2002). A method for the detection and quantification of bacteria in human carious dentine using fluorescent *in situ* hybridisation. *J. Dent.* **30**, 359-363.
- Berney, M., Weilenmann, H.-U., and Egli, T. (2006). Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). *Microbiology* **152**, 1719-1729.
- Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., and Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. *Appl. Environ. Microbiol.* **73**, 3283-3290.
- Bunthof, C.J., and Abee, T. (2002). Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. *Appl. Environ. Microbiol.* **68**, 2934-2942.
- Chalfie, M. (1995). Green fluorescent protein. *Photochem. Photobiol.* **62**, 651-656.
- Chattopadhyay, P.K., Price, D.A., Harper, T.F., Betts, M.R., Yu, J., Gostick, E., Perfetto, S.P., Goepfert, P., Koup, R.A., De Rosa, S.C., *et al.* (2006). Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat. Med.* **12**, 972-977.
- Comas-Riu, J., and Vives-Rego, J. (2002). Cytometric monitoring of growth, sporogenesis and spore cell sorting in *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*). *J. Appl. Microbiol.* **92**, 475-481.
- Cronin, U.P., and Wilkinson, M.G. (2007). The use of flow cytometry to study the germination of *Bacillus cereus* endospores. *Cytometry A* **71**, 143-153.
- Cronin, U.P., and Wilkinson, M.G. (2008). Physiological response of *Bacillus cereus* vegetative cells to simulated food processing treatments. *J. Food Protect.* **71**, 2168-2176.
- Cronin, U.P., and Wilkinson, M.G. (2009). The growth, physiology and toxigenic potential of *Bacillus cereus* in cooked rice during storage temperature abuse. *Food Control* **20**, 822-828.
- Davey, H.M. (2011). Life, death, and in-between: meanings and methods in microbiology. *Appl. Environ. Microbiol.* **77**, 5571-5576.
- Davey, H.M., and Kell, D.B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol. Rev.* **60**, 641-696.
- Dixon, B.R., Bussey, J.M., Parrington, L.J., and Parenteau, M. (2005). Detection of *Cyclospora cayetanensis* oocysts in human fecal specimens by flow cytometry. *J. Clin. Microbiol.* **43**, 2375-2379.
- Doherty, S.B., Wang, L., Ross, R.P., Stanton, C., Fitzgerald, G.F., and Brodkorb, A. (2010). Use of viability staining in combination with flow cytometry for rapid viability assessment of *Lactobacillus rhamnosus* GG in complex protein matrices. *J. Microbiol. Methods* **82**, 301-310.
- Douesnard-Malo, F., and Daigle, F. (2011). Increased persistence of *Salmonella enterica* serovar Typhi in presence of *Acanthamoeba*

- castellanii*. Appl. Environ. Microbiol. 77, 7640-7646.
- Edgar, R., McKinstry, M., Hwang, J., Oppenheim, A.B., Fekete, R.A., Giulian, G., Merrill, C., Nagashima, K., and Adhya, S. (2006). High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes. Proc. Natl. Acad. Sci. U.S.A. 103, 4841-4845.
- Egli, T. and Köttsch, S. (2015). Flow cytometry for rapid microbiological analysis of drinking water: from science to practice, an unfinished story. In Flow Cytometry in Microbiology: Technology and Applications, M.G. Wilkinson, ed. (Norfolk, UK: Caister Academic Press), pp. 175-216. <https://doi.org/10.21775/9781910190111.09>
- Entis, P., Fung, D., Griffiths, M., McIntyre, L., Russell, S., Sharpe, A.N., and Tortorello, M.L. (2001). Rapid methods for detection, identification, and enumeration. In Compendium of Methods for the Microbiological Examination of Foods, F.P. Downes, and K. Ito, eds. (American Public Health Association, Washington, DC, USA), pp. 89-126.
- Favrin, S.J., Jassim, S.A., and Griffiths, M.W. (2003). Application of a novel immunomagnetic separation bacteriophage assay for the detection of Salmonella enteritidis and Escherichia coli O157:H7 in food. Int. J. Food Microbiol. 85, 63-71.
- Ferrari, B.C., and Bergquist, P.L. (2007). Quantum Dots as alternatives to organic fluorophores for *Cryptosporidium* detection using conventional flow cytometry and specific monoclonal antibodies: lessons learned. Cytometry A 71, 265-271.
