Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting

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Abstract

With the increased awareness of the problems associated with the growth dependent analysis of bacterial populations, direct optical detection methods such as flow cytometry have enjoyed increased popularity over the last few years. Among the analyses discussed here are: (1) Bacterial discrimination from other particles on the basis of nucleic acid staining, using sample disaggregation to provide fast reliable enumeration while minimizing data artefacts due to post sampling growth; (2) Determination of basic cell functions such as reproductive ability, metabolic activity and membrane integrity, to characterise the physiological state or degree of viability of bacteria; and (3) The use of single cell sorting onto agar plates, microscope slides or into multi-well plates to correlate viability as determined by cell growth with fluorescent labelling techniques. Simultaneous staining with different fluorochromes provides an extremely powerful way to demonstrate culture heterogeneity, and also to understand the functional differences revealed by each stain in practical applications. Analysis of bacterial fermentations showed a considerable drop (20%) in membrane potential and integrity during the latter stages of small scale (5L), well mixed fed-batch fermentations. These changes, not found in either batch or continuous culture fermentations, are probably due to the severe, steadily increasing stress associated with glucose limitation during the fed-batch process, suggesting ‘on-line’ flow cytometry could improve process control. Heat injured cells can already show up to 4 log of differences in recovery in different pre-enrichment media, thus contributing to the problem of viable but non-culturable cells (VBNC’s). Cytometric cell sorting demonstrated decreasing recovery with increasing loss of membrane function. However, a new medium protecting the cells from intracellular and extracellular causes of oxidative stress improved recovery considerably. Actively respiring cells showed much higher recovery improvement than the other populations, demonstrating for the first time the contribution of oxidative respiration to intracellular causes of damage as a key part of the VBNC problem. Finally, absolute and relative frequencies of one species in a complex population were determined using immunofluorescent labelling in combination with the analysis of cell function. The detail and precision of multiparameter flow cytometric measurements of cell function at the single cell level now raise questions regarding the validity of classical, growth dependent viability assessment methods. © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Microbial cytometry: bulk vs. single cell measurements

Because of the importance of microbiology to human health, methods have been developed to enumerate bacteria, to identify them and to look at the impact of physical, chemical or biological interventions. Bulk measurements, based on detection of changes in turbidity, conductivity, or gas pressure of liquid media (Fig. 1) have become popular for bacterial detection because of their speed and relative simplicity. Selective growth media can allow some degree of bacterial differentiation, but species- and strain-specific detection still requires cell isolation followed by biochemical, immunological and/or genetic characterisation. Although immunological and genetic differentiation can be applied directly to certain bulk samples, pre-enrichment steps involving separation of organisms and/or incubation to increase available biomass are usually necessary to generate adequate signals.

The cornerstone of microbiology has always been single cell analysis. Colonies derived from single cells have been examined by plating since its development by Koch more than a century ago. If the high amplification factor of $10^{9-12}$ is the strength of plating, the dependence on growth is its weakness. In Koch’s and Pasteur’s time, microscopy represented the only means for analysis of growth at the single cell level; today, direct single cell measurements by image or flow cytometry methods can rapidly provide information about growth, and also allow assessment of population heterogeneity. The spatial resolution of the microscope not only allows the characterisation of cell morphology, but also the position of bacteria within a sample. This can yield information about the development of colonies or biofilms or about symbiotic interactions. However, the large amount of data processing required in computerised image analysis limits sample throughput, and analysis of large numbers of cells may be more readily accomplished by flow cytometry (FCM) of cell suspensions. Spatial resolution of FCM is minimal, although differences among samples taken from different sites may provide some spatial information.

A number of books that explain flow cytometry in its wider context are available. A quick introduction to the technique can be found in Ormerod’s books (Ormerod, 1994; Ormerod, 1999). The most comprehensive book is Shapiro’s Practical Flow Cytometry (Shapiro, 1995) which devotes a section to Microbiology. There are also a number of recent review articles about microbial cytometry (Fouchet et al., 1993; Pinder et al., 1993; Pore, 1994; Davey & Kell, 1996; Nebe-von Caron & Badley, 1996; Porter et al., 1997; Vives-Regó et al., 1999)

Apart from the explicit description in Shapiro’s book, the historic development of the technology is best summarised by Melamed et al. (1990). Modern microbial cytometry began with the analysis of cell replication processes (Hutter, 1974; Hutter et al., 1975; Paau et al., 1977; Bailey et al., 1977; Steen et al., 1982), which is still an important factor in the control of fermentation processes (Muller et al., 1995; Muller et al., 1997).

