REVIEW ARTICLE

Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities

Susann Müller[1] & Gerhard Nebe-von-Caron[2]

[1]Department of Environmental Microbiology, UFZ – Helmholtz Centre for Environmental Research GmbH, Leipzig, Germany; and [2]Inverness Medical International, Bedford, UK

Correspondence: Susann Müller, Department of Environmental Microbiology, UFZ – Helmholtz Centre for Environmental Research GmbH, Permoserstrasse 15, 04318 Leipzig, Germany. Tel.: + 49 341 235 1318; fax: + 49 341 235 1351; e-mail: susann.mueller@ufz.de

Received 8 July 2009; revised 26 January 2010; accepted 28 January 2010.
Final version published online 10 March 2010.

DOI:10.1111/j.1574-6976.2010.00214.x

Editor: Bernardo González

Keywords
single cell; heterogeneity; flow cytometry; cytomics; population dynamics; community structure.

Abstract

The still poorly explored world of microbial functioning is about to be uncovered by a combined application of old and new technologies. Bacteria, especially, are still in the dark with respect to their phylogenetic affiliations as well as their metabolic capabilities and functions. However, with the advent of sophisticated flow cytometric and cell sorting technologies in microbiological labs, there is now the possibility to gain this knowledge at the single-cell level without cumbersome cultivation approaches. Cytometry also facilitates the understanding of physiological diversity in seemingly likewise acting populations. Both individuality and diversity lead to the complex and concerted actions of microbial consortia. This review provides an overview of the state of the art in the field. It deals with the handling of microorganisms from the very beginning (i.e. sampling, and detachment and fixation procedures) and goes on to discuss the pitfalls and problems in analysing cells without any further treatment. If information cannot be gained by specific staining procedures, phylogenetic technologies, transcriptomic and proteomic approaches may be options for achieving advanced insights. All in all, flow cytometry will be a mediator technology to gain a deeper insight into the heterogeneity of populations and the functioning of microbial communities.

Introduction

Several references provide an idea about the live thriving role of microorganisms that are the prevailing organisms of our biosphere. For example, 3.6×10^{29} microbial cells with a total cellular carbon content of about 3×10^{11} tons are documented as living in the oceans. Bacteria are responsible for 98% of the primary carbon production and are important mediators in all biogeochemical cycles (Sogin *et al.*, 2006). As another example, the human intestinal microbiota was described recently as being composed of 10^{13}–10^{14} microorganisms, with a collective genome containing at least 100 times as many genes as the human genome (Gill *et al.*, 2006).

The direct microscopic observation of such small 'animalcules' by Leeuwenhoek in 1676, as described in his letters to the British Royal Society, has undoubtedly been one of the key events in science of the last few centuries. His observation of single cells facilitated the understanding of the single-cell nature of bacteria and biology in general, as eventually demonstrated for plants and animals by Schleiden and Schwann in 1837 and 1838. The fact that one bacterium can give rise to an entire culture or colony has provided microbiologists with a single-cell analysis system with outstanding detection sensitivity. Colonies derived from single cells have been examined using the plating techniques developed by Koch more than a century ago. The strength of this technique, the high amplification factor, is also its weakness, the dependence on the ability of the cells to grow. As we now understand, $>$ 98% of the microorganisms in our environment cannot be kept in culture (Keller & Zengler, 2004), and so culture-independent technologies are required that can characterize them precisely. With cytomics, a discipline is now emerging that uses single-cell analytics based on microscopic and flow cytometric techniques to unravel a cell's position, function and physiological state within a community.

The single-cell concept was born at a time when the likes of Pasteur and Koch discovered bacteria as origins of

FEMS MICROBIOLOGY REVIEWS

processes such as fermentation and disease. While Pasteur's work 'On the organized particles existing in air' in 1862 already suggested the origins of particles in putrefaction, it was an Irish scientist by the name of John Tyndall who, in 1877, disproved the theory of spontaneous generation after even excluding spore germination from his experiments. To support his claim, he built a dust-free box that contained the basic elements of a modern flow cytometer, a light source illuminating a path through which cells pass and their detection by the scattering of light – as detected by the human eye.

Although Koch had already developed objective image cytometry by means of photography, flow cytometry (FCM) really came to life in the late 1940s. Driven by the need to identify bacterial aerosols in warfare, the next generation of flow cytometers started with a design similar to Tyndall's (Gucker *et al.*, 1947; Ferry *et al.*, 1949; Gucker & O'Konski, 1949), the only difference being that the light source was now a Ford headlamp and detection was performed using a photodetector. The next period of more intensive FCM in microbiology was started in the mid-1970s by Hutter (1974); Hutter *et al.* (1975), Paau *et al.* (1977), Slater *et al.* (1977) and Bailey *et al.* (1977), and as early as 1979, the technology was already pushed to detect large viruses (Hercher *et al.*, 1979). Hutter & Eipel (1978) were the first to undertake a complex study on the viability, total protein and cell cycle of bacteria, yeast and moulds, and the autofluorescence of algae. They already utilized the power of multiparameter measurements possible with FCM, a feature neglected in most of the more recent studies. At the same time, Steen used a modified microscope that he developed into a flow cytometer more geared for microbial applications (Steen & Lindmo, 1979; Steen & Boye, 1980; Steen, 1983). He carried out fundamental work in bacterial replication and subsequently drug susceptibility (Steen *et al.*, 1982, 1986) and also applied immunofluorescence (Steen *et al.*, 1982). Since the 1980s, the number of articles applying FCM in microbiology seems to be growing exponentially. Comprehensive information on physics, optics and electronics as well as companies manufacturing instruments can be obtained from Shapiro (2002).

Although single-cell techniques in microbiology are highly relevant in many aspects (e.g. Allemand & Maier, 2009), their application to studies on bacterial population dynamics clearly represents a challenge. As compared with mammalian cells, bacteria are only 1/10 of the diameter; thus, the cell surface is only 1/100 and cell volume 1/1000, which has implications for the signals derived from them. Nevertheless, bacterial FCM enables cell states to be visualized and allows the growth, death, replication, cell division, metabolism and surface phenomena to be followed by the investigator, considerably enhancing the ability to understand and control cell physiology. In addition, a huge assortment of fluorescent probes is available commercially, their numbers having rapidly increased recently. Davey & Kell (1996) provide a comprehensive review of this topic. Summaries are presented by several other authors too (Fouchet *et al.*, 1993; Álvarez-Barrientos *et al.*, 2000; Vives-Rego *et al.*, 2000), and Howard Shapiro's Cytometry manual is now also available online against a free registration with Beckman-Coulter (see also http://www.coulterflow.com/bciflow/practical.php).

The central appreciation from performing microbial analysis at the single-cell level is that there is rarely – if ever – such a thing as a homogenous population. Biological systems are based on heterogeneity as a way of creating stability (Gefen & Balaban, 2009); therefore, diversity seems to be the key to survival (Avery, 2006). Heterogeneity within microbial communities is well accepted because knowledge is present on independent microorganisms that communicate via molecular interactions such as signalling molecules (Patankar & Gonzalez, 2009) or increase stability of systems by shared substrate pathways (Pandey *et al.*, 2009). Lateral gene transfer among bacteria is also a well-described cause for community stability (Ochman *et al.*, 2000). However, heterogeneous states of cells within pure cultures are less accepted. Therefore, it needs to be stated that sources of variation additionally comprise cell cycle-dependent events as well as cell states that are related to the age of a cell. Bacteria are not immortal, but show age-dependent variations in physiology. Also, the kind of surroundings impacts the cells' physiology, causing diverse individual adaptation mechanisms that enable to survive adverse environmental conditions. As a result, there are variations in the individual enzyme activity, structure and charge of the cells' membrane and/or wall and/or the contents of various metabolites, for example the synthesis of storage products to safeguard maintenance metabolism (Müller *et al.*, 2010). In addition, some individuals of many bacterial species are able to form spores or cysts. This variability is best described by the term 'heterophysiology' as it is far more than a question of inherited traits, but describes the mechanical, physical and biochemical functions and interactions of living organisms not only among themselves but also in their interaction with the environment. The ability of an organism to respond to changes in microenvironmental conditions and to maintain homeostasis is a strong indicator of its resilience and capacity to tolerate environmental perturbations. Systems biology (Baquero & Lemonnier, 2009; Gefen & Balaban, 2009; Lopez *et al.*, 2009) combines the information of the different approaches, collecting and linking data on the cell's capacities, states and activities and their inter-relationship with its surroundings (Conners *et al.*, 2006).

Following these processes not only on a bulk level but also on the individual scale is an obligation. Cytomics, and

especially FCM, can be used to disintegrate a microbial community or population into subsets of cells with shared structural or physiological properties within complex and dynamic networks. The key to enhanced knowledge in this field will be high resolution, and quantitative and reliable cytometric data analysis. The central role of FCM is its ability to generate population statistics as it can measure the relevant numbers and features of cells, identify subpopulations/subcommunities with similar properties, find (rare) events and separate organisms of interest to connect individual-based knowledge with advanced methods of molecular biology and protein biochemistry, obtaining even advanced information on individual cell composition and state. It is therefore an essential tool to gain a holistic understanding of a complex process. One has to do the differentiation first, which has been phrased so beautifully by Goethe in his quest to gain that unifying understanding:

"to find you in infinity of space and time
one must first divide and then combine,"

which is nowadays the central theorem of scientific studies such as systems biology.

Sample handling

Detachment

Bacteria usually grow in colonies and biofilms in nature; only a minor part can be observed as planktonic cells (Nadell *et al.*, 2009; Pamp *et al.*, 2009). Although most of the benchtop experiments in microbiology are performed with suspension cultures, natural samples have the constraint of being agglutinated. Single-cell techniques, however, require suspensions of single cells. This involves not only the application of the right fixatives but also requires efficient detachment of cells from surfaces such as soil or the disintegration of aggregates or flocs. Often, chemical and physical techniques are used, such as application of pyrophosphate solutions (Riis *et al.*, 1998), shaking and sonication procedures (Buesing & Gessner 2002; Boenigk, 2004) or a combination of alkaline hydrolysis and shear force (Völsch *et al.*, 1990). Chelating agents such as sodium pyrophosphate weaken hydrogen bonds, van der Waals, electrostatic and chemical forces that attach a cell to the soil particles. Shaking or sonication releases bacteria that are entrapped in micropores or channels. While sonication can increase cell recovery for FCM as well for culture-based methods by several orders of magnitude (Banks & Walker, 1977), it can also lead to selective loss of filamentous organisms and the loss of flagella structures, the latter most notably by the loss of motile function when observed under the microscope (G. Nebe-von-Caron, unpublished data; Lotz *et al.*, 1977).