- Fleet, G.H. (1999). Microorganisms in food ecosystems. Int. J. Food Microbiol. 50, 101-117.
- Flint, S., Drocourt, J.-L., Walker, K., Stevenson, B., Dwyer, M., Clarke, I., and McGill, D. (2006). A rapid, two-hour method for the enumeration of total viable bacteria in samples from commercial milk powder and whey protein concentrate powder manufacturing plants. Int. Dairy J. 16, 379-384.
- Gauthier, C., St-Pierre, Y., and Villemur, R. (2002). Rapid antimicrobial susceptibility testing of urinary tract isolates and samples by flow cytometry. J. Med. Microbiol. 51, 192-200.
- Golan, A., Kerem, Z., Tun, O.M., Luzzatto, T., Lipsky, A., and Yedidia, I. (2010). Combining flow cytometry and *gfp* reporter gene for quantitative evaluation of *Pectobacterium carotovorum* ssp. *carotovorum* in *Ornithogalum dubium* plantlets. J. Appl. Microbiol. 108, 1136-1144.
- Grootaert, C., Boon, N., Zeka, F., Vanhoecke, B., Bracke, M., Verstraete, W., and Van de Wiele, T. (2011). Adherence and viability of intestinal bacteria to differentiated Caco-2 cells quantified by flow cytometry. J. Microbiol. Methods 86, 33-41.
- Gunasekera, T.S., Veal, D.A., and Attfield, P.V. (2003). Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk. Int. J. Food Microbiol. 85, 269-279.
- Guttikonda, S., Tang, X.L., Yang, B.M., Armstrong, G.D., and Suresh, M.R. (2007). Monospecific and bispecific antibodies against *E. coli* O157 for diagnostics. J. Immunol. Methods 327, 1-9.
- Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., and Egli, T. (2008). Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res. 42, 269-277.
- Harkins, K.R., and Harrigan, K. (2004). Labeling of bacterial pathogens for flow cytometric detection and enumeration. Curr. Protoc. Cytom. 11, 11.17.
- Hartmann, H., Stender, H., Schäfer, A., Autenrieth, I.B., and Kempf, V.A.J. (2005). Rapid identification of *Staphylococcus aureus* in blood cultures by a combination of fluorescence *in situ* hybridization using peptide nucleic acid probes and flow cytometry. J. Clin. Microbiol. 43, 4855-4857.
- Herzenberg, L.A., De Rosa, S.C., and Herzenberg, L.A. (2000). Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine. Immunol. Today 21, 383-390.
- Hewitt, C.J., and Nebe von Caron, G. (2004). The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. Adv. Biochem. Eng. Biotechnol. 89, 197-223.
- Hibi, K., Abe, A., Ohashi, E., Mitsubayashi, K., Ushio, H., Hayashi, T., Ren, H., and Endo, H. (2006). Combination of immunomagnetic separation with flow cytometry for detection of *Listeria monocytogenes*. Anal. Chim. Acta 573-574, 158-163.
- Holm, C., and Jespersen, L. (2003). A flow-cytometric Gram-staining technique for milk-associated bacteria. Appl. Environ. Microbiol. 69, 2857-2863.
- Holm, C., Mathiasen, T., and Jespersen, L. (2004). A flow cytometric technique for quantification and differentiation of bacteria in bulk tank milk. J. Appl. Microbiol. 97, 935-941.
- Honda, J., Okubo, Y., Imamura, Y., Kusaba, M., Saruwatari, N., and Oizumi, K. (1997). Flow cytometric detection of cytomegalovirus antigen in peripheral blood cells after bone marrow transplantation. Br. J. Haematol. 99, 415-417.

- Hussein, H.S., Thran, B.H., and Redelman, D. (2002). Detection of *Escherichia coli* O157:H7 in bovine rumen fluid and feces by flow cytometry. *Food Control* 13, 387-391.
- Ibanez-Peral, R., Bergquist, P.L., Walter, M.R., Gibbs, M., Goldys, E.M., and Ferrari, B. (2008). Potential use of quantum dots in flow cytometry. *Int. J. Mol. Sci.* 9, 2622-2638.
- Ivniński, D., Abdel-Hamid, I., Atanasov, P., and Wilkins, E. (1999). Biosensors for detection of pathogenic bacteria. *Bios. Bioelectron.* 14, 599-624.