**Fig. 1.** Cytometry as bulk or single cell measurements. Bulk measurements are usually easy to perform and less expensive. In most cases, cell growth is required to generate enough signal. Direct single cell measurements on the other hand tend to be more complex. Post-sampling growth is not required and the measurement can reflect the true heterogeneity of microbial populations.
2. Detection and counting of microorganisms

All microbial detection systems that rely on cell replication are limited by the requirement to grow bacteria in an artificial environment. Lack of symbiotic partners or an unsuitable micro-environment may result in inaccurate plate counts from natural samples. Also, some cells will only grow anaerobically, some only aerobically, and some under both conditions. Therefore representative counts, which ideally detect healthy, injured, dormant, and ‘viable but sometimes-non-culturable’, as well as truly dead, bacteria can only be obtained by direct optical methods.

In counting, one must first differentiate the bacteria from other particles in a sample. This can best be achieved by using DNA specific stains in combination with intrinsic light scatter measurements. Special care must be taken to avoid interference by DNA fragments and micelles that can stain nonspecifically. Dual labelling with two fluorochromes that exhibit differential membrane permeability allows for more stringent discrimination of bacteria as ‘DNA surrounded by an intact cell membrane’.

Due to the statistical counting error ($n^{-0.5}$) it is necessary to investigate more than 100 events to achieve a statistical coefficient of variation (CV) less than 10%. At 1000 events, the CV reaches 3%; above 5000, it goes below 1%. Thus, because of the speed of analysis and the degree of automation, flow cytometry is preferable to image cytometry for enumeration, unless spatial information is required.

There are three basic counting methods. The first, fixed volume counting or volume integration, is used in most haematological analysers. The volume measurement is achieved by two electrodes or optical sensors which determine the position of the sample in a capillary tube or loop or equivalent small fixed volume; the cell count is initiated when the sample passes the first sensor and terminated when it passes the second, providing a count per-unit-volume. The second method, time integration, assumes a constant volumetric flow of sample with time; this is achieved in practice by using constant flow (syringe) pumps to deliver the sample. The most generally applicable method, ratiometric counting, is based on spiking samples with reference particles, such as fluorescent beads. A known concentration of beads is added to the sample. The sample volume analysed can be determined from the number of beads counted while a sample is run; the cell count is then determined by dividing the number of cells counted by this volume. Ratiometric counting can be used with any flow cytometer; it corrects for system dead times and for the variations in flow rate that can occur in instruments that use differential pressure to deliver samples. It has become widely adopted, since almost all flow cytometer manufacturers now produce ‘counting beads’ of known concentration for their instruments. The tight cluster of counting beads on the cytometer’s data display can also serve as an on-line alignment control, particularly important when measuring environmental samples that are more likely to block the flow path.

Single cell suspensions are essential for accurate counting. Cell aggregates only give rise to a single colony in plating or a single observed event in cytometry. If, for example, one cell in a triplet is positive for a dead cell marker, the whole aggregate will be registered as dead but the two viable cells will produce a colony when sorted onto agar plates.

Cells can be disaggregated by chemical or mechanical methods. Mechanical methods have a broader application spectrum but can lead to problems when used with filamentous organisms and yeasts. The application of a shear force by forced flow through needles can lead to problems with clogging and is very tedious to perform. The application of a shear force within homogenisers is difficult to perform with small volumes often causing problems with foaming and sample carryover. Ultrasonic treatment is the most convenient disaggregation method, but it is important to apply reproducible energy levels. The geometry of the set-up and the material of the sample container must be taken into consideration. When using a probe, energy can be lost to ice-cold water surrounding the sample container or to air-bubbles trapped at the bottom of pointed vessels. Transmittable energy in an ultrasonic water-bath is affected by the water level, temperature dependent dissolved gas concentration, and the presence of contamination. Plastic or elastic container materials such as polypropylene will absorb energy in both systems. The following example describes a counting method for a dental application, based on a period of sonication, followed by single
colour DNA staining to investigate the effects of sonication (Fig. 2).

2.1. Example: Disaggregation and single colour staining of bacteria for cytometric analysis

Sonicator: 3 mm exponential probe at 23 kHz (MSE Soniprep 150).
Instrument settings: 1 and 2 μm amplitude for various time length.
Sample container: Disposable polystyrene 7 ml flat bottom containers (#129B Sterilin, Stone, UK) sonicated against air. The probe tip was 5 mm below the liquid surface of a 2 ml sample.

Samples: 24 h bacterial plaque scraped with a wooden applicator, dissolved in 5 ml of Dulbecco’s phosphate buffered saline (DBS), filtered through a 50 μm nylon filter and divided into two 2 ml samples.

