Introduction

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Microbiologists continually strive to develop methods which can enumerate bacteria, yeasts and mould populations and to distinguish the various physiological states of key microorganisms in industrial fermentations, waste treatment plants, water supply systems, during bioremediations or in clinical applications. Traditional methods have largely relied on various non-selective and selective media followed by more sophisticated analysis using PCR or immunological serotyping. The advent of genetic methodologies such as PCR and its variants has enabled a huge step forward in terms of both rapid and highly specific analysis of cells in various matrices. When combined with bioinformatics and phylogenetic analysis the transformation of these data has enabled new and exciting relationships to be drawn regarding species and strain evolution. However, while these technologies are highly useful, a key aspect that has not been clarified is the viable properties of these cells at the point of analysis. Indeed a key question for microbiologists is whether we can replace viability counting on agar plates with something faster but which can also generate a range of data enabling a better understanding of all bacterial cells within a given population. One such technology which promises to deliver further advances towards these objectives is flow cytometry abbreviated as FCM or FC. Flow cytometry is indeed a multi-parametric technique and a flow cytometer brings together various physical, biological, chemical and engineering disciplines to deliver a unique analytical package for the study of cell biology.

While FCM holds primacy in human clinical applications especially for immunological analysis it has now begun to make serious inroads into a new and challenging area; microbiology, in all its manifestations and complexities. In this text a number of authors have brought together a wealth of experience demonstrating the power and limitations of FCM as it currently stands within microbiology today. The book commences with a contribution in Chapter 1 from probably the most eminent scientist in the area of flow cytometry, not just microbial flow cytometry, Professor Howard Shapiro. This scientist who has already written a seminal text in the area elegantly describes the evolution of cytometry from the early microscopes of Antonie van Leeuwenhoek to the staining techniques of Paul Ehrlich and the development of the first true flow cytometers and casts an eye forward to its future position within microbiology based on technological advances and the ability to provide equipment which is simple to operate and is cost-effective. Thereafter the various chapters deal with individual areas of microbiology where FCM applications have advanced the basic understanding of the science through analytical innovation driven by FCM and/or its more advanced aspect, cell sorting. To the microbiologist the ability to use FCM to visualize, enumerate and analyse a population of cells into subpopulations of varying physiological status followed by the use of cell sorting to then actually physically isolate and sort specific cells for further analysis is truly amazing! Traditional microbiology has relied on separate techniques to examine growth (plate counting), physiology (biochemical testing) and morphology (light or fluorescence microscopy) of microorganisms of interest. These techniques while well accepted