Fixation

Determination of bacterial cell states using FCM includes the stabilization of the cell itself, its morphology, structure and surface characteristics. Frequently, characterization of bacterial physiology is restricted to fixed cell samples. However, common fixation procedures (e.g. formaldehyde or glutaraldehyde), routinely used for eukaryotic cells, often cause agglutination as well as increased autofluorescence, which complicate the flow cytometric analysis of bacteria. Agglutination is often caused by the slow action of these fixatives, enabling the bacteria to produce exopolysaccharides or other high molecular weight compounds to protect themselves against such chemicals. The highly complex structures lead inevitably to aggregation. On the other hand, formaldehyde is also known to stabilize and close membranes of single cells by intensely cross-linking surface proteins. This can be a useful technique to prevent probes from going into cells, if wanted. On the other hand, formaldehyde can be used to seal cells to prevent leakage (or pumping) of probes after staining living cells (Achilles *et al.*, 2006). Using ethanol as a fixative agent, lipids of the cells membrane are known to be eluted, thus changing the composition of the cell structure. Nevertheless, for some bacterial species, formaldehyde in combination with ethanol is recommended in case the samples have no or a minor autofluorescence. Samples thus fixed can be stored at $-20\,^{\circ}C$, but the number per millilitre should be below 10^9 cells mL^{-1} to prevent agglutination. This procedure has been proven to be successful for some FISH protocols (Wallner *et al.*, 1993; Herrmann *et al.*, 1997). Additionally, 10% NaN_3 is recommended for the preservation of aerobically cultivated bacterial cells. This compound inhibits proteins of the respiratory chain and therefore stops all energy-dependent processes within the cells. The compound also has an osmotic fixation effect, preventing cellular molecular degradation processes very effectively. Both ethanol–formaldehyde and NaN_3 are reported to preserve bacterial cell states for months (Müller, 2007). Anaerobically grown bacteria require other fixation procedures. Molybdenum and hydrogen peroxide can be used for sulphate reducers, a procedure that was found to stabilize cells for at least 2 weeks (Vogt *et al.*, 2005). Anaerobically growing natural bacterial communities can be fixed with solutions based on metal solutions in combination with sodium azide. The fixation efficiencies of aluminium, barium, bismuth, cobalt, molybdenum, nickel and tungsten salts were evaluated by a flow cytometric measurement of the DNA contents as a bacterial population/community stability marker (Günther *et al.*, 2008). It needs to be mentioned that bivalent metals will have an impact on the cellular RNA contents of the anaerobically grown bacteria and will prevent a successful cell labelling via FISH, a PCR

application or subsequent mRNA isolation after cell sorting. In fact, alkaline hydrolysis by barium hydroxide has been used to systematically remove RNA originally in HeLa cells (Stöhr & Petrova, 1975) and also in bacteria (Völsch *et al.*, 1990).

Cell vs. particle measurements

The third point that should be taken care of is that most solutions such as buffers or media do contain particles of size and density characteristics similar to bacteria, which can produce misleading signals when analysed using FCM. In its simplest form, these signals can just be bubbles or micelles. They tend to form continuous distributions, but discrete particle distributions may also occur. An example for this was shown for bacteria grown under anoxic, sulphide-producing conditions. Live bacteria were analysed by FCM, but additional clusters of events were recognized. The high reactivity of the mediums' sulphide in the presence of oxygen resulted in the sulphides oxidizing to sulphur within the few minutes of sample harvesting and analysis at the flow cytometer, forming microparticles in the range of 1 μm. When such inorganic particles exhibit light scatter signals similar to cells, they can cluster to distinct, bacteria-like subdistributions in a histogram. To prevent artefacts such as this, the following treatments are suggested: (1) removal of hydrogen sulphide from the sample by flushing with an oxygen-free nonreactive gas (e.g. nitrogen, helium), or alternately, (2) treatment of samples in an anaerobic chamber before measurement; and (3) performing controls by which the sulphide-containing medium is replaced immediately by a sulphide-free fixation buffer (Hübschmann *et al.*, 2007).

To separate cells from electronic background noise and abiotic particles, it is recommended to include nucleic acid stains. However, care has to be exercised due to the lipophilic nature of some of the dyes. They frequently also stain abiotic particles, which is why their datasheets often recommend to confirm the specificity of the stain in complex environments. It has to be kept in mind that in natural environments, particle-free solutions are not the norm. Also, the statement that a solution is sterile does not mean that it is particle free.

Cell count measurements

Many investigations require enumeration of bacteria and determination of the impact of physical, chemical and biological interventions on their cell number. Bulk measurements such as changes in turbidity, conductivity or gas pressure of liquid media have become popular for bacterial quantification because of their ease of handling and their high sensitivity and relatively low cost. However, there are many situations where they can either provide no reliable information on cell number because individual cell growth may overlay the increase in cell number or bulk techniques cannot be applied because of very low cell densities or increased presence of abiotic particles in the solutions. Opaque or coloured media or solutions also do not allow reliable turbidity measurements.

Manual direct microscopic single-cell count measurements tend to be more reliable, but are elaborate and depend considerably on the person who performs this task and her ability to identify the bacteria. Also, the statistics of the microscopic counting techniques are limited by the number of cells counted, which tend to be low compared with FCM counts. On the other hand, direct counting does not require postsampling growth and is therefore free of growth-related distortions. Bacterial cell number is very often determined by counting CFUs after plating them on agar plates on distinct media or by most probable number (MPN) estimations. However, most commonly used cultivation-based methods for detecting and enumerating bacteria have serious limitations as some individuals in a population or even species are generally unable to grow under the test conditions, for instance, because they need symbiotic partners or special microenvironments, unknown growth factors or other so far unexplored conditions.

Changes in cell number can be determined reliably and with high statistical confidence by FCM. Some cytometers are equipped with volumetric measurement possibilities measuring either a fixed volume or with a mechanically controlled volume flow to obtain counts in a specified volume. Many cytometers do not contain such equipment, and in these cases, monodisperse latex beads of an appropriate size, fluorescence intensity and number should be used to determine cell numbers. Depending on the design, alignment and cleanliness, some flow cytometers can resolve cell numbers solely from light scatter characteristics and by triggering on light scatter. However, depending on the level of interference by signals derived from the instrument or the sample, the cells need to be stained, for example using nucleic acid dyes such as ethidium bromide (EB), propidium iodide (PI), Syto-9, Syto-13 or 4′,6-diamidino-2-phenylindole (DAPI, Völsch *et al.*, 1990; Monfort & Baleux, 1992; Lebaron *et al.*, 1998b; Comas-Riu & Vives-Rego, 1999; Servais *et al.*, 1999; Trousselier *et al.*, 1999; Vives-Rego *et al.*, 1999; Vogt *et al.*, 2005). Even minimized chip-based microfluidic devices (*on-chip* FCM) are available that are described to count bacterial numbers within relatively short time ranges (six parallels in 30 min for 2000 cells each, Sakamoto *et al.*, 2005). The advantage here is that the risk of biohazard is low because the devices are closed systems and disposable.

Scatter

Bacterial populations can be characterized to some extent by analysing their forward scatter (FSC) as well as their side

scatter (SSC). The general assumption is that FSC is correlated to cell size and SSC represents cell density or granularity.

The relationship between light scatter and cell morphology can be complicated. The problem arises from the chosen size of the angle that is used to measure the incoming light scattered by the cells' features. As a result, the FSC signal of the cells does not always correlate linearly with their real size. Even small variations in the scatter angle geometry of the cytometer impact the scatter signal of a cell, as can be visualized using programs such as MIEPLOT (http://www.philiplaven.com/mieplot.htm). For example, the scatter behaviour of latex beads (2, 4, 6 and 8 μm, respectively) with a fixed refractive index of 1.42 in water against light scatter angles of 4, 9 and 12° results in a ranking of 2, 4, 6 and 8 μm at 4°, but 2, 6, 4 and 8 μm at 9° and 2, 4, 8 and 6 μm at 12°. Thus, it is not surprising that there are different relationships between latex bead size and analysed FSC using different instruments. Measuring latex beads of 3–8 μm, the order of 4, 3, 6, 5, 8 and 7 μm was obtained on a particular Becton Dickinson FACS-Scan, whereas it was 4, 3, 5, 6, 7 and 8 μm on a Becton Dickinson LSR. The correct order was preserved on a Coulter XL (Becker *et al.*, 2002). Therefore, the assumption that the ability to detect a 0.5-μm latex bead would indicate sufficient sensitivity for detecting a 1 μm bacterium cannot be generally upheld (Nebe-von-Caron, 2009). Satisfactory FSC correlations have been achieved on an Ortho Cytofluorograph using UV illumination (Robertson *et al.*, 1998), and Foladori *et al.* (2008) on their Apogee A40 (UK). However, with regard to FSC, it should be kept in mind that the performance of most cytometers is insufficient to analyse the small differences in the signals or to obtain any signal at all due to lack of critical alignment and instrument noise. Another point to be mentioned is that some applications may require an extensive treatment of the cell wall to channel for example large marker molecules such as antibodies or enzymes into the cell. Such procedures frequently destroy the surface scatter characteristics of bacterial cells.

On the other hand, SSC performances between instruments are sufficiently similar between different instruments to allow comparisons (Nebe-von-Caron, 2009). However, cytometer operators should be aware of the effect of the surrounding liquid, for example the sheath and the sample diluent and their relative flow rates, as scatter is a function of the refractive index change, for example the difference to the surrounding medium (see http://www.photometer.com/en/abc/show.html?q=Refractive%20index). The use of refractive index-matched particles has shown a good correlation on the Apogee A40 (Foladori *et al.*, 2008). However, any experienced cytometrist would expect bacteria to be fairly variable at a single-cell level. The ability of bacteria to surprise by changing their refractive index by environmentally caused changes in cell composition and in the extreme by inclusion bodies or spore formation will outmanoeuvre the investigator. To make matters worse, light scatter is a function of wavelength. In theory, it yields higher signals at a lower wavelength, but in practice, there are several exceptions, for example due to changes in wavelength-dependent absorption phenomena. To summarize, the scatter signal of a cell may change due to the refractive index of the sample and the sheath solutions, the wavelength and, additionally, the geometry of the lasers and the optics used in a particular flow cytometer.

Nevertheless, scatter measurements provide *online* and *real-time* information and no staining procedures need to be involved. Looking at pure cultures, different types of cells may be detected if they display different morphological characteristics. Scattering signals at different angles can even allow visualization of different bacterial species, which was shown to be possible for a binary culture where one of the strains produced a bacteriocin that acted against the second strain in the culture (Hechard *et al.*, 1992). Also, some authors used the scatter information to follow the action of antibiotics. Degeneration of cells after application of ceftazidine, ciprofloxacin and gentamycin was shown by loss of the light scatter signal (Walberg *et al.*, 1997). On the other hand, similar antibiotics and others can lead to filament formation because of impaired cell division, which results in doublets or chains of bacteria, increasing or changing the FSC (Suller & Lloyd, 1998; Wickens *et al.*, 2000). SSC was described to be a useful cell volume predictor in aquatic environments (Marisol *et al.*, 2007) and is also known to be a simple approach to obtain information about increasing cellular density if additional intracellular products are synthesized. Some authors were able to analyse the accumulation of metabolites that are used as reserve material and an energy source in bacteria. Such inclusion bodies can be poly-β-hydroxybutyrate (PHB) granules produced as an overflow product by carbon excess and limitation in nutrient composition or in response to limitations in the energy metabolism (Srienc *et al.*, 1984). Analysis of heterologous or homologous protein accumulation when overexpressed in genetically modified bacteria is another application. Thus, light scatter can be used to study the levels of synthesis and accumulation and thus provide information on the metabolic and physiological states of cells (Fouchet *et al.*, 1993).

Cell sorting

The two most important milestones in FCM after its first description by Kamentsky *et al.* (1965) were the introduction of fluorescence into the measurement independently by Göhde (Dittrich & Göhde, 1969), Kamentsky & Melamed (1969) and Van Dilla *et al.* (1969) and the application of cell sorting by Fulwyler (1965). Together, they formed the core

of modern FCM and gave rise to the term 'Fluorescence Activated Cell Sorter' or FACS™ (introduced by Herzenberg; Hulett *et al.*, 1969), which later became the trade name used by Becton Dickinson. The acronym EPICS™ from Coulter standing for 'Electronically Programmable Individual Cell Sorter' describes the sorting process more accurately as sorting is not limited to fluorescent signals, and most people trigger their instruments on light scatter. As the analysis and sort functions are by no means limited to the application of cells and are used increasingly for the analysis of bead arrays and other particles, the acronym PAS™ originally used by Partec (Germany) for Particle Analysis System is probably the most appropriate description for the technology. Apart from destructive 'cell zappers', which destroy unwanted cells by photodamage, there are also virtual cell sorters in image-based systems available that register the location of events as well as magnet-based sorter systems such as the Miltenyi MACS™ (Germany) that separate bacteria out of matrices inaccessible for FCM or are useful for pre-enrichment.