Staining: A 20 μl sample was diluted at various time intervals with 80 μl DBS. A total of 20 μl aliquots of diluted sample were mixed with 20 μl 0.66 μm yellow–green fluorescent beads (Polysciences, Warrington, PA, USA) for counting, 10 μl ethidium bromide (EB) or propidium iodide (PI) (Sigma, Poole, UK) at 1 mg/ml in distilled water, to stain nucleic acid, 150 μl DBS containing 0.1% azide, and 0.05% Tween 20. After 15 min, samples

Fig. 2. The effect of sonication on the enumeration, membrane integrity and recovery of dental plaque bacteria.
were diluted with 1.8 ml DBS and measured using an EPICS XL flow cytometer, with the first tube (EB) giving total number of bacteria and the second tube (PI) reflecting the fraction with permeabilised cytoplasmic membranes.

**Cell cultures:** Sonicated cells were dilution plated onto brain heart infusion agar (Oxoid Ltd., UK) supplemented with blood and grown for 48 h in 5% CO₂ at 37°C.

**Results:** (Fig. 2) It is readily seen that despite any disaggregation caused by sample handling, sonication caused a significant increase in ratiometric and dilution plate counts. The initial decline of the percentage of dead cells in Fig. 2 results from dispersion of aggregates. The subsequent increase in PI positive cells indicates cell permeabilisation by the sonication treatment. The decline in the ratio of total intact cells to plate counts in Fig. 2a also indicates injury amongst the intact cell population. Both graphs in Fig. 2 show an optimum recovery of cells when using a sonication time of 2 min at a 2 μm amplitude. From the decrease of total cells and the relative increase of permeabilized cells, it is apparent that sonication times above 10 min caused destruction of intact cells. The decrease of dilution plate counts also suggests that cell damage may occur. Sonication at 1 μm appears to give a wider window of constant recovery, but is more difficult to carry out, and the longer sonication time required makes it less feasible in practice. In addition, despite the disaggregation caused by pipetting the samples, sonication generated an average increase of counts by a factor of 4–7 for cytometric counts and 6–8 for plate counts of smooth surface plaque. Specific detection of *Streptococcus sanguis* by immunofluorescence showed a 12.2-fold increase compared to a 6.3-fold increase in total counts, demonstrating species specific variation in aggregation. Sonication also improved signal-to-background fluorescence and yielded a tighter intrinsic light scatter cluster.

The large number of bacteria analysed by flow cytometry increases counting precision and accuracy. Using a careful pipetting technique, and adding of 0.05% Tween 20 to the sample to minimize cell aggregation, it was possible to achieve counting variations within 1% of the expected numerical counting error. The sensitivity of optical systems in detecting cells is limited by the concentration of the cells and the signal intensity separating the cells from extraneous particles and other background noise. In order to visualise cell clustering it is desirable to have at least 100 cells with the desired properties; thus, if there is one organism per μl in the final volume, 100 μl should be sampled. Relative frequency can affect the measurement. For example, after a 3 log reduction in count, with a desired cell subpopulation now present at an event frequency of 0.1%, 100,000 events must be analysed to detect 100 that exhibit the desired properties. If the signal-to-noise ratio of the measured parameter is not high enough, the labelled cells of interest fall within the tail of the much larger distribution of unlabelled events, and become undetectable. Signal-to-noise ratio also limits the speed of measurement, as with increased sample volume throughput, the sample variances increase. When sample flow rate is increased, background fluorescence increases due to the presence of more free fluorochrome in the observation region. Using flow rates below 10 μl/min results in a practical sensitivity of approximately 10³ cells ml⁻¹ within a 10-min acquisition time. Lower concentrations require long observation times or pre-enrichment by physical or biological means.

**Limitations:** Filamentous cells and some yeasts are very sensitive to sonication treatment and may not survive even a 10 s treatment in an ultrasonic water-bath. Sonication in the presence of stains can lead to uptake of membrane impermeant dyes such as PI and loss of antibody fluorescence and of surface structures such as flagella. We have previously found that that mechanical disaggregation is not always a sufficient enough treatment to disaggregate samples such as old plaque.

### 3. Classification of cell functionality.

Viability is the key cell function investigated in microbiology. Multi-colour flow cytometric analysis allows differentiation of stages far beyond the classical definition of viability, which is usually defined by demonstrable reproductive growth (Fig. 3). The fact that complex cellular functions, other than growth, can be detected, has lead to a shift in the interpreta-
tion of the term “viability”, thus generated the term of “viable but non-culturable cells”. The use of the functional criteria such as reproductive growth, metabolic activity and membrane integrity should help to clarify the situation with regards to the measured property and its interpretation.

Reproductive growth (as the most stringent proof for viability) requires both metabolic activity and membrane integrity. Direct cytometric measurement of replication is a very rapid way to assess reproductive growth, and has been shown by Cohen and Sahar (1989) to provide antibiotic susceptibility information from patient samples within 2 h of sampling.