- Jarzembowski, T., Wiśniewska, K., Józwiak, A., Bryl, E., and Witkowski, J. (2008). Flow cytometry as a rapid test for detection of penicillin resistance directly in bacterial cells in *Enterococcus faecalis* and *Staphylococcus aureus*. *Curr. Microbiol.* 57, 167-169.
- Joux, F., and Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteria at singlecell level. *Microbes Infect.* 2, 1523-1535.
- Jung, Y.S., Frank, J.F., and Brackett, R. E. (2003). Evaluation of antibodies for immunomagnetic separation combined with flow cytometry detection of *Listeria monocytogenes*. *J. Food Protect.* 66, 1283-1287.
- Kadkhoda, K., Manickam, K., Degagne, P., Sokolowski, P., Pang, P., Kontzie, N., and Alfa, M. (2011). UF-1000i flow cytometry is an effective screening method for urine specimens. *Diagn. Microbiol. Infect. Dis.* 69, 130-136.
- Kalyuzhnaya, M.G., Zabinsky, R., Bowerman, S., Baker, D.R., Lidstrom, M.E., and Chistoserdova, L. (2006). Fluorescence *in situ* hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. *Appl. Environ. Microbiol.* 72, 4293-4301.
- Kempf, V.A.J., Trebesius, K., and Autenrieth, I.B. (2000). Fluorescent *in situ* hybridization allows rapid identification of microorganisms in blood cultures. *J. Clin. Microbiol.* 38, 830-838.
- Kempf, V.A.J., Mändle, T., Schumacher, U., Schäfer, A., and Autenrieth, I.B. (2005). Rapid detection and identification of pathogens in blood cultures by fluorescence *in situ* hybridization and flow cytometry. *Int. J. Med. Microbiol.* 295, 47-55.
- Kennedy, D., Cronin, U.P., and Wilkinson, M.G. (2011). The response of *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* to simulated food processing treatments using fluorescence activated cell sorting and plate counting. *Appl. Environ. Microbiol.* 77, 4657-4668.
- Kennedy, D. and Wilkinson, M.G. (2015). Application of flow cytometry to the detection of pathogenic bacteria. In *Flow Cytometry in Microbiology: Technology and Applications*, M.G. Wilkinson, ed. (Norfolk, UK: Caister Academic Press), pp. 91-108. <https://doi.org/10.21775/9781910190111.05>
- Kenny, J.H., Zhou, Y., Schriefer, M.E., and Bearden, S.W. (2008). Detection of viable *Yersinia pestis* by fluorescence *in situ* hybridization using peptide nucleic acid probes. *J. Microbiol. Methods* 75, 293-301.
- King, D.N. (2007). A critical evaluation of a flow cytometer used for detecting enterococci in recreational waters. *J. Water Health* 5, 295-305.
- Kolb, A.W., and Brandt, C.R. (2004). Enhanced isolation of low frequency herpes simplex virus recombinants using green-fluorescent protein and FACS. *J. Virol. Methods* 115, 73-81.
- Langerhuus, S.N., Ingvarsen, K.L., Bennedsgaard, T.W., and Røntved, C.M. (2013). Gram-typing of mastitis bacteria in milk samples using flow cytometry. *J. Dairy Sci.* 96, 267-277.
- Lautenschlager, K., Hwang, C., Liu, W.-T., Boon, N., Köster, O., Vrouwenvelder, H., Egli, T., and Hammes, F. (2013). A microbiology-based multi-parametric approach towards assessing biological stability in drinking water distribution networks. *Water Res.* 47, 3015-3025.
- Lazcka, O., Campo, F.J.D., and Munoz, F.X. (2007). Pathogen detection: A perspective of traditional methods and biosensors. *Bios. Bioelectron.* 22, 1205-1217.
- McCarthy, M., and Culloty, S. (2011). Optimization of two immunofluorescent antibodies for the detection of *Escherichia coli* using immunofluorescent microscopy and flow cytometry. *Curr. Microbiol.* 62, 402-408.
- McClelland, R.G., and Pinder, A.C. (1994). Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Appl. Environ. Microbiol.* 60, 4255-4262.
- McSharry, J.J., Costantino, R., Robbiano, E., Echols, R., Stevens, R., and Lehman, J.M. (1990). Detection and quantitation of human immunodeficiency virus-infected peripheral blood mononuclear cells by flow cytometry. *J. Clin. Microbiol.* 28, 724-733.