Droplet sorting is the most widely used method of single-cell sorting. It is based on the formation of droplets downstream of the measurement zone, which are assigned a positive or a negative charge depending on the desired direction and magnitude of deflection in an electric field generated by deflection plates. Droplet sorting allows direct deposition of a single cell onto an agar plate, into a well filled with the appropriate liquid medium or coculture system or into other devices for further treatment or investigation. The power of this approach was exploited at an early stage in industrial applications for strain improvement (Betz *et al.*, 1984). It is also useful for the assessment of bacterial replication potential and confirmation of biochemical differentiation (Nebe-von-Caron *et al.*, 1995, 2000). It is also possible to sort dilution curves for MPN calculations (Stephens *et al.*, 2000).

The flow diversion sort principle, originally implemented by Partec (Germany) by producing a pressure wave to divert the flow of cells into a separate channel, has the advantage of being entirely enclosed to reduce the risk to the operator by aerosols of hazardous materials (Dühnen *et al.*, 1983) and the risk to the sorted material from becoming contaminated. Becton Dickinson developed a similar system using a 'catcher tube' that moves into the flow stream to divert the cells of interest into a container, and flow switching is a popular sort mode in microfluidic cytometry chips. As sort systems, they tend to be very reliable as they do not suffer so much from mechanical blockages because of the absence of the flow-restricting orifice or nozzle, but they are of limited use when it comes to the handling of single cells or high cell numbers.

The most important task before starting the sorting process is to ensure the timing between the measurement and the progression of the cell to the point of mechanical separation. With droplet sorters, it is easily optimized by running a test sort onto a microscope slide with various delay times or by *online* monitoring of the sort stream, as implemented in the Accudrop™ system of the Becton Dickinson instruments. Although using larger particles for sorting makes the visualization easier, it is recommended to check the sort timing with particles with signals equivalent to the particle of interest, particularly in sorters where the measurement is taken inside a flow cell. Unlike with jet in air sorters, the particles are accelerated in the nozzle downstream from the point of measurement. A high recovery mode reduces the sorting speed to avoid having an unwanted cell contained in a droplet. Thus, when sorting to achieve high purity, a wide safety time window is set around the cell of interest. When an unwanted cell is measured so shortly before or after the wanted cell that it could end up in the sorted drop or drops, the sort will be aborted. Threshold or trigger settings are critical to the purity of the sorting process, as the sort logic cannot make this abort decision if the unwanted particle is not detected by the system. A threshold set to high, indicated by a straight line at the base of a population, leads to variable sort purity (Porter *et al.*, 1993). Generally, there should always be a reasonable separation between the detectable events and the trigger level. Coincident cells and aggregated cells are additional problems that cannot necessarily be removed by pulse processing due to the wide dynamic range of the signals. The only way to remove their occurrence is by disaggregation methods and spacing out the cells further either by reducing the event rate to acceptable coincidence levels or by increased transition velocity through the laser beam. Performing coincidence checks by mixing small beads with complementary fluorescence such as red and green is recommended to identify the number of double-positive beads generated by coincidence at a given sample and sheath flowrates.

The potential influence of the sort process itself on cell viability or structure can be a serious problem. Sheath containing azide or sodium fluoride can damage the cells. The effect of pressure on dissolved gases, pH, etc. has to be considered. However, lack of vital cell recovery may not always be caused by inappropriate sort settings (see Functional information). It is also conceivable that particularly already injured cells become more susceptible to handling in general, which has to be considered in the experimental design. Additionally, the sequential processing of the samples, the time needed for the sorting procedure itself as well as potential instrument drifts have to be considered. Therefore, it is good practice to run control sorts at multiple time points to verify consistency. Nevertheless, the sorting process appears to have nearly no impact on fixed or preserved cells as comparable proteome patterns of sorted and

nonsorted bacterial cells were obtained (deviation about 2%; S. Müller, unpublished data).

System cleanliness and absolute sterility are important requirements for low background noise for the measurement and for sterile sorting. In the droplet sorters, this is easiest accomplished by in-line sheath filtration directly in front of the flow cell, for example, disposable, but self-venting Sterivex^{TM} filter cartridges from Millipore for instruments operating below 40 psi sheath pressure and a thorough cleaning of tubing downstream of the filter units. Preventing the contamination of the sorted sample then still requires a clean environment in the sort chamber and appropriate aseptic handling techniques.

Regarding the biohazard considerations, most operators and manufacturers run their droplet sorters within a laminar flow cabinet to comply with the safety legislation. Nevertheless, a thorough risk assessment, a description and testing of the control measures are necessary. To appreciate the problem, the reader might want to refer to the biosafety resource material on the ISAC website featuring guidelines and presentations (http://www.isac-net.org/index.php?option=com_content&task=view&id=743&Itemid=46).

There are developments under way looking towards the improvement of flow cytometric devices. They span from commercial solutions such as multiscatter devices able to detect very small bacteria just by analysing their surface and granularity characteristics using different light scatter angles to single-cell genomics and proteomics in microfluidic chips. While some of these might not (yet) be applicable to microbiology, the microfluidics in the classical cytometer form surely are. Such approaches open the gate to systems biology, to the control and prediction of microenvironmental processes and to a multifaceted understanding of microbial ecology. An integrated microfabricated cell sorter for bacteria was presented by Fu *et al.* (2002), focusing on recovery and labelling efficiencies of bacteria.

Structural information

Structural information on bacterial cells includes all information that can be quantified by stoichiometric staining of distinct cell components with fluorescent probes after fixation or preservation. To date, this includes mainly the DNA and RNA contents, protein contents, lipophilic storage products and, to some extent, membrane composition and fluidity. Additionally, accumulation of other metabolites such as polyphosphates can also be quantified. All this information is necessary to basically understand the proliferation, growth, physiology and metabolism of bacterial cells. What is especially helpful when obtaining structural information is that fixed cells can be used, therefore providing convenient techniques to analyse cell states closely without staying day and night in the laboratory, bioreactor facility or the field.

Analysis of structural information is especially useful for bioprocess engineering whose efficiency depends ultimately on the level of understanding and control of the physiological state of the bacterial population that is exploited. Characterization of these states via fluorescence monitoring, evaluation of the data obtained and subsequent regulation of the process regime by controlling the surrounding microenvironmental conditions are among the most challenging (and important) tasks in single-cell analytics (Scheper *et al.*, 1987). Although bioprocess control is still not being widely applied at the cellular level, some processes are now being adjusted by FCM (Hewitt & Nebe-von-Caron, 2001; Müller *et al.*, 2001b; Hewitt & Nienow, 2007).

Similar studies on natural bacterial community capacities are scarce. Uncritical application of the countless fluorescent techniques in order to understand the operation of individuals within natural communities will definitively lead to misunderstanding or to only limited information. Not only does the heterogeneity of individuals of a distinct species and their ability to change cell wall structure and permeability as well as the formation of capsules, etc. need to be considered but also the variability due to the many taxa involved. The distinct structural and physiological characteristics of the involved taxa prevent joint quantitative staining approaches. Furthermore, the number of fluorescent probes that can be applied is limited, because of the small size of the bacterial cells and the need to penetrate cell walls without destroying the cells' structure. These are some of the reasons that hamper obtaining reliable information on bacterial consortia, a fact that certainly calls for novel approaches allowing the characterization of complex communities while still gripping the individual scale (see Community dynamics).

Nucleic acids

As early as 1958, Schaechter *et al.* (1958) made observations on bacterial growth and division behaviour. This was subsequently followed by the work of Donachie, Cooper and Helmstetter, who presented an extensive narrative portrayal of the pattern of DNA replication (Helmstetter, 1967, 1968, 1969; Donachie, 1968; Helmstetter & Cooper, 1968; Cooper, 1969, 1979, 1990, 2006). They showed that the time for DNA replication (the C phase) and the time necessary for cell division (the D phase) were relatively invariant in a certain species. There is also a third period B defined as the period between the birth and the initiation of the C phase. This phase reduces if growth conditions improve until these are nearly optimal for swift growth. In such a situation, B disappears. When analysed by FCM, a DNA distribution pattern evolves that is similar to the patterns always

obtained during eukaryotic cell cycle investigations. Therefore, populations behaving in such a way are reported to present eukaryotic-like cell cycle behaviour. If growth rates are even higher, DNA synthesis can be continuous during the division cycle. This behaviour is called uncoupled DNA synthesis. These events are precisely documented for *Escherichia coli* by Cooper (1991). Another phase in bacterial cell cycle progression is obvious when growth conditions are limiting. Some bacterial species have been found not to divide after finishing replication and remain at the end of the C phase until better conditions occur. Such behaviour was found in many species (Lebaron & Joux, 1994; Ackermann *et al.*, 1995; Müller *et al.*, 2000a, b; Müller & Babel, 2003); therefore, a further period was suggested within the bacterial cell cycle. The time between the end of replication and the start of the D phase was determined to be the pre-D phase (Müller, 2007). These are the basic proliferation activities that can be easily followed by the DNA patterns obtained from FCM.

Bacterial growth is related to cell cycle events (proliferation) and therefore to distinct quantities of cellular DNA. The analysis of these quantities is useful to obtain the status information of a species. Bacterial DNA quantities, besides coded information, are an effective tool for understanding the life cycle and survival behaviour of microorganisms. The DNA patterns mirror the number of chromosomes ($=n$) within individuals and the state of an individual in the cell cycle. The resulting DNA distribution patterns provide valuable information: an asynchronous growing population (a typical way of growth in nature) always contains individuals with different chromosome contents. The individuals are grouped to subsets of cells with C_n and C_{2n} chromosome contents (eukaryotic-like cell cycle) or C_{xn} (uncoupled DNA synthesis). Furthermore, the number of subpopulations and the number of individuals comprising the subpopulations create a characteristic pattern that is determined by the kind of species investigated, the kind of substrate(s) supplied as well as the microenvironmental conditions available (for a review, see Müller, 2007). This is independent of the fact that some species do not basically contain only one chromosome encoding all genetic information (without the plasmids) and many copies of it, but two or three chromosomes of different sizes and information (Trefault *et al.*, 2004; Dubarry *et al.*, 2006; Pohlmann *et al.*, 2006; Perez-Pantoja *et al.*, 2008).

To understand why cell proliferation patterns are so useful for determining bacterial physiological states, individual life strategies of these organisms need to be kept in mind; these are multiplication and survival. Under optimal microenvironmental conditions, bacteria will multiply quickly, but with a decreasing velocity, the growth conditions become more restrictive. This will alter individual numbers of chromosomes (Müller, 2007).