In many cases, growth itself cannot be measured due to irreversible DNA damage, fastidious growth requirements, lack of symbiotic partners or extremely slow growth. Detection of metabolic activity, which provides presumptive evidence of reproductive growth, is easier but metabolism even in the absence of growth may produce undesirable effects such as food spoilage, the accumulation of toxins or the transfer of genes. In cases of injury, dormancy or extreme starvation, metabolic function may be transiently undetectable. Its measurement can also be complicated due to extrusion pumps, which at the same time are powerful indicator of functioning cell metabolism.

In the absence of metabolic activity, it is still possible to determine membrane integrity by either dye retention or dye exclusion. Metabolic activity and proliferation depend on an intact cytoplasmic membrane, which separates the cell from its environment. Cells with intact membranes are presumed capable of metabolic activity/repair and to be able to reproduce unless their DNA is damaged beyond repair or they can not generate a positive energy balance. Cells without an intact membrane can not maintain or generate the electrochemical gradient which generates membrane potential, and can be classified as dead cells; as their internal structures are freely exposed to the environment they will eventually decompose. In product safety applications, one would ideally want all bacteria counted to be dead

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![Viability Concept](image)

**Fig. 3. The Viability Concept.**
based on this criterion of loss of membrane integrity, because, in some cases, accumulation of metabolites from organisms which metabolise but do not grow can be sufficient to render a product unfit or toxic.

3.1. Reproductive growth

Proliferation can easily be demonstrated by counting bacteria against reference beads. With counting errors below 5% (even within sub-populations in a mixture) minor changes can be detected. The major limitation on accuracy is the ability to obtain single cell preparations. Cell tracking, using a covalently bonded fluorescent label (Fig. 4), can be used to demonstrate culture heterogeneity as well as cell division. Growth can be followed by an increase in intrinsic light scatter or volume with biomass accumulation followed by halving fluorescence intensity. Important factors are high initial labelling intensity and low coefficients of variation to allow visualisation of multiple division cycles.

3.2. Metabolic activity

Demonstrations of the activities of enzymes such as dehydrogenases or esterases provide indications of metabolic activity. They show the capacity of a cell to have synthesised these enzymes in the past and its ability to having maintained them in active form. However, the substrate cleaving reactions by which activity is demonstrated are typically not energy dependent. The ability to transport molecules across the membrane for membrane energisation, active efflux, or pH regulation requires active energy metabolism. Membrane potential (MP) is the most ‘abused’ vitality parameter in microbial flow cytometry. The transmembrane electrical potential gradient, which in microorganisms is typically on the order of 100 mV, with the interior negative, originates from

![Fig. 4. Reproductive growth demonstrated by cell tracking. Listeria innocua was starved for 3 days and then covalently labelled with dichloro-carboxyfluorescein-diacetate-succinimidylester. Cells were measured at 60 min. (dot plot) and 180 min. (contour plot) growth in tryptic phosphate broth at 25°C. The contour plot shows some cells with still the same fluorescence as at 60 min but increased light scatter signal whilst the divided cells have returned to lower light scatter signals and half the fluorescence intensity.](image)
selective permeability and the active transport of ions across the cytoplasmic membrane. MP measurements are usually performed using so-called ‘distributional probes’, lipophilic dyes that can readily pass the cell membrane and accumulate according to their charge. Signals from those probes are also dependent on cell volume and on any changes in binding sites for the dyes. The active extrusion of cationic probes like rhodamine 123 can pose a problem when trying to measure the MP, but such extrusion is a stand-alone measure of metabolic activity. The detection of electrical depolarisation using the anionic probe bis-oxonol (BOX) overcomes that problem (Fig. 5), but the problem of signal variation with cell size remains. Ratiometric, largely size-independent, MP measurements have recently been described by Novo et al. (1999).

Detection of biosynthesis, particularly of macromolecules, provides the strongest evidence of normal metabolic activity. Nucleic acid synthesis was one of the earliest measurements in fluorescence based flow cytometry of microorganisms (Hutter, 1974) and has been used as one of the earliest detection criteria for antibiotic susceptibility (Steen et al., 1982). In the environmental field cell elongation under antibiotic pressure (Direct Viable Count) has become popular (Joux & Lebaron, 1997). Its biggest advantage is the fact that the measurement does not require a fluorescent channel, but uses light scatter. This leaves more channels of a multiparameter instrument available for measurements using specific reagents such as antibodies or rRNA probes.

3.3. Membrane integrity

Membrane integrity can be detected by dye-retention or dye-exclusion. In the dye retention method, cells are incubated with a non-fluorescent substrate, which is cleaved by intracellular enzymes to a fluorescent product that is retained in cells only when the cytoplasmic membrane is intact. Dye retention measurements may be inaccurate due to lack of enzyme activity, poor substrate uptake, or active dye extrusion, and also because some cells can retain product in vacuoles even after cytoplasmic membrane permeabilisation.