are quite slow and heavily labour-intensive with quite a considerable lag time before a complete data profile can be generated. A new and exciting development, which is only recently reaching the field of microbial cytometry, is imaging flow cytometry. This technique combines flow cytometry with advanced cell imaging enabling rapid differentiation of individual cells within a flow stream on the basis of shape/integrity/morphology. Using this type of flow cytometry, imaging analysis of morphology and the degree of uptake of fluorescent metabolic stains and/or expression of GFP constructs to enable monitoring of cellular responses at genetic level can be undertaken. This type of flow cytometer such as the Image Stream X Mark II from Amnis Corp, (Seattle, WA, USA) acquires a range of images for an individual cell in a flow stream using various imaging modes. The image quality is enhanced by using 40–60× microscopy objectives and it can acquire six images of each cell. Most reported applications of imaging flow cytometers involve mammalian cells. However a number of papers reporting the progression of bacterial infection of mammalian cells have provided interesting data on the possibilities for other non-medical microbiology applications. These cytometers have a reported flow through of ~300 cells per second. While the imaging system is purported to generate 60,000 images of 10,000 cells in about 30 seconds and 600,000 images of 100,000 cells approximately 5 minutes (Basiji *et al.*, 2007). In Chapter 2, Professor Kenji Yasuda outlines the development of on-chip imaging flow cytometry systems for analysis of bacterial behaviour in terms of single cell movement and growth kinetics. It is clear from this work that imaging flow cytometry may not be far behind in its implementation at on-chip level with numerous potential applications at basic and applied levels. A key feature of FCM is the ability to gain a range of data based on multi-parametric analysis, this feature is especially of interest when applied to understanding bacterial population heterogeneity. The traditional 'plate and wait' approach to bacterial population enumeration is already recognized as being too limited in its scope and merely allows certain bacterial cells which satisfy particular growth criteria to be enumerated. The use of enrichment and/or recovery media combined with more sophisticated techniques such as PCR enables a better composite profile of a bacterial population. However it is clear that multi-parametric FCM is superior in many aspects to many of the traditional techniques. In a number of Chapters the application of FCM to understanding population dynamics is discussed from a range of applications. Firstly, from the point of view of Environmental Bioprocessing where a new insight into understanding the key populations of the microbial consortia within biotreatment plants is discussed in Chapter 3 by Mónica Herrero and Mario Díaz. These authors outline the major advances that have been made when FCM and cell sorting have been combined with genetic analysis to isolate key bacterial species responsible for biodegradation reactions. The ability of FCM and cell sorting to isolate putative or candidate bacteria with potentially important metabolic activities which have even yet to be grown or identified by conventional technologies is also outlined. As these authors state, the numbers of unidentified or unculturable bacteria species in soil or water is immense but it is clear that cell sorting will advance our understanding of the environmental microflora. This theme of using FCM to gain a deeper understanding or fingerprinting of complex microbial communities has been developed by Susann Müller and Kathleen Schleinitz in Chapter 4. In this chapter an experimental case study is described in which the complexities involved in degradation of m-xylene by successive microbial populations are revealed using FCM. These and other authors outline a recurring theme related to analysis of microorganisms in complex samples (soil, water, food) by FCM, namely the requirement to release maximum number of bacterial cells without affecting their physiological status.

All sample preparation steps for microbial FCM analysis must generate an analyte solution free of interfering particles, with minimum auto-fluorescence and contain populations of cells capable of being analysed by the particular cytometer in use. To date, no optimal sample preparation method has been established for general use in analysis of matrices such as soil, food or environmental sources. Publications generally describe in-house sample preparation

methodologies which in many cases includes filtration and centrifugation (including density gradient centrifugation). However, antibody based systems such as Immunomagnetic Separation (IMS) show promise when looking to concentrate/isolate specific target populations in complex matrices. While this approach has been developed for some commercialized PCR and FCM based technologies it is still a very difficult technical proposition to clean up samples, release the total microbial population and capture sufficient cells for FCM analysis. An evaluation of commercial systems which recovered *E. coli* 0157:H7 from spinach using IMS capture found that efficiencies ranged from ~1 to 63%. This variation was dependent on extraction medium, sample volume, presence of background microflora and the use of spiking (Chen *et al.*, 2014). Thereafter a 4- to 6-hour enrichment was required for detection either by PCR or FCM (Chen *et al.*, 2014). Buzatu *et al.*, (2013, 2014); Wilkes *et al.*, (2012) describe the evolution of a commercial flow cytometric system for the rapid detection of *E. coli* 0175H7 in foods such as spinach and outline in great detail the technical issues involved including overcoming matrix interference and cell recovery steps. It is evident that for direct rapid FCM analysis for a range of pathogens in foods much work still remains to provide simple, cost-effective, high efficiency methods for capture of cells from complex matrices and which eliminate or minimize a cell enrichment step.

While FCM can analyse samples rapidly the data are only as good as the sample presented for analysis. In Chapter 5 the detection of pathogenic bacteria by FCM in a range of samples including food, environmental and clinical origins are discussed. The themes both Deirdre Kennedy and myself as authors have sought to highlight are the methodologies required for isolation of target bacteria from diverse samples and the necessity to set up the cytometer properly by running an extensive series of control cell populations. For all scientists or practitioners in the field of microbial cytometry it is vital to have first ascertained where live, dead and damaged target cells occur on a typical cytograph for the strain of interest. This can be carried out initially in media and thereafter in samples which have been cleaned up by various methodologies as outlined in this chapter. It is also necessary to understand that species and strain-related differences may well occur in the uptake of many common stains used in FCM analysis and some stains may prove toxic to cells thus producing erroneous data. Hence, a series of experiments should be undertaken to ensure that cell viability or metabolic activities are not affected by the staining procedures. In the case of cell sorting, the sorting procedure may also affect viability and should also be checked prior to embarking on extensive microbial studies.