Many commercially available flow cytometers are limited in their suitability for analysing bacterial proliferation if they cannot discriminate the bacteria by means of light scatter as a correlating parameter or lack the light source to use the appropriate DNA probes. With a wider selection of excitation sources and probes becoming available, the situation is bound to improve in the future. Such probes should label DNA stoichiometrically and not interact with other cellular components. DAPI and probes of the Hoechst family fulfil those criteria and label A/T-rich regions of DNA in bacteria, and when excited with a multiline-UV laser, yield reliable DNA distributions, usually already at concentrations in the nanomolar and up to the micromolar range (Andreatta *et al.*, 2004; Vogt *et al.*, 2005). DAPI is bright, stable and minimally affected by DNA conformation (Button *et al.*, 1993; Schut *et al.*, 1997a, b). Additionally, this probe is easy to combine with others, which are excitable at higher wavelengths, to label further structures or functions within the bacterial cell. It was possible to analyse small 1–2 Mb genome cells from fresh- and seawater as well as low DNA cells ('dims' with an apparent DNA content of 0.1 Mb) according to their chromosome contents, after permeabilization with Triton X (Button & Robertson, 2001). The high resolution of the probe for the analysis of proliferation activity is shown in Fig. 1. *Muricauda ruestringensis* B1, a Gram-negative, marine bacterium and a member of the *Flavobacteriaceae* family, was morphologically characterized to have a pleomorphic appearance (Fig. 1a, arrow) and every so often an asymmetric cell division (Müller *et al.*, 2001a). It showed uncoupled DNA synthesis under optimal (e.g. on peptone DSM 514 medium, first row) and a eukaryotic-like cell cycle under suboptimal growth conditions (e.g. within a biofilm, third row). Depending on the kind of substrates and growth rates, several subpopulations with multiple chromosome equivalents were obvious, which visualized specific states of the population within the growth cycle. The light scatter characteristics also changed during the different growth stages and cultivation procedures (compare vertical ledger lines).

If bacteria are growing, the RNA content is much higher than the DNA content in a way that using just nucleic acid probes might provide an inaccurate indication of cell cycle activity unless RNAse treatment is performed. Nucleic acid probes include TO-PRO, the TOTO dye family (Guindulain *et al.*, 1997), EB and PI (Bailey *et al.*, 1977; Hutter & Eipel, 1979) and Sytox Green (Veldhuis *et al.*, 1997). Nucleic acid probes of the TOTO family are described to be highly sensitive to ionic strength, which is a disadvantage when applied to samples from natural environments (Marie *et al.*, 1996). Pico Green is highly selective for dsDNA as compared with RNA and is used to segregate high from low nucleic acid-containing species within natural marine communities (Fernandez *et al.*, 2008). These probes and others are well described by Shapiro (2002).

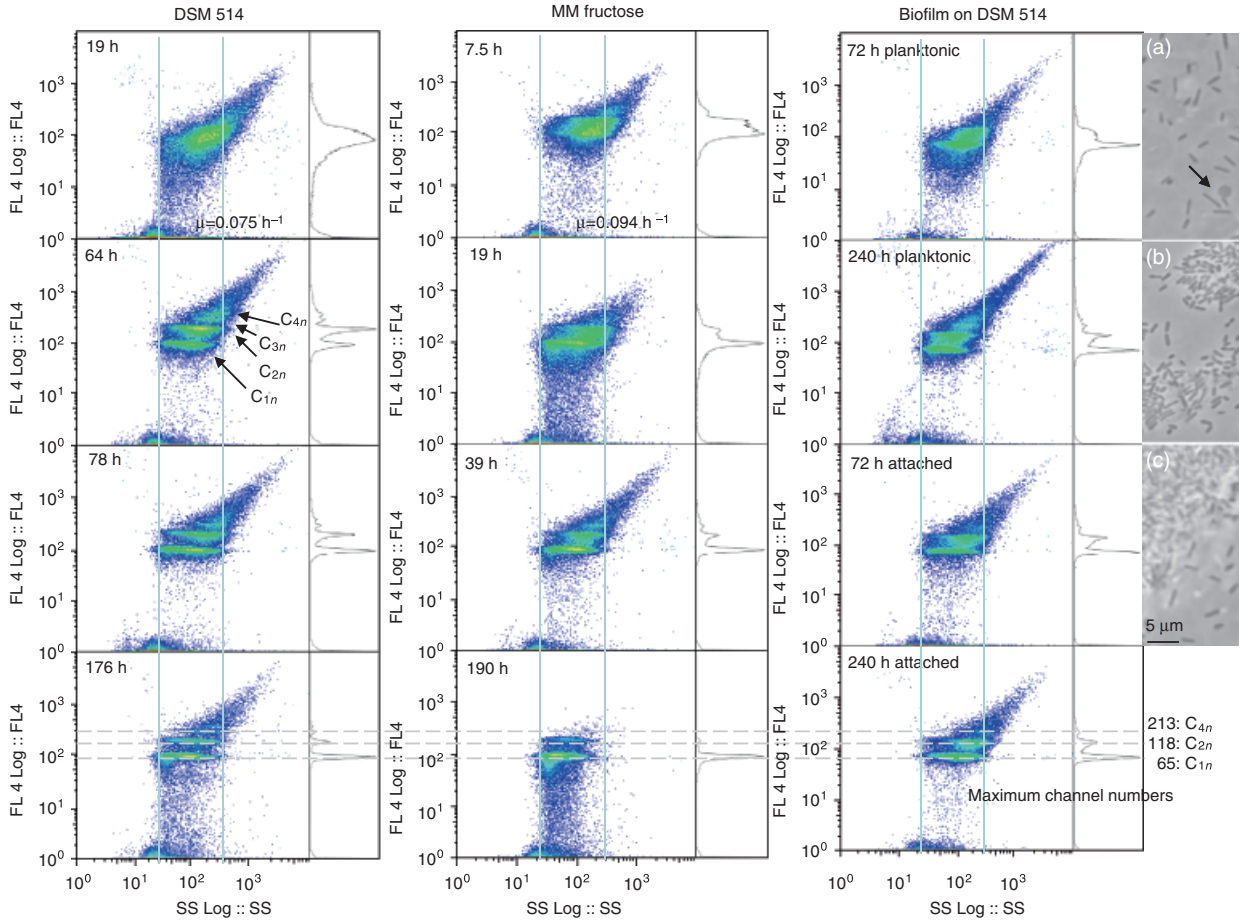

Fig. 1. *Muricauda ruestringensis* B1 was cultivated aerobically in 500-mL batches (175 r.p.m., 25 °C) on DSM 514 medium (Bacto Marine Broth) with 1 g L^{-1} yeast extract and 5 g L^{-1} peptone as carbon and energy sources (first row). The second row represents the strain grown under the same cultivation conditions; however, modified seawater bouillon X2 (minimal medium, SWB-X2) with 1 g L^{-1} fructose as a sole carbon and energy source was used. The growth rates of these batches were estimated to be $\mu = 0.199$ and 0.075, respectively. For biofilm formation, the strain was cultivated on glass slides that were placed in a glass chamber filled with DSM 514 medium and stirred for oxygen entry. The cells were harvested, treated and analysed as described by Günther *et al.*, 2009 using a MoFlo Cell Sorter (Beckman-Coulter). The .fcs-files were plotted and interpreted using the program FLOWJO (Tree Star Inc., Switzerland, *x*-axis stands for SSC, *y*-axis stands for DNA contents). The mode values of the DNA distributions point to the appearance of symmetric or asymmetric cell division. The vertical lines indicate an increase in scatter signals during exponential growth (first histograms left and middle rows). Planktonic as well as attached cells did not show much of uncoupled DNA synthesis when grown in the biofilm chamber. The phase-contrast microphotographs were obtained using an Axioskop, Zeiss, Germany; software: OPENLAB 3.1.4, Improvision, Lexington. Pleomorphic planktonic cells are shown in (a), start of biofilm growth is shown in (b). Mushroom-like structures were already observed after around 70 h of cultivation and longer, as shown in (c).

If living cells are investigated, the probes used should meet the criteria of low cytotoxicity. This is, however, easily tested by cell sorting on agar plates or into liquid media. Dyes such as Syto 13 were claimed to reduce the culturability of *Micrococcus luteus* to $< 2\%$ (Resina-Pelfort *et al.*, 2001). Hoechst 33342 was found to be useful for following cell cycle behaviour in living bacterial cells (Joux *et al.*, 1997; Shi *et al.*, 2007).

Generally, the user needs to remember that the DNA-staining techniques presented here were always elaborated for specific bacterial strains and distinct microenvironmental conditions. Calibration of every technique to the target species is the precondition for stoichiometric and quantitative analysis and interpretation of DNA patterns.

Storage materials and membrane structures

Storage materials in bacteria evolve mainly in response to restricted growth conditions or in genetically manipulated organisms with the aim of overproduction of the product of interest. A growing range of microorganisms is used in modern biotechnology industries for the synthesis of such

valuable materials, which may be pharmaceuticals, antibiotics, enzymes, metabolites and various other compounds. The efficiency in the synthesis of the compounds of interest, however, do not progress undisturbed at linear or exponential synthesis rates as it may occur in pure chemical processes (Sonnleitner, 1991). This is the point where FCM is increasingly involved in controlling biotechnological processes successfully. A population of microorganisms consists of individuals of different physiological states, each of them efficient to a certain, but different degree in producing products of interest. This means that some cells in a population may not act as an active catalyst although they are alive, whereas others are highly productive. This represents the usual heterogeneity of a bacterial population, which may be caused by different physiological cell states, by cell cycle-dependent metabolism of products and even by small genetically caused differences, which can also be the cause of plasmid loss or evolution of new substrains (Degelau *et al*., 1992).

Introducing foreign genes into bacterial cells enables the synthesis of desirable products such as proteins, lipids and a wide variety of other biologically active compounds. The heterogeneous cellular accumulation of the resulting products can be followed by analyses of light scatter, stained products of interest and proliferation activity as was already described more than 30 years ago (Scheper *et al*., 1984). As early as the mid-1980s, FCM has been used to follow plasmid propagation in recombinant organisms (Seo *et al*., 1985). In some cases, high expression rates of cloned genes, or extremely large plasmid contents, led to reductions in the FCM-analysed growth rates of the recombinant cells. It has been observed that some of the recombinant cells revert during prolonged cultivation, either (in the case of plasmids) through defective partitioning during cell division or through changes in the plasmid structure (Seo & Bailey, 1985).

FCM intracellular compound quantification such as the analyses of protein and RNA contents were found to resolve underlying physiological cell state-correlated processes in bioreactors and were used for modelling of such microbial systems (Borth *et al*., 1998; García-Ochoa *et al*., 1998; Porro *et al*., 2009). Another well-known and already heavily investigated biological synthesis is that of the hydrophobic PHB, which can be used as a precursor for diverse biocompatible plastic materials. PHB granules evolve often as a result of overflow metabolism and can easily be stained with Nile Red (Ackermann *et al*., 1995; Müller *et al*., 1995). For some species, it has been found that under growth-limiting conditions, the bacterial cells first proceed though the DNA replication program, thereby safeguarding their genetic information by doubling their chromosome contents. Others produce the compound cell cycle coupled, obviously because there are energy limitations in the metabolism (Müller *et al*., 1995, 1999). FCM was also used to identify bacteria capable of polyphosphate accumulation. The ability to store polyphosphates as granules was found to be widespread among microorganisms and is an important activity of bacteria in wastewater plants. The granules can be visualized by DAPI (Tijssen *et al*., 1982), which stains cells with polyphosphate contents $> 400\,\mu mol\,g^{-1}$ dry weight when applied at a concentration of at least 18 μM (5–50 $\mu g\,mL^{-1}$; Streichan *et al*., 1990; Klauth *et al*., 2006). DAPI staining depends on a polyphosphate-mediated metachromatic reaction, which causes a shift in the emitted fluorescence from blue to a bright yellow-green. Another dye for polyphosphates is 9-aminoacridine, which has properties similar to DAPI (Kulaev & Kulakovskaya, 2000). Recently, a novel fluorescent staining technique was developed using the antibiotic tetracycline's green fluorescence (Günther *et al*., 2009). As polyanions, the polyphosphate chains need counter ions to neutralize the negative charge, with Mg^{2+}, Ca^{2+} and K^{+} being the most important (van Groenestijn *et al*., 1988; Nelson, 1998). When tetracycline is bound to the diamagnetic divalent cations, its fluorescence intensity is enhanced, with excitation and emission maxima at 390 and 515 nm at pH 7.5, respectively (Lee *et al*., 2003).