Exclusion of propidium iodide (PI) is the method of choice; this avoids the problems associated with enzyme activity stains. Demonstration of exclusion of more complex molecules, such as ethidium homodimer, can be difficult if the dye is contaminated with monomeric ethidium, which is membrane permeant. TO-PRO-3, a red-excited dye, classified by a number of people as a dye-exclusion marker, can stain intact bacteria (Nebe-von Caron, unpublished results), as does ethidium bromide (EB). Therefore, extreme care must be taken when interpreting the uptake and exclusion of such stains. The best way to understand stain exclusion properties is to use the stains in combination. The example shown in Fig. 6, combining the nucleic acid stains PI and EB with a marker of esterase activity clearly demonstrates the principle of this approach.

3.4. Multicolour labelling

The simultaneous supravital staining for total cell detection might require inhibition of extrusion pumps with azide or the use of extremely membrane permeable DNA stains like SYTO9 (used in the

Fig. 5. Staining of viable and heat fixed plaque with bis-oxonol (BOX) and propidium iodide. The double staining technique allows the identification of permeabilized cells which are by default also depolarised, thus serving as internal control. Whilst the heat fixed cells appear high in quadrant 2, the initially dead cells (region E) show only a weak bis-oxonol fluorescence.
Fig. 6. Correlation of dye exclusion properties of for DNA stains and esterase activity. Dual staining combinations of a dental plaque sample using red fluorescent DNA stains propidium iodide (PI) and ethidium bromide (EB) with esterase activity as determined by cleavage of di-chloro-carboxyfluorescein diacetate succinimidylester (CCFAS). The exclusive staining of either CCFAS or PI confirms the dye exclusion property of PI, whilst the coexpression of EB and CCFAS indicates the supravital staining properties of EB. Appropriate combinations, for example, To-Pro-3 and CCFAS, can be used for similar comparisons.

BAC-light viability stain from Molecular Probes (Eugene, OR)). Fig. 7 illustrates the use of a three colour combination to detect membrane integrity and membrane potential simultaneously, employing EB for total cell staining, rhodamine 123 (RH123) to indicate MP, and PI for the detection of membrane permeabilization, to demonstrate population heterogeneity in efflux-negative E. coli. An even more complex picture emerges when dealing with actively pumping cells, as is illustrated in Fig. 8. Multi-colour analysis, combined with single cell sorting directly onto agar plates, allows the correlation between the functional staining and the growth potential of the cells to be established.

Membrane potential measurements shown in Fig. 7 reveal the population heterogeneity in a heat stressed sample; however, the RH123 staining does not work on pumping cells (i.e., cells with an active efflux mechanism). The dye combination of bis-oxonol, ethidium bromide and propidium iodide (BEP-stain), shown in Fig. 8, therefore has a wider range of applications. It is important to remember that the uptake of BOX and PI in gram negative cells requires permeabilisation of the outer membrane, e.g., by treatment with 5 mM EDTA.

Sorting of bacteria from different functional stages reveals that, in most cases, all but the permeabilized cells are capable of recovery. In the example used in Fig. 8, 35% of the electrically depolarised cells grew direct on Nutrient Agar plates, and even more on special resuscitation media, or when sorted into liquid media. This shows that depolarisation indicates decline in cell functionality, but certainly not cell death.

Pump activity, here used as a measure for metabolic activity, is perhaps the major factor behind the controversy over ‘universal’ supravital stains. The influence of the buffers, washing steps, temperature and the availability of stored energy should not be underestimated. When cells which possess active extrusion pumps are metabolically compromised, pump activity ceases prior to electrical depolarisation; this in itself can provide a highly sensitive indicator of cell stress.
Fig. 7. Simultaneous staining for membrane integrity and membrane potential. *Escherichia coli* D634 was grown over night in brain heart infusion, washed and resuspended in PBS. Cells were heat stressed for 5 min at 60°C and 50 μl of cells were resuspended in 500 μl PBS-Tween at 25°C, containing 0.1 μM Rhodamine 123 and 5 μg/ml EB and PI each. The X-axis of the left contour plot represents 630 nm (red) DNA fluorescence generated by either EB or PI, the Y-axis represents the 575 nm (orange fluorescence) spectral overlap of EB unquenched by PI, thus cell integrity. The debris in region (A) (unstained cluster bottom left) are excluded from picture (B). The right contour plot shows the correlation of membrane integrity (Y-axis) versus the membrane potential stain Rhodamine 123 (525 nm) on the X-axis. Of the 90% Rhodamine negative cells (C2 = 9%) only 46% (C3) are permeabilized.