A key issue for microbial FCM is the necessity to selectively stain and/or label target cells within a complex microflora. To date, the stains commercially available do not allow for any major species and strain selectivity of the extent required for routine pathogen analysis. In contrast to mammalian FCM, the range of satisfactory commercial antibodies suitable for FCM analysis is still quite limited. In part, this may be due to market forces which are still not driving the development of commercial antibodies probably due to the limited commercial application of microbial FCM. A major technical obstacle is the necessity to determine unique antigenic properties of a range of various species and strains and also to find epitopes which can also be used classify live, dead and damaged cells. Especially when these cells are present at very low levels in complex samples and preferably without an enrichment step. In Chapter 6, the application of FCM to detection and study of spore formers such as *Bacillus* or *Clostridia* species is outlined in detail by Ultan Cronin. Currently spore formers in foods are a major issue as they can survive almost all current conventional heating and dehydration technologies and subsequently germinate on storage of the product causing various illnesses, some fatal. The issue of rapid methods to detect spore formers is of major interest to the Dairy and Infant Formula industries who are currently using slow, laborious and non-selective quality control methods. However as the author explains there are a number of morphological and physiological states in the spore to vegetative cell life cycle that may provide avenues which can be exploited for FCM detection. Hence, in this chapter, the potential for FCM – when combined with

immuno-labelling, staining and/or nucleic acid probes – to offer the possibility to generate rapid and selective assays for spore formers and vegetative cells is highlighted. The range of applications of FCM and cell sorting to the study of yeasts and fungi is explored extensively in Chapter 7 by Tim Overton. Yeasts and fungi play a major role in industrial production of high value products such as beers, wines and industrial enzymes. Hence, bioprocess control using on/at line FCM technology would clearly be of economic value. In the medical field, the use of FCM for rapid detection of yeast or fungal infections and the identification of optimal drug based therapeutic strategies is already providing new insights into drug–host cell interactions. The use of yeast surface display (YSD) in combination with FCM and cell sorting is a major biotechnological application for this technology for recombinant protein production and is outlined in detail.

Another important group of industrial microorganisms are lactic acid bacteria (LAB) principally involved in the production of a range of fermented dairy foods including cheese, yogurt and fermented milks. Indeed, LAB also play a key role in the generation of particular flavours in wines through the malo-lactic fermentation process. In Chapter 8, authors, Doolan, Hickey and Wilkinson outline the development of FCM and cell sorting to the study of LAB during cheese production and ripening along with some other food applications. LAB contribute to acidification of cheese products during their viable state thus ensuring their safety, while their enzymes generate flavour compounds during the non-viable state which proceeds to extensive cell autolysis and release of intracellular enzymes which hydrolyse the casein and lipid content of the cheese to generate typical flavours. In the selection of LAB cultures for cheese production a key factor is the ability of cells to release enzymes through early autolysis. The traditional methodology to establish strain related autolytic profiles relies heavily on the use of viable cell counts on solid media with a three day incubation step. This is usually combined with data obtained from measurement of released intracellular enzymes such as lactate dehydrogenase (LDH) or peptidases such as Pep X into aqueous cheese extracts which act as markers of autolysis. However, while these methods are sufficiently useful to allow determination of variations in autolysis it was not clear until recently whether release of enzymes was possible or even influenced by differences in cell permeabilization properties. In this chapter the important contribution of FCM towards the establishment of rapid methods for estimation of cell viability and the establishment of the importance of strain related permeabilization properties in intracellular enzyme release is outlined. It is also clear that the application of FCM and cell sorting will provide new methodologies for selection of LAB cultures for particular industrial processes based on the ability to select strains or even strain subpopulations which perform key reactions within a food system. In addition, the potential for FCM to unravel and provide a deeper insight into the potential interactions between a human host cell in the gastrointestinal tract and a probiotic LAB culture are only just becoming to be realized.