Another information to record cell states is membrane fluidity by assessing the physicochemical vigour of the bacterial bilayer using the dye 1,6-diphenyl-1,3,5-hexatriene (DPH; Müller *et al*., 2000b). Changes in membrane fluidity are known to be important indicators of potential environmental perturbances and can be detected using such probes (Herman *et al*., 1994). However, the cell membrane incorporation of DPH molecules is very low in bacterial membranes so that movement of the phospholipids cannot be followed by polarization measurements as it was done in human and animal cells (Levanon *et al*., 1979). Hence, only the amount of dye that was able to invade the membrane was measured by FCM.

Additionally, fluorescent aliphatic probes such as nonylacridine orange can be very useful for monitoring certain properties of membranes (Petit *et al*., 1992; Mileykovskaya & Dowhan, 2000). Epand & Kraayenhof (1999) reported that physical properties of the membrane may affect the behaviour of the probe chemically. Some probes can be sensitive to the polarity of the chemical environment and it is desirable that a probe should not affect a cell membrane by its structure. Despite some limitations, such probes can be useful tools in combination with different located (and bound) quenchers or energy transfer partners. Aliphatic fluorescent probes were also used to label liposomes that encapsulated antibiotics in order to study strongly resistant *Pseudomonas aeruginosa* as the mediator of chronic pulmonary infections (e.g. Sachetelli *et al*., 2000). The application of various fluorescent phospholipids to both Gram-positive and Gram-negative bacteria is well described,

but not readily used in microbial FCM. The interesting point here is that the authors followed the topology of the probes movement over the cell membrane and their location, which is done mainly with imaging technologies (Huijbregts *et al.*, 1996; Christensen *et al.*, 1999).

Fluorescein isothiocyanate-labelled antibiotic nystatin A1 was used to follow ergosterol synthesis and membrane stability in yeast. Ergosterol is the main sterol component in membranes of *Saccharomyces cerevisiae* and is reported to be responsible for the start of proliferation and the survival of the cells under stress situations (Müller *et al.*, 1992). Other fluorescent-labelled antibiotics such as vancomycin can be used to study cell wall synthesis (Papadimitriou *et al.*, 2007).

Functional information

Functional parameters are measured on live cells. This has advantages but also serious disadvantages. An advantage is that physiological information can be obtained quasi *on line* after a very short staining procedure, which allows obtaining quick information on cell states and therefore enables the investigator to act immediately. The disadvantage is that viable bacterial cells usually have mechanisms to protect themselves against the fluorescent probes, which are regarded as hazardous compounds. This means that dye extrusion in different forms is a common phenomenon in live cell-staining procedures (see Concerning efflux pumps). Also, dyes may act toxically on bacterial metabolism and viability and therefore alter the original bacterial cell state. The investigator needs to be sure of the action of the dye. And last but not least, several techniques are available for functionality and viability measurements in bacteria; however, most of them were developed for species important in health research and biotechnology. Almost nothing is known of the behaviour of environmental isolates or members of natural communities that may react in completely different ways in comparison with established techniques in bacterial FCM. This is unlike the situation in eukaryotic single-cell research fields where the basic mechanisms of metabolism and cell structure are well understood. Therefore, if an investigator needs to follow functionality in bacteria at the single-cell level, he should carefully adapt his technique to the organism he wants to investigate. Investigation of functionality requires several controls.

For many microbiologists, the term viability is synonymous with the ability of a cell to replicate – or even worse, to replicate on an agar plate. The advantage of bacterial detection using this method with the extremely high intrinsic amplification factor is that it has provided microbiologists with an attractive tool in biological research for a long time. However, with the advance of a direct single-cell examination using cytometric measurements, the discrepancy between the existence of cells and their ability to replicate under given conditions has become increasingly apparent and stimulated the debate on the phenomenon of 'viable but nonculturable' bacteria – which in most cases are still shown to be culturable. Thus, it is important to differentiate the measurement into stages of functionality probed by the procedure in question such as reproductive growth (see Nucleic acids), membrane potential (see Membrane potential), metabolic activities such as biosynthesis and enzyme activities (see Storage materials and membrane structures, Membrane potential, Labelled substrates and Concerning efflux pumps) or membrane integrity (see Membrane integrity), as summarized in Table 1. The differential information can be obtained mainly from vital cells, but in some applications, also from dead cells. Numerous viability tests have been found to be relevant in health protection and consumer safeguarding (e.g. Berney *et al.*, 2008; Vital *et al.*, 2008) in the biotechnological industry by analysis of the physiological and metabolic states during product formation processes as well as the ability to recognize the cell death of production strains (Hewitt & Nebe-von-Caron, 2001; Hewitt & Nienow, 2007) and in medicine because of their ability to assess the susceptibility of bacteria against antibiotics. In the clinic, FCM is increasingly being used to rapidly facilitate therapeutic decisions of therapy instead of using common methods such as estimation of the minimum growth inhibitory concentration of the compounds or the reductions in CFUs after exposure. (Ordóñez & Wehman, 1993; Mason *et al.*, 1995; Braga *et al.*, 2003).

The most common and widely used techniques are the functional live/dead cell approaches, which are based on assessment of the membrane's integrity as a valuable target (Lloyd & Hayes, 1995). There are four main classes of dyes that can be used for this information. First, there are dyes that penetrate both intact and permeabilized cells. Examples are SYTO® 9 (Lebaron *et al.*, 1998a, b), Hoechst 33342 (Achilles *et al.*, 2007) and acridine orange (McFeters *et al.*, 1991). With regard to the acridine orange probe, one should be aware of the excimer state of this dye when bound to single-(red) or double-stranded nucleic acids (green), which have also been interpreted as dead (red) and live (green) staining, respectively. However, as quickly growing bacterial cells often contain high amounts of single-stranded RNA, these cells may fluoresce red despite being alive, whereas dead cells do not always degrade the DNA very quickly and therefore appear green. Second, dyes such as Sytox Blue, Sytox Green, YOYO-1, TOTO-1, TOPRO 3, ethidium homodimer-1 (EH-1) and PI (Gant *et al.*, 1993; Roth *et al.*, 1997; Comas & Vives-Rego, 1998; Williams *et al.*, 1998) are claimed to be unable to penetrate intact cells, but strongly stain permeabilized cells, which therefore are considered to be dead. As will be shown in the section on Membrane

Table 1. Overview on the basic approaches to describe viability states in bacteria in the order of stringency

Cell function	What is measured	How	Caveats	Measurable on fixed cells
Membrane integrity	The ability of the cell membrane to exclude or retain molecules	Impermeable probe with intracellular target	Can be temporarily, disturbed by stress on the membrane	No
Membrane potential proton motive force	The accumulation/dissipation of charged molecules responding to the distribution of charge across the membrane	Charged membrane-permeable anionic or cationic dyes	Interference by dye extrusion pumps, signal can also depend on cell size and availability of unspecific dye-binding sites	No
Enzyme activity	The presence of functional enzymes	Fluorogenic substrates that become fluorescent	Active extrusion systems	No
Substrate turnover	Uptake and storage of substrates	Fluorescent labelled substrates or analogues	Active extrusion systems	No
Pumps	Active transport in and out of the cell	Destaining in the presence of probes	Is low in nonactive cells	No
Cell responses	Activation of a particular pathway	Promoter-coupled fluorescent protein synthesis	Handling of genetically engineered cells	Yes
Cell division	Increase in cell number	Counting fixed volumes or against reference beads	Cells may not separate but grow in filaments	Yes
Cell proliferation	Number of chromosomes	DNA specific stain (in case of viable cells together with efflux inhibitors)	Need of UV excitation for specific UV probes	Yes

Cells may be metabolically active, but may not replicate, and productivity may be indicated by metabolic activity alone even in the absence of reproductive growth. Many of these approaches can be combined. Application of the same probes to different species may result in dissimilar information, which is due to the characteristic features of a strain or a species. Therefore, controls are mandatory.

integrity, there are many exceptions. The third group contains nonfluorescent enzyme probes that are converted into fluorescent compounds by the appropriate enzyme activities in the cells and are retained if the cells are still intact, the fluorescent products are insoluble or the extrusion pumps are inhibited (see Enzymes and Concerning efflux pumps). Fourth, dyes such as rhodamine 123 (Rh 123), bis-oxonols and carbocyanines (see Membrane potential) show charge-dependent distributions and therefore label cells under distinct, limited physiological conditions (Kaprelyants & Kell, 1992; Mason *et al.*, 1995; Comas & Vives-Rego, 1997; Nexmann Jacobsen *et al.*, 1997; Suller & Lloyd, 1999; Gauthier *et al.*, 2002; Haidinger *et al.*, 2003; Papadimitriou *et al.*, 2007; Want *et al.*, 2009). All these dyes can be combined easily and are also commercially available as kits. In order to understand the functional information conveyed by their staining behaviour, they actually have to be combined and compared directly against each other, ideally in combination with cell sorting.

Membrane integrity

Several papers have reported the use of PI as a measure of compromised membranes. As PI has been described extensively in the literature to penetrate injured membranes and cell walls, the dye is often applied to distinguish living from dead microorganisms. Nebe-von-Caron and colleagues used combinations of membrane integrity and several other DNA, membrane potential and enzyme probes to differentiate among stressed, sublethally injured or otherwise 'viable but nonculturable' cells. The combination of PI with other exclusion probes such as EB (Nebe-von-Caron *et al.*, 1998), TOPRO 3 or Sytox Green shows that these probes vary in their exclusion mechanisms, and thus convey varying information. Importantly, results can be obtained in *real time*, 1–2 min after samples are taken, enabling informed decisions to be taken about a biotechnological process (Hewitt *et al.*, 2000; Hewitt & Nebe-von-Caron, 2001, 2004). These applications have been successfully used by many other investigators. Verthé & Verstraete (2006) used PI for the analysis of a bacteriophage-infected *Enterobacter aerogenes* strain as an evaluation of phage therapy functioning. Temporary permeabilization by electroporation was also described (Garcia *et al.*, 2007).

Only a few reports are available about membrane integrity studies within environmental communities, with most of them focusing on wastewater treatment plants (Hoefel *et al.*, 2005). In some cases, the viability of environmental bacteria is described to be assessed using commercially available kits that rely on the PI-based assessment of dead cells. Usually, a combination of SYBR Green or Syto® 9 and PI is used to analyse dead cell numbers. The green nucleic

acid probes lead to energy transfer to the red PI fluorescence in case of double staining (Barbesti *et al.*, 2000). However, the results obtained can be very contradictory. Fig. 2 shows examples where sonicated drinking water samples were labelled with Syto® 13 (5 μM) and PI (15 μM; a, b) as well as Sytox Green (5 μM) and PI (15 μM; c). There is a large unspecific fluorescence of the dissolved probes (blue gate; a). For these extremely small cells, dye micelles are a serious interference problem in such samples and can give rise to double-positive events by coincidence or aggregation with the cells. Energy transfer can be observed from Syto® 13 to PI, shown by the low green fluorescence of the PI-stained cells (b). Interestingly, there seems to be neither energy transfer nor displacement between Sytox Green and PI (c) in this example even though they are both supposed to be DNA dyes, indicating different binding sites or the significantly higher affinity of the Sytox Green.