Fig. 8. Functional assessment by multicolour staining and cell sorting. *Salmonella typhimurium* stored for 25 days on nutrient agar at 4°C was re-suspended in DBS containing 0.1% peptone, 0.1% sodium succinate and 0.2% glucose. 10 μl sample was diluted in 200 μl DBS containing 4 mM EDTA, sonicated for 10 s in the hot spot of an ultrasonic water bath and incubated with 2 μl PI and EB at 0.5 mg/ml each and 2 μl *bis*-oxonol at 100 μg/ml for 30 min at 25°C. Cells were directly sorted on Nutrient Agar plates.
Limitations: No staining technique can give a guaranteed answer about a bacterial cell’s reproductive viability. Even membrane permeabilisation can be temporary, as can be demonstrated when looking at PI uptake of cells subjected to heat, electro- or chemoporation. Therefore, it is important to allow adequate time for repair of the membrane after such treatments. In addition, the potential for bacteria to recover and reproduce depends on the conditions experienced after the measurement took place. If, for example, the DNA is damaged beyond repair, no growth will occur; growth will also not be possible if the damage/stress caused by the culture conditions requires more energy than the cell can safely generate for its repair.

4. Practical applications of the multi-colour assessment of cell functionality.

The staining combinations described above have been used to investigate biotechnological processes and deliberate cell injury. This section gives a brief summary of those applications.

4.1. Biotechnological processes

During the course of any biotechnological process it is essential to monitor cell proliferation and viability. Accurate estimates of viable biomass concentration are essential so that informed decisions on process control can be made. This allows products to be harvested at optimum concentrations, and the activation of inducible systems to be initiated at the correct time, so that high product yields can be achieved. A high fraction of dead or dormant cells present during any part of a bio-process will be detrimental; it is therefore important to have accurate information on the physiological states of individual cells within a population. Multi-colour flow cytometry is capable of providing such information, rapidly, with a high degree of accuracy, and thus has a number of advantages over more traditional methods. Classical microbiological techniques routinely used for monitoring cell proliferation and viability can cause problems. Optical density, dry cell weight and manual cell counts give an indication of reproductive growth, but provide no further information on cell physiological state. Indeed, dry cell weight data is usually only available a considerable time after the sample is taken, too late for alterations to be made to process control. Additionally, such measurements made during the early stages of a fermentation, when biomass concentrations are low, are known to be inaccurate. Optical density measurements rarely take into account changes in cell size or in background medium composition. Manual cell counts are tedious to perform, and, for statistical accuracy, rely on an equal distribution of cells within the counting chamber, a situation almost impossible to achieve. Classically, a cell is deemed to be viable when reproductive growth has been demonstrated. Dilution plating relies on post sampling growth using non-selective media, and results often take in excess of 12 h to obtain, again too late for changes in process control to have any meaningful effect. Importantly, stressed, injured or otherwise so-called ‘viable but non-culturables’ cells often go undetected. Traditional staining techniques used with microscopy suffer from the same problems associated with manual cell counts. Also, it is the user who makes the decision on whether a cell has taken up the stain and hence, on its physiological state. This leads to inconsistencies between temporally spaced samples when different personnel have made the measurement. Mathematical models used to predict biomass production during fermentation processes are often inaccurate, since the assumption, based on classical microbiological measurements, that a bacterial population is homogenous with respect to its physiological state, and hence with respect to its ability to divide, is erroneous.

4.1.1. Fermentation

Multi-staining flow cytometric techniques have been used for the ‘at-line’ study of bacterial fermentations (Hewitt et al., 1998). It has been shown during the latter stages of small-scale (5L), well mixed fed-batch fermentations that there is a considerable drop in cell viability, about 20% (Fig. 9), as characterised by cytoplasmic membrane depolarisation and permeability. These phenomena are thought to be due to the severe and steadily increasing stress associated with glucose limitation during the fed-batch process. Such severe effects are not found in either batch or continuous culture fermentations.
Fig. 9. Cell sample taken after 36 h during a high cell density fed-batch fermentation with E. coli W3110 stained with propidium iodide and bis-oxonol. Three main sub-populations of cells can be distinguished, corresponding to healthy cells (A), no staining, cells with no membrane potential (B), stained with bis-oxonol; and cells with permeablised membranes (C), stained with both propidium iodide and bis-oxonol (after Hewitt et al. (1999a); Hewitt et al. (1999b)).

Therefore ‘on-line’ flow cytometry can potentially be used to improve process control.