In the final chapter, Thomas Egli and Stefan Kotzsch outline a fascinating journey from laboratory-scale work on water quality using FCM to replace or enhance traditional methodologies for drinking water quality to the eventual full scale acceptance and implementation of newly developed FCM based standards of quality control. Their work has been so successful that FCM is now used as a key quality control technology for drinking water quality in major cities of Switzerland such as Zurich. In this Chapter the authors have outlined the obstacles they faced both technical, regulatory and attitudinal towards the use of FCM for water monitoring. However, they also show how a 'bottom up' approach works best when laboratory analysts take on board and are first convinced by the new technology and then drive the regulators towards adoption of the technology, provided all validation steps are satisfactorily achieved. Currently, FCM-based drinking water quality testing is written into Swiss Federal regulations and the technology is progressing towards on-line quality testing. This is a situation that the pharmaceutical, food and allied healthcare industries should strive for, and in my view, this will become a reality in certain of the industrial processes provided they are amenable to rapid sample preparation, have access to species/strain

related discriminatory staining or antibody/DNA based tagging procedures and the availability of cheap, simple and perhaps specifically modified microbial flow cytometers.

A key advantage to flow cytometry for the microbiologist is the instant output of data which can quantify various cell subpopulations. Increasingly cytometers are providing us with the ability to manipulate these data and develop statistically valid outputs which will certainly enhance acceptability for publications and for uptake by the regulatory community. However, what may be of benefit is the integration of modelling and predictive growth responses, already available in the literature or as accessible databases from regulatory authorities, for a range of pathogens into the data analytics package of a cytometer. This must of course be based on using samples which allow the capture and analysis of a particular strain or species, for example by immuno-magnetic separation (IMS). For example, in Food Microbiology there is quite a body of work on modelling of growth patterns of pathogens, e.g. *Listeria monocytogenes* under different conditions of temperature, pH and salt concentrations. These data could also be supplemented with FCM profiles using differential staining to provide an enhanced database for major bacterial species of interest. These data could then be available on a flow cytometer analytics package such that, when working with specific cultures obtained from samples with particular compositional/storage parameters, the analyst may be able to relate certain FCM profiles with the statistical probability of the preponderance of a particular physiological status, e.g. the higher or lower likelihood of the presence of cell subpopulations in a VBNC state. These decisions could be based on certain pre-programmed criteria from a combination of predictive modelling, FCM profiling and other physiological data available within the cytometer's analytics package. Another factor critical to the uptake of microbial flow cytometry is development of specially adapted cytometers for microbial detection. Fortunately, this is also becoming a commercial reality with a range of engineering and electronic modifications and pre-selected gates such as those described for the RAPID-B model 9013 flow cytometer for detection of microbial pathogens (Wilkes *et al.*, 2012).

Finally, I wish to make the reader aware of the limitations of this book which for various logistical reasons has not dealt with other areas of microbial flow cytometry such as the exciting work on monitoring marine microbiology conducted by Dr Gerald Gergori and colleagues in Marseille, the work on use of cytometry for monitoring microbial toxins by Francis Ligler and colleagues in the USA. It would be totally remiss of me to not acknowledge the pioneering and ongoing research undertaken into microbial flow cytometry undertaken by Chris Hewitt and Gerhard Nebe-von-Caron, whose work has inspired many traditional microbiologists, including myself, to take the step into microbial cytometry. These workers and others, including J. Paul Robinson at Purdue University, have laid the foundations and hopefully all others can build on them to develop a unified, robust methodology which provides a unique insight into the microbial cell and its interactions with humans in all its manifestations both good and bad.

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