PI uptake in the presence of 5-(and-6)-caboxyfluorescein diacetate (cFDA) in compartmented *Bifidobacterium* species (Amor *et al.*, 2002) produced segregated double-stained cFDA/PI cells. Large quantities of these cells were reproducibly culturable on agar plates, a phenomenon attributed to transiently injured or dead (compartmented, probably as part of a hyphae-like morphology), but recovering, cells. Hiraoka & Kimbara (2002) managed to load *Comamonas testosteroni* with calcein-AM and PI and observed truly double-labelled cells as demonstrated by microscopy. Double-positive populations may arise either from measurement coincidences, cell aggregates, chain-forming cells or cells that are already compartmentalized (Nebe-von-Caron *et al.*, 2000; Amor *et al.*, 2002; Papadimitriou *et al.*, 2007), thus unsurprisingly producing colonies after cell sorting. To find false double-positive signals by aggregation, Syto® 9 in combination with PI will in this case produce double labelling [live (green) and compromised (red) cells aggregated] in comparison with exclusive compromised (only red by energy transfer) or live cells (only green by impermeability) to PI. Insufficient PI concentration can also give rise to double-positive populations (Barbesti *et al.*, 2000).

On the other hand, if Sytox Green and PI are combined, only dead cell staining can be expected. However, as was shown for polymyxin treatment, 78% of the cells appear positive for Sytox Green, but only 1.2% have taken up PI. While the sorted PI-positive cells yielded only one colony, 92 out of 96 sorted bacteria positive for Sytox Green grew well on the blood agar plate, illustrating the use of cell sorting to interpret membrane integrity information correctly (Fig. 3). Most people would expect uptake of exclusion probes to be a consequence of cellular injury. In *Mycobacterium fredericksbergense* and in *Shingomonas* sp., an increase in PI-stained cells was found up to nearly 50% during the early exponential growth phase (Shi *et al.*, 2007). Sorting of these cells onto agar plates revealed their culturability. The cell membrane integrity is known to be temporarily affected during periods of fast cell size growth and, as a side effect, to be particularly sensitive to a variety of antibiotics due to physical cell wall reconstruction at new cell poles or sites of cell division (Mailaender *et al.*, 2004; Cabeen & Jacobs-Wagner, 2005). Therefore, reconstruction of the cell wall during (fast) cell growth may result in a short, but temporary opening of the cell wall. Additionally, the induction of

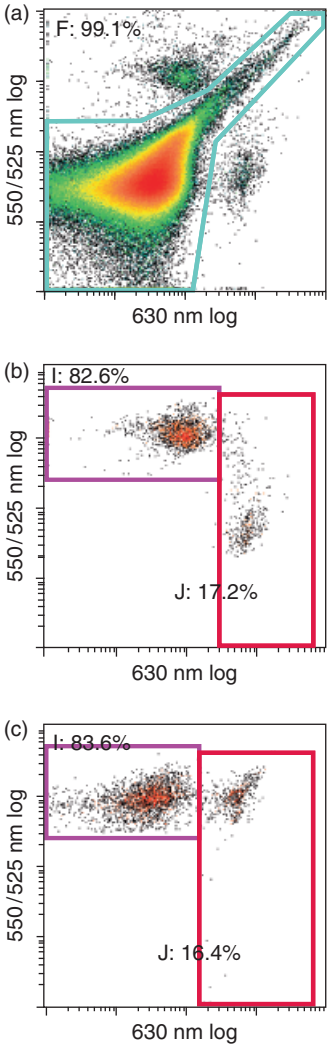

Fig. 2. Drinking water samples were labelled with Syto® 13 (5 μM) and PI (15 μM; a, b) as well as with Sytox Green (5 μM) and PI (15 μM; c). The micelle fluorescence of the dissolved probes (blue gate; a) was virtually removed by gating. Energy transfer was observed from Syto® 13 to PI, shown by the low green fluorescence of the PI-stained cells (b). Interestingly, there appears to be neither energy transfer nor displacement between Sytox Green and PI (c) in this example despite the fact that they are both supposed to be DNA dyes, indicating different binding sites or the significantly higher affinity of the Sytox Green.

porins under increasingly benefiting growth conditions can make cells susceptible to fluorescent probes.

PI might present the most stringent indicator for membrane integrity, but care has to be taken to consider the limitations of such an approach. The commercial kits were developed and validated for selected medically or biotechnologically relevant strains (Haugland, 2005) and unsurprisingly found to work satisfactorily in *E. coli* in Shi's paper on exponential growing cells with $< 2\%$ PI-stained cells. However, the utility of membrane integrity measurements as a universal indicator for bacterial cell death even with PI should not be presupposed. Temporary permeabilization effects and the impacts of solvents should always be considered (Nebe-von-Caron *et al.*, 1998), particularly in natural environments where secretion of pore formers to inhibit competing organisms is a common practice. Careful testing of the reliability of viability assays is thus recommended for their application outside of standardized procedures. Intelligent controls such as quenching or energy transfer and ultimately single cell sorting can be one way of validating the staining patterns, but even more important is the thorough standardization of the staining protocols (see Standardization).

Membrane potential

The assessment of variations in membrane potential using charged and slightly lipophilic dyes such as the carbocyanines [e.g. as $DiOC_6(3)$] allows both the bacterial cells' rapidly changing physiological behaviour and the ecotoxic potential of certain substances towards their cell membranes to be detected. It is well known that many substances and substrates are membrane-active and burden the cell energetically. Consequently, changes in the cells' energetic states can be analysed by measuring their individual membrane potential fluorescence intensities, together with solute transport, ATP synthesis and pH homeostasis (Monfort & Baleux, 1996; Müller *et al.*, 1996; Ratinaud & Revidon, 1996). The action of membrane active dyes is based on their binding to the negatively charged inner bilayer of a bacterial membrane. If a cell has sufficient membrane potential, a ring-like membrane labelling can be observed in the case of $DiOC_6(3)$. The intensity of the cells' fluorescence is not only dependent on the proton motive force but also on the total surface of the membrane. Therefore, most users estimate the membrane potential of a bacterial cell by creating a ratio of fluorescence intensity and cell size if labelled with cationic probes. If the proton motive force is not active, the cell usually shows no fluorescence intensity. However, sometimes, the fluorescence can be caused by unspecific binding of the dye to lipophilic compounds of the cell. Others (Novo *et al.*, 1999; Shapiro, 2000) have shown that more accurate measurements of membrane potential may be achieved in the case of using $DiOC_2(3)$ in determining the emission ratio of red to green fluorescence using, in this case, the potential-independent green fluorescence (due to high concentrations of the dye) to correct for variations in red aggregate accumulation. In an attempt to overcome the problems in dye extrusion pumps (see Concerning efflux pumps), the measurement of depolarization was hoped to be a suitable alternative to investigate cell function (Nebe-von-Caron *et al.*, 1998). Bis-oxonol ($DiBAC_4(3)$) was successfully used for that purpose, but seems to work better on Gram-negative cells, thus leaving the successful combination of $DiOC_6(3)$ and PI to Gram-positive bacteria. To prevent false-positive information by unspecific lipophilic binding of membrane, potentially active probes, ionophores such as valinomycin or gramicidin or others always need to be applied to verify hyperpolarization and/or depolarization caused by different concentrations of the ionophores. These are the ultimate control agents in measuring membrane potential activities. Gramicidin forms pores inside the membranes, whereas valinomycin is a lipophilic potassium-selective/proton antiport system causing hyperpolarization before the proton motive force is destroyed (Harold & Baarda, 1967). Also, valinomycin has been applied as a control agent with Rh 123 measurements. In this case, the ionophore was found to increase the Rh 123 uptake in some species, which was, however, status dependent (Porter *et al.*, 1995a).

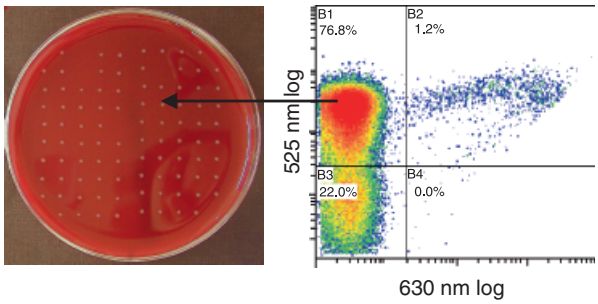

Fig. 3. Sort control for cell injury by polymyxin for a strain of *Escherichia coli* as detected by Sytox Green measured at 525 nm and PI measured at 630 nm. At the given concentration of polymyxin, 78% of the cells appear to be positive for Sytox Green, but only 1.2% have been taken up PI. PI-positive cells only yielded one colony out of 96 sorted bacteria. Ninety-two positive colonies for Sytox Green-stained cells were detected during growth on the blood agar.

Enzymes

Fluorogenic probes are often and successfully used to determine metabolic activity states in bacteria and are also frequently applied in combination with membrane integrity probes. The general principle is that they are nonfluorescent to begin with, but become fluorescent by the action of

certain intracellular enzyme activities and normally retained by their charge. Representatives are calcein-AM (Porter *et al.*, 1995a, b), fluorescein diacetate (for esterases), cFDA (Riis *et al.*, 1995; Kawai *et al.*, 1999), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, for dehydrogenases, Kaprelyants & Kell, 1992), 6,8-difluoro-4-methylumbelliferyl phosphate, fluorescein diphosphate, 4-methylumbellyferyl phosphate (DiFMUP, FDP and MUP, respectively, for phosphatases; Nedoma & Vrba, 2006; Duhamel *et al.*, 2009) and fluorescein di-β-galactopyranoside (FDG, for β-galactosidase, Nolan *et al.*, 1988; or for encapsulated *E. coli* cells in agarose microbeads: Nir *et al.*, 1990). They therefore label cells that show metabolic activity, indicating that the cells have a functional enzyme system. The pH dependence on the fluorescein probes can be a problem because of the often lower intracellular pH, which reduces the fluorescence intensity. This can be overcome by selecting another version of the dye (dichloro-carboxyfluorescein-diacetate-succinimidylester; Nebe-von-Caron *et al.*, 2000), but deliberate acidification of the extracellular pH can reduce background fluorescence from free dye and increase the stringency of the functionality information.

Sensor-based development of fluorescence is another kind of application. There is ongoing interest in microbiology to detect specific cell-associated molecules such as distinct proteins of certain functions. One way to achieve such a goal is to use reporter enzymes. For instance, the expression of foreign genes in bacterial cells, or the stability of transformed plasmids, can generally be monitored using reporter genes with FCM. Consequently, β-galactosidase-positive cells were described to exhibit a level of fluorescence that is measurable in individual cells by single-cell techniques. These systems allow FCM monitoring of the gene expression even when the gene product is difficult to assay. Some authors described a high efflux of the resulting cleaved fluorescent product after a certain time (Alvarez *et al.*, 1993). This is obviously a primary problem in single-cell β-galactosidase or other fluorescence multiplying enzyme assays where leakage of the intracellular fluorescent marker is omnipresent. A number of methods have been developed to prevent this movement (Poot & Arttamangkul, 1997). In animal cells, this efflux was avoided by treating the cells in the cold under hypotonic shock (Nolan *et al.*, 1988). To overcome an easy leakage in bacteria, lipophilic derivatives were developed. Retention of intracellular produced fluorescent dye is enhanced for example by 5-(and-6)-carboxyfluorescein succinimidylester (Ueckert *et al.*, 1997), which was selected for its ability to become covalently bound inside the cell and CTC (Walsh *et al.*, 1995; Joux *et al.*, 1997; Posch *et al.*, 1997; for anaerobic bacteria: Bhupathiraju *et al.*, 1999), which forms insoluble formazan crystals every so often inside the cell and can alter its scatter characteristics. C_8FDG and C_{12}FDG were described to penetrate cells easily because of their hydrophilic characteristics and to retain after cleavage (Miao *et al.*, 1993; Chung *et al.*, 1995).