4.1.2. Bio-remediation

The same techniques have been used by Boswell and colleagues (Boswell et al., 1998) to evaluate the toxic effects of four heavy metals (copper, cadmium, uranium and nickel) on an Acinetobacter sp. used for the bio-remediation of contaminated wastewaters. It was demonstrated that all four metals differentially permeablised the cytoplasmic membrane of the exposed bacterial population, whilst both copper and cadmium also generated an intermediate population of intact cells with depolarised membranes (Fig. 10). This has important implications for the final bio-remediation process since, for continued metal accumulation, cells must remain intact and metabolically active.

4.2. Heat injury

Heat treatment is the most common method of food preservation, causing a significant reduction in bacterial cell numbers. The correct application is critical to render a product safe or to achieve a required lengthy shelf life. The critical parameters of the process are the absolute temperature and the period of treatment. Whilst heat transfer in food materials is inherently very complex, bacterial heat injury simulation studies can readily be carried out in...
a temperature controlled water bath. To generate reproducible conditions, bacteria from an overnight culture were used as an inoculum for an exponential culture. At a set optical density the culture was exposed to the appropriate heat treatment. The degree of injury achieved was compared by recovery difference in a standardised agar ±2.5% sodium chloride.

Initial attempts to correlate recovery from heat injured Salmonella cells with membrane integrity measurements by flow cytometry (PI exclusion) failed. Even worse, none out of several hundred sorted single cells recovered on the agar plate, even when classical plate counting only suggested a one log reduction. The discrepancy between culturability and cell function remained also in the light of the multicolour staining approach. Heat injury gave rise to a loss of membrane function with time (see Fig. 11), but sorting single cells directly into agar plates yielded no recovery. As indicated above, injury could be quantified to some extent by osmotic stress, using salt, and this stress was assumed to be high when sorting a bacterium onto an agar plate with only 3 nl of liquid. Indeed, cell growth could be detected when sorting into liquid. However, investigations into the recovery of heat injured cells demonstrated that recovery was heavily dependent on the pre-enrichment medium used (Stephens et al., 1997) and discrepancies of up to four decades could be observed using media currently on the market. This, coupled with the fact that the cells still showed metabolic function after the heat injury, confirmed the viable non culturable cell (VBNC) problem to be a ‘growing issue’.

Apart from osmotic stress, oxidative stress was considered to be the biggest hurdle to overcome. Therefore, a new medium, based on buffered peptone water, was developed that minimised the amount of reactive oxygen products in the medium to avoid extrinsic oxidative damage from the medium, and also forced the cells to switch to anaerobic metabolism to avoid intrinsic oxidative damage from cell metabolism. This medium showed superior performance to all other commercial media in standard most probable number (MPN) dilutions and was compared for recovery of subpopulations as discriminated by the multicolour BEP staining (Fig. 12).

To look at low recovery using a sorting approach,

![Fig. 11. Effect of heat injury on the physiological states of *Salmonella typhimurium*. Heart infusion broth was inoculated with an overnight culture of *S. typhimurium* and grown in a shaking water bath to an OD of 0.3 at 600 nm. Bacteria were heat injured at 51.5°C for different lengths of time and 200 µl diluted in 9 ml of buffered peptone water at room temperature. Cells were allowed to equilibrate for more than 30 min before they were stained with EB/PI (5 µg/ml) and BOX (0.5 µg/ml) for 15 min and measured in the flow cytometer.](image)
Fig. 12. Recovery of *Salmonella* injured for 30, 40 and 60 min at 51.5°C and sorted into 96 well plates with Buffered Peptone Water and Salmonella Enrichment Broth with recovery supplement (SPRINT). To cope with low levels of recovery, 100, 10 and 1 cell was sorted for equal number of wells for each cluster and numbers of live cells were calculated and estimated as for most probable number dilutions. The recovery of permeabilized cells shown in (A) is potentially due to cells with remaining pores in the membrane at the time of staining or the aggregation of permeabilized with unstained (pumping) cells. The ratio of recovered cells in SPRINT and BPW in (B) indicates that for shorter injury times the improvement in recovery is equal for all groups, whilst the advantage for the pumping cells becomes more and more pronounced with prolonged injury.
it was necessary to change the sorting strategy from single cell sorting to “Most Probable Number” (MPN)-sorting in order to avoid sorting hundreds of 96-well plates. Even then, the reference media had to be in the upper range of the performance scale in order to demonstrate growth. Thus the recovery was compared to one of the better buffered peptone water products. Another limitation in the experiment was the potential of sorting cell aggregates. As mentioned above, sonication provides a good method to ensure cell separation, but was not applied here in order to minimise the risk of additional damage which could have caused deviations from the results obtained by normal procedures.