- Martinon, A., Cronin, U., and Wilkinson, M. (2011). Development of defined microbial population standards using fluorescence activated cell sorting for the absolute quantification of *S. aureus* using real-time PCR. *Mol. Biotechnol.* 50, 1-10.



- Sincock, S.A., and Robinson, J.P. (2001). Flow cytometric analysis of microorganisms. *Methods Cell Biol.* **64**, 511-537.
- Stein, D.C. (1998). Chapter 7 - Principles of bacterial pathogenesis. *Prin. Med. Biol.* **9**, 85-97.
- Stender, H., Fiandaca, M., Hyldig-Nielsen, J.J., and Coull, J. (2002). PNA for rapid microbiology. *J. Microbiol. Methods* **48**, 1-17.
- Subires, A., Yuste, J., and Capellas, M. (2014). Flow cytometry immunodetection and membrane integrity assessment of *Escherichia coli* O157:H7 in ready-to-eat pasta salad during refrigerated storage. *Int. J. Food Microbiol.* **168-169**, 47-56.
- Summers, H.D., Holton, M.D., Rees, P., Williams, P.M., and Thornton, C.A. (2010). Analysis of quantum dot fluorescence stability in primary blood mononuclear cells. *Cytometry A* **77**, 933-939.
- Sunwoo, H.H., Wang, W.W., and Sim, J.S. (2006). Detection of *Escherichia coli* O157:H7 using chicken immunoglobulin Y. *Immunol. Lett.* **106**, 191-193.
- Thöne, F., Schwanhäusser, B., Becker, D., Ballmaier, M., and Bumann, D. (2007). FACS-isolation of *Salmonella* infected cells with defined bacterial load from mouse spleen. *J. Microbiol. Methods* **71**, 220-224.
- Tortorello, M.L., Reineke, K.F., Stewart, D.S., and Raybourne, R.B. (1998). Comparison of methods for determining the presence of *Escherichia coli* O157:H7 in apple juice. *J. Food Protect.* **61**, 1425-1430.
- Tracy, B.P., Gaida, S.M., and Papoutsakis, E.T. (2010). Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. *Curr. Opin. Biotechnol.* **21**, 85-99.
- Trouillet, S., Rasigade, J.-P., Lhoste, Y., Ferry, T., Vandenesch, F., Etienne, J., and Laurent, F. (2011). A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells. *J. Microbiol. Methods* **86**, 145-149.
- Ueckert, J., Breeuwer, P., Abee, T., Stephens, P., von Caron, G.N., and ter Steeg, P.F. (1995). Flow cytometry applications in physiological study and detection of food borne microorganisms. *Int. J. Food Microbiol.* **28**, 317-326.
- Untermann, F. (1998). Microbial hazards in food. *Food Control* **9**, 119-126.
- Vora, G.J., Meador, C.E., Stenger, D.A., and Andreadis, J.D. (2004). Nucleic acid amplification strategies for DNA microarray-based pathogen detection. *Appl. Environ. Microbiol.* **70**, 3047-3054.
- Want, A., Hancocks, H., Thomas, C., Stocks, S., Nebe-von Caron, G., and Hewitt, C. (2011). Multi-parameter flow cytometry and cell sorting reveal extensive physiological heterogeneity in *Bacillus cereus* batch cultures. *Biotechnol. Lett.* **33**, 1395-1405.
- Wilkes, J.G., Tucker, R.K., Montgomery, J.A., Cooper, W.M., Sutherland, J.B., and Buzatu, D.A. (2012). Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. *Food Microbiol.* **30**, 281-288.
- Wilkinson, M.G. (2015). *Flow Cytometry in Microbiology: Technology and Applications* (Norfolk, UK: Caister Academic Press). <https://doi.org/10.21775/9781910190111>
- Wilkinson, M.G. (2016). *Flow Cytometry and Food Microbiology: challenges, opportunities and progress to date*. *Tecnicas de Laboratorio*, **417**, 722-728.
- Winson, M.K., and Davey, H.M. (2000). Flow cytometric analysis of microorganisms. *Methods Enzymol.* **21**, 231-240.
- Zahavy, E., Ber, R., Gur, D., Abramovich, H., Freeman, E., Maoz, S., and Yitzhaki, S. (2012). Application of nanoparticles for the detection and sorting of pathogenic bacteria by flow-cytometry. *Adv. Exp. Med. Biol.* **733**, 23-36.