Labelled substrates

In microbiology, there has been longstanding interest in the application of probes for visualizing specific metabolic activities in living cells. Whereas in living animal cells, the activity of metabolic enzymes has been followed by siRNA or FIVH (fluorescent in vivo hybridization) approaches (Jacobson *et al.*, 1995; Bouzakri *et al.*, 2006; Chan *et al.*, 2006; Ho *et al.*, 2006; Yanagihara *et al.*, 2006), serious difficulties limit the application to living microorganisms (Knemeyer *et al.*, 2003; Couzin, 2004; Pernthaler & Amann, 2005; Albertini *et al.*, 2006). Many molecules capable of labelling specific intracellular metabolites or larger molecules such as mRNA (e.g. antibodies or oligonucleotide probes) appear to be too large to pass microbial cell walls and membranes of living cells. Therefore, a good alternative to follow distinct metabolic activities is to apply specific fluorescent substrates as well as fluorogenic or surrogate substrates that become fluorescent after the action of specific intracellular enzymes and to follow the substrates' uptake and fate within the individual cell (Muthian *et al.*, 2000; Masami *et al.*, 2003; Achilles *et al.*, 2004, 2006, 2007). Such approaches do not compete with the typical fluorogenic probes for viability analysis as is described above (see Enzymes). In contrast to this, for example the fluorescent glucose analogue 2-NBD glucose [2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] has been used by Natarajan & Srienc (1999, 2000) to develop a much-referred method for single-cell analysis of active glucose uptake in *E. coli*. This fluorescent substrate enabled one to estimate the kinetics of substrate uptake in this organism. NBD can also be attached to other substrates. To do so, the user needs to take care of the unchanged chemical characteristics and specificity of the substrate after the labelling. This includes experiments with regard to the analysis of ongoing competitive inhibition of uptake mechanisms or of first steps in metabolism. In the case of lipophilic substrates, the octanol–water coefficient should be estimated additionally in comparison with the unlabelled substrate. Applications of NBD substrates such as toluene are currently under investigation (Sträuber *et al.*, 2010). In most cases, the fate of the NBD in the cell after uptake and/or the first metabolic step is very often unclear.

Another way to follow selected intracellular enzyme activities can be the application of fluorogenic surrogate substrates (Manafi & Kneifel, 1991). However, this is no trivial task. Günther *et al.* (2007) described the use of surrogate substrates that develop, besides chromogenic, fluorescent products or intermediates upon transformation by intracellular catabolic enzymes that are indicative of specific pathways. The resulting fluorescence intensities

were, however, too low to be detectable by FCM, the result of the action of further enzymes present in the organism or, in the range of the autofluorescence of the cells. Also, the fluorescent products are very often pumped out of the living cells; therefore, the cells need to be sealed, for example by formaldehyde, or the pumps need to be knocked out by appropriate chemicals. Last but not the least, the user needs to keep in mind that bacteria display an unimaginable broad variance of metabolic pathways that are not at all known or described to date. Applying such substrates to isolates from natural environments whose characteristics are not known can therefore be a challenging task.

Concerning efflux pumps

A clear statement needs to be made with regard to dye pumping. Bacteria contain a number of active and passive mechanisms to protect themselves against toxic assault. Passive mechanisms include increase and decrease of fluidity or stability of the cell membranes or the cell walls, which occasionally may prevent labelling of other intracellular constituents. One of the active mechanisms is the ability to immediately transport toxic compounds out of the cells (Martinez *et al*., 2009a, b). The expressions of such energy-dependent efflux pumps are known to cause antibiotic resistance (Hsieh *et al*., 1998). Molenaar *et al*. (1992) and Konings *et al*. (1997) described different excretion systems such as toxin/proton antiport systems as well as ATP-driven ABC-like excretion systems for cations and anions for some lactic acid bacteria. The efflux of dyes such as the fluorescent pH indicator BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) was found to correlate with the ATP concentration of the cells and could be prevented by orthovanadate, an inhibitor of P-type ATPases and ABC transporters. Simply adding EDTA was described to lower the activity of EB pumping in *P. aeruginosa* by dismantling the outer membrane barrier in this way (Germ *et al*., 1999). EB pumping was also used to follow physiological states and stress in *E. coli* (Jernaes & Steen, 1994). Several models have been postulated for pump functioning of multidrug transporters in bacteria to explain their broad specificity for chemically unrelated compounds (Konings *et al*., 1997). Cell substrate concentrations can be regulated via such systems. As an example, cellular toluene concentration is, among other mechanisms, reduced by a protein belonging to the resistance nodulation cell division family, HAE1 in *Pseudomonas putida* strains. An overview concerning various efflux systems is provided by Paulsen *et al*. (1996) and Saier (2000). The action of efflux pumps can be diminished or inhibited, for example, by adding verapamil, NaN_3 or the protonophore *m*-chlorophenylhydrazone. In the case of verapamil, the compound is described as competing to Hoechst 33342 via binding to the same drug interaction site (Krishan, 1987; Borges-Walmsley *et al*., 2003).

Live marker proteins

Other bioreporter systems encode detectable markers such as fluorescent proteins with their different fluorescent intensities and stability characteristics. In biotechnology, analysing the segregational instability of plasmid-containing expression systems is one of the main applications of fluorescent proteins such as the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria*. Their potential utility as markers for gene expression in a variety of bacterial strains has been highlighted by Czechowska *et al*. (2008). The mutagenesis and engineering of this protein into chimeric proteins has already been described by Roger Tsien in 1998, who provided an overview of the structure, absorbance and fluorescence properties and already a vision to molecules of altered fluorescence wavelength, which are quite commonly used nowadays (Tsien & Prasher, 1998; Campbell *et al*., 2002). An advantage of using the intrinsic fluorescent protein markers is that the sometimes complicated complex staining procedures of the bacteria can be omitted. In addition, the activities of different promoters can be monitored simultaneously using different green and red fluorescent proteins (Hakkila *et al*., 2003). Using such marker systems, the performance of heterologous expression systems can be rapidly evaluated, facilitating the optimization of screening technologies programmes or induction strategies (Patkar *et al*., 2002). Using single gene copies instead of plasmid insertions, the expression intensity of the GFP can be used to determine quantitative expression as it is no longer a question of plasmid copies. Cells can be sorted from a low and a high intensity of expression (Bongaerts *et al*., 2002). Fluorescence measurements of intrinsic proteins have also been combined with measurements of other cellular characteristics, such as light scattering, for instance by Hedhammar *et al*. (2005) in analyses of recombinant bacterial cells forming protein inclusion bodies. Applications of such biotechnologically relevant approaches at near-industrial scales are nevertheless rare, and their widespread use probably requires the development of more combined techniques for determining other bacterial structural or functional characteristics, using specific probes, in addition to fluorescent proteins. This was done in an experiment shown in Fig. 4. The sample was taken from a study of gene expression in *Bacillus cereus* pSB357 GFP-transfected strains in germination. All cells showed similar narrow forward angle light scatter behaviour (NFALS); however, they differed in the green fluorescence to a high degree. If one looks at the density plots of red (PI) vs. green fluorescence (GFP), the sample shows several clusters of

various GFP expression intensities and also cells that have already lost their membrane integrity. Looking at the single cells immediately raised several questions about plasmid stability, copy number, variation in metabolism, variation in size, signal contribution of permeabilized cells, etc. What it also shows quite obviously is the inadequacy of bulk measurements in such a case as it demonstrated that the cells under investigation were clearly heterogeneous showing differential gene expressions.

Community dynamics

Major parts of the biosphere exist as oligotrophic (nutrient-depleted) habitat, with the ocean as the environment with the highest cellular production rate. This is mainly due to the growth of oligotrophic, often extremely small bacteria that proliferate by growing slowly, even at nanomolar concentrations of growth substrates (Schut *et al.*, 1997a, b). Bacteria also predominate soil ecosystems, some of them described to obtain high-affinity, broad specificity substrate uptake systems and able to metabolize mixed substrates (Schut *et al.*, 1997a, b). Nevertheless, most of the bacteria are still unknown and their large, diverse physiology is uncharacterized. The lack of knowledge is mainly due to the difficulty in isolating them from the environment and growing them in a pure culture for closer phylogenetical, physiological and biochemical investigations (Pace, 1997).

There are not only large gaps in the description of the structure and function of environmental bacteria. The cells' performances depend on their intrinsic metabolic abilities mirrored by the activities and types of metabolic pathways and enzymes involved, which determine the substrates' flux rates through the metabolic sequences. These rates might vary depending on the concentrations and bioavailability of the chemicals and nutrients and also depending on the possible abiotic conversion of metabolites and pathway intermediates. Neighbouring microorganisms will have an important influence on flux rates because they might scavenge intermediates with a high or a low affinity, thus altering the concentration of the intermediates. Therefore, dynamic intra- and interspecies cross metabolization can be very complex.

The constantly changing mixture of substrates and intermediates makes it nearly impossible to follow the action of a distinct species, let alone the action of individuals. What is known till today about the function of bacterial communities is based mainly on studies of a few pure microbial cultures such as *P. aeruginosa* and *E. coli* (Pamp *et al.*, 2009). Yet, pure cultures are highly artificial because ecosystems always consist of different taxa that generally use different strategies to gain energy and survive. Some ongoing steps were carried out using new cultivation techniques as was done by Keller & Zengler (2004), who cultivated different

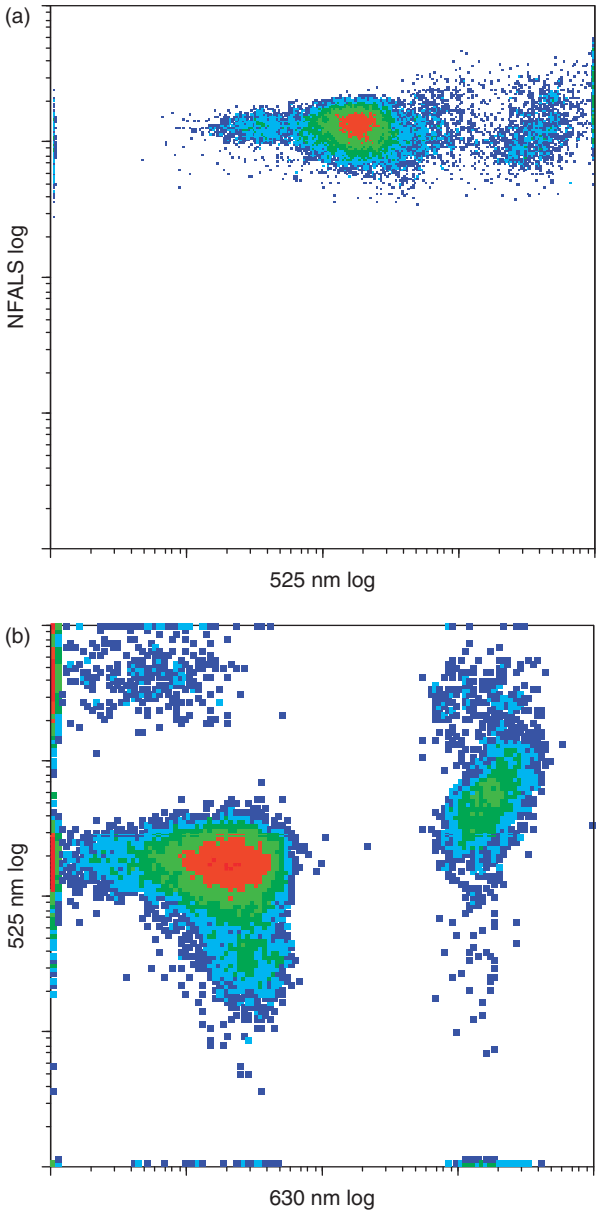

Fig. 4. GFP expression (525 nm, band pass) in germinating *Bacillus subtilis* pSB357 counterstained with PI (630 nm, long pass). The NFALS vs. GFP already indicated that three subpopulations exist, but the counterstain reveals two further PI-positive populations.

pure bacterial colonies in gel microcapsules together in columns fed with different kinds of substrates and by Ferrari *et al.* (2005) using soil substrate membrane systems.