As can be seen from Fig. 12, the recovery of the cells injured for 90 min increases 21- and 37-fold for the depolarised and de-energised sub-populations, but nearly 400-fold for the pumping, thus still respiring, cells. The increased recovery of the permeabilized cells at 40 min suggests that the recovering cells are from the same sub-group, but, as described above, either still temporarily permeabilized or aggregated with dead cells. The fact that the cells still respiring after the injury process are the ones benefiting most from the oxidative protection suggests that, in normal cultures, those cells destroy themselves by intrinsic oxidative damage. The deenergized and depolarised cells would be expected to benefit from the link of onset of respiration with oxidative protection, as described by Gort and Imlay (1998).

5. Combined identification, counting and viability assessment

Identification of bacteria is classically based on morphology, nutritional requirements and metabolic reactions, which are only of limited use in direct single cell analysis. Serological methods based on antigenic differences have been used for a long time, and, more recently, genetic methods have been introduced into the field of bacterial identification. The advantages of methods using rRNA probes are the capability for broader classification and the ability to design probes from databases without the need for isolation of the bacteria. However, these methods require permeabilisation of the cell and the presence of sufficient metabolic activity/ribosomal target material. This limits their use in combination with functional assessment of the cells. Whilst the development of monoclonal antibodies is more complicated, immunofluorescent labelling of surface antigens can be performed without disruption of the cell membrane, and therefore combined, for example, with membrane integrity measurements, as described below and shown in Fig. 13. This allows the isolation of antigenically defined organisms for subsequent cultivation. Other multi-colour combinations have used of four different antibodies simultaneously (Hutter, 1992), or combined rRNA probes and antibodies (Wallner et al., 1997).

5.1. Example: Sample preparation for counting intact and permeabilized antibody labelled cells in 24 h dental plaque

Plaque samples were obtained by scraping the smooth surface or the gingival margin of the upper incisors using a wooden applicator stick. Samples were dissolved in 2 ml Dulbecco’s phosphate buffered saline (DBS), sonicated as described above for 2 min at 2 μm amplitude, and spiked with reference beads for counting.

Bacteria 5 × 10^6 were loaded into the well of a 96-well filter plate 0.2 μm polycarbonate (Porvair Filtronic, Sheperton, UK) and washed twice with 200 μl DBSwater (0.1% Azide; 0.5% BSA; 0.5% Ovalbumin; 0.05% Tween 20) by sucking the liquid through the plate. 200 μl of antibody supernatant or 200 μl DBSwater containing 1 μg purified antibody was added and incubated for 30 min at 25°C followed by two more washing steps in DBSat.

Cells were resuspended in 200 μl DBSat containing 1 μg fluorescein (FITC)-conjugated rabbit–anti-mouse antibody, incubated for 30 min 25°C, and washed twice in DBSat.

After final resuspension in 200 μl DBSat containing 10 μg/ml EB and PI cells were transferred into 600 μl tubes for measurement in the Coulter EPICS Elite.

Natural samples are the best examples to demonstrate heterogeneity and the power of cytometric analysis of bacteria. As the method is free of the distortions of post sampling growth, it can accurately detect and enumerate bacteria in mixtures of healthy,
Fig. 13. Antibody based analysis of dental plaque and its verification by cell sorting: Dental plaque was collected in DBS, spiked with reference beads, sonicated and labelled with antibody anti *S. sanguis* 4715 followed by rabbit-anti mouse*FITC F(ab)_2*. Cells were briefly sonicated after staining to minimise the risk of aggregation. Cells were sorted based on membrane integrity and absence or presence of antibody label. Selected colonies were identified by API, based on morphology and haemolytic capacity. 99.4% of the antibody positive cells were correctly identified. 3.6% appeared false negative due to the sonication treatment required to disaggregate the antibody labelled cells.
injured, stressed, aerobic, anaerobic and facultatively anaerobic bacteria that coexist, for example, in dental plaque. The disaggregation of the bacteria not only increases total and plate counts, but can also alter the relative proportions of selected species if they behave differently with regard to aggregation.

6. Summary

Direct analysis of bacteria on a single cell level will continue to enhance our understanding of microbial populations, their heterogeneity and complexity. Flow cytometry in combination with single cell sorting has allowed the interpretation and verification of the properties of a number of fluorescent stains. In biotechnological applications, the multicolour staining approach provides important physiological information at the individual cell level about process efficiency that is almost impossible to obtain in any other way.

The requirement of single cell suspensions for flow cytometry poses limitations on its applicability to microbiological research, but the technology has still advantages over image cytometry with regards to speed of analysis and automation of sample handling. However, the philosophy between the two techniques based on a fluorescent probe approach is the same. Image cytometry is the method of choice in cases of sample collection on filter membranes or if spatial information is required. Hopefully, cytometry will become as essential a tool in microbiology in this millennium as the plastic petri dish became in the last.

References