Monitoring of natural microbial processes requires reliable and sensitive diagnostic tools for the identification and quantification of individual cells that are active *in situ*. It is widely accepted that these tools have to be cultivation-independent. However, established methods such as micro-autoradiography FISH (Roselló-Mora *et al.*, 2003), stable

isotope probing of nucleic acids or quantitative PCR detecting key genes of metabolic pathways or their transcripts (for reviews of these methods, see Radajewski *et al*., 2003; Scow & Hicks, 2005; Talbot *et al*., 2008) are often elaborate and time-consuming; especially when working with slow-growing microorganisms, they require a priori knowledge of the pathways to choose the appropriate primers and probes, and they are not quantitative with regard to the active cell numbers in a consortium. The *in situ* PCR technique, already developed by Hodson *et al*. (1995), has the disadvantage that the cells need to be permeabilized to allow the loading of the enzymes concerned. Since then, only a few further applications of the technique have been described (Porter *et al*., 1995b; Tolker-Nielsen *et al*., 1997), and to our knowledge, for FCM, no successful application has been published.

For a long time, the majority of bacteriologists have not been aware of the possibilities that FCM offers (Steen, 2000). Knowledge on natural communities using FCM can be obtained by antibody, lectin or aptamer (not yet applied for bacteria, Yang *et al*., 2003) applications; nanoparticles may also be an option in future (Akin *et al*., 2006). FISH approaches will also be discussed below. A more global picture over the ongoing dynamics of the whole community is gained when DNA/nucleic acid patterns are analysed. Studies following the nucleic acid dynamics within communities do exist (Rutten *et al*., 2005; Zubkov & Burkill, 2006), but remain a considerable challenge (Vaulot *et al*., 1995; Fuchs *et al*., 2007). Additionally, cell sorting of interesting subcommunities is a very powerful tool for resolving natural communities. Furthermore, cell sorting allows to control fluorescent-based knowledge and to yield profound information by including subcommunity-directed analyses of the submetagenomes or submetaproteomes.

Diversity resolution of communities

The morphological diversity of microorganisms is low, although many species show pleomorphic forms during different stages of the cell cycle. However, in general, the morphology cannot be used to identify bacteria reliably. On the other hand, bacteria possess a high metabolic diversity that enables them to live and proliferate under diverse and edge-cutting environmental conditions. As a result, the molecular diversity of proteins, lipids and nucleic acids of microorganisms is very high. These three classes can be used to identify bacteria on very different levels of specificity. Informative and highly conservative macromolecules such as the rRNA molecules (Woese & Fox, 1977) and even so-called signature sequences of proteins (Gupta & Griffiths, 2002) are or can be used to classify bacteria phylogenetically. In FCM, mainly two highly specific tools are used for individual microbial cell identification: antibodies or lectins that recognize and bind to specific surface epitopes or molecules and single-strand nucleic acid probes that hybridize specifically to complementary strands of target nucleic acids (DNA or RNA).

Ab labelling

Differentiation of microorganisms using FCM and antibody staining has been a widely applied technique since several decades (Ingram *et al*., 1982; Phillips & Martin, 1983, 1985; Sahar *et al*., 1983; Barnett *et al*., 1984; Libertin *et al*., 1984; Völsch *et al*., 1990). Libertin and colleagues were the first to use sorting in combination with immunofluorescence for the detection of *Pneumocystis carinii* for microscopical confirmation of the organism, a principle revisited nearly 10 years later for the analysis of *Cryptosporidium* sp. (Versey *et al*., 1993). Generally, monoclonal (very expensive) or polyclonal antibodies are used, which can be directed to specific strains or molecules by binding on the organism's surface. In the past, the binding of this first specific antibody has been visualized by adding a fluorescently labelled secondary antibody specific for the primary antibody. The advancement of antibody conjugation kits, however, has made it possible to directly label the primary antibodies easily, making it easier to perform multicolour experiments and reducing sample manipulation steps, therefore reducing additional sources of artefacts. The secret of a good antibody does not lie in the fluorescent enhancement by attachment of fluorescent enhancement systems like the biotin–strept–avidin complex or the use of enzyme substrates that precipitate [Tyramide System Amplification (TSA®) reaction], but in the careful generation and purification of the antibody.

Antibody labelling is a preferred technique to detect bacteria, fungi or protozoa in clinical, food or drinking water samples because the labelling process does not require denaturation of the cells. It is also useful when cells are present at very low concentrations (Sakamoto *et al*., 2005). It can also be used to study the expression of surface structures relevant for immune recognition or adhesion or of other functional interest. An example is the use of FCM to screen specific antibody binding to *P. aeruginosa* (Hughes *et al*., 1996). Antisera of several synthetic peptide conjugates against the outer membrane protein F (OMPF) molecules were tested with regard to their ability to bind and in this way detect the pathogen. The authors pointed out that only a third (in the best case, about 50%) of the cells were labelled and suggested a cell cycle-dependent variation of the surface-exposed epitopes of OMPF. Another study on *Helicobacter pylori* reported that the binding of both polyclonal and monoclonal antibodies against the surface-exposed heat-shocked protein 60 differed, although the immunoblotting showed an equally expressed protein in all the strains investigated. This was described to be caused by

cultivation condition-dependent surface exposure of the protein, seemingly a common behaviour that should always be kept in mind (Yamaguchi *et al.*, 1996). *Mycobacterim tuberculosis* antibody labelling potentially allowed for a more sensitive detection of changes in growth patterns (Nader *et al.*, 1991). There are also studies on the application of antibodies to investigate lipooligosaccharide distributions on *M. tuberculosis* surface epitopes that are speculated to have impacts on the virulence of the strains (Ozanne *et al.*, 1996). Aerobiological studies were performed by the detection of the aerosol-transmitted bacterium *Francisella tularensis* using monoclonal antibody binding and staining with a phycoerythrin-conjugated secondary antibody (Henningson *et al.*, 1998). In addition, the authors have determined the viability states of the pathogen by the application of Rh 123.

There are also very early descriptions of the use of FCM and antibody surface binding to *E. coli* epitopes. This organism was used as a genomic library for genes of the oil-degrading microorganism *Acinetobacter calcoaceticus* (Minas *et al.*, 1988). The stable expression of foreign antigens and their export to the outer cells' surface in the right orientation, their successful immunolabelling and the subsequent sorting of positive strains were an impressive start of more advanced approaches in microbiology at the time. Another application was the analysis of surface-expressed Fab fragments (Francisco *et al.*, 1993). Epitope mapping of antibodies using the bacterial surface expression of antigen protein fragments, followed by antibody-based flow cytometric sorting, is used currently as a systematic validation procedure of antibodies for epitope specificity and cross reactivity (Rockberg *et al.*, 2008). In this study, the Gram-positive *Staphylococcus carnosus* was used as a host for the display of peptide libraries created by random fragmentation of the PCR-amplified gene encoding the corresponding antigen – a technique that may have future potential for whole proteome-like analyses. The innovation is that not only can positive and strongly antibody-binding regions be detected but also the negative ones that react nonspecifically.

Fluorescent-labelled antibodies can also be applied to follow the abundances and dynamics of bacteria in artificial or natural consortia. However, it should be considered that antibody production and/or specificity verification is restricted to the prior cultivation of the target organism. An example for such an application is the quantification of *Nitrosomonas* serotypes in activated sludge of wastewater treatment plants. Although only 0.1–2% of this organism is present, the authors were able to detect 90% of these. This high detection level was achieved by labelling the sample simultaneously with PI. In this way, unspecific non-cell-related fluorescent events were excluded from the measurement. Because the serotypes were available as pure cultures, their signal could be used additionally as a threshold and calibration signal (Völsch *et al.*, 1990). Applying antibodies to species within mixed populations or even to communities of unknown composition means that the user needs to take special care of unspecific staining and cross reactivity. Even discrimination of well-characterized members of binary cultures was described to be hampered by cross reactivity and agglutination (Endo *et al.*, 1998). Agglutination of surface antibody-labelled bacteria may lead to double-stained false-positive signals in a histogram. In such a case, a consecutive staining and analysis procedure is recommended, which was successfully applied within a four-membered bacterial consortium able to degrade chlorosalicylate (Pawelczyk *et al.*, 2008).

Antibody binding to intracellular structures and functional genes is not widely used in bacterial FCM. This may be due to the loss of a healthy scatter signal after a rigorous treatment of the cell wall/membrane to induce entry of the large protein molecules into the cell necessary for signal enhancement. However, a healthy scatter trigger signal is required to separate a population or a community from the noise signal of the flow cytometer. Nevertheless, antibody-surface-stained cells can be combined relatively easily with the labelling of further intracellular structures such as DNA and storage material contents. Quenching or FRET mechanisms can be avoided in this case. Because proliferation provides information on the growth and, therefore, on the metabolization of substrates or intermediates, strain differentiation by antibody labelling combined with proliferation activity is described to enable substrate routes through bacterial artificial consortia (Pawelczyk *et al.*, 2008).

Lectin labelling

Lectins have specificities to surface sugars such as *N*-acetylglucosamine (e.g. *Tritium vulgaris* agglutinin, WGA), *N*-acetylgalactosamine (e.g. *Glycine max* agglutinin, SBA), galactose (e.g. *Griffonia simplicifolia* I agglutinin, GSI), glucose (e.g. *Pisum sativum* agglutinin, PSA) and mannose (e.g. *Canavalia ensiformis* agglutinin, Concanavalin A). Lectins can be conjugated with all fluorescent dyes that are suitable for direct protein detection. Agglutination studies between the lipopolysaccharide layer of the bacterial outer membrane and the labelled lectins are necessary in the first place to verify the specificity of the staining. Inhibition of agglutination should be proved by the addition of free partner sugars of the respective lectins or the addition of divalent cations. Also, various lectins may agglutinate with different strengths to the same sugar class. Many bacterial species have different sugar residues on the cell surface such as nitrogen-fixing bacterium *Azospirillum brasilense*, which was described to bind nearly all of the above-described lectins (Yagoda-Shagam *et al.*, 1988). Additionally, the presence of sugar residues on the bacteria's surface may

depend on the growth conditions, influencing therefore the agglutination strength and the intensity of the label in the end (Heine *et al.*, 2009). Lectins were also used to differentiate Gram-positive and Gram-negative bacterial strains within a binary culture that grew in interdependence of a shared substrate (Müller *et al.*, 2000a, 2002). Specific binding of labelled lectins isolated from the serum of mammals was used for virulence studies of changes in pathogen surfaces (Devyatyarova-Johnson *et al.*, 2000).

16S rRNA gene labelling

The application of fluorescently labelled 16S rRNA gene-targeted oligonucleotide probes (by FISH) to microbial cells is a widely used and well-established technique for the differentiation and abundance analysis of microorganisms in mixed culture or of uncultivable microorganism within communities. These probes allow specific phylogenetic identification of microorganisms starting from a domain via a phylum or a class down to a genus or a species. These synthetic nucleic acid probes have several advantages. Large rRNA sequence databases are available publicly and the identification of target regions on the rRNA molecules is facilitated by comparative sequence analysis (for a review, see Amann & Kühl, 1998). With regard to single-cell studies, the 16S rRNA gene molecule is interesting because it is amplified and therefore provides a natural enhancement of a fluorescent signal when targeted. *Escherichia coli* is described to contain 10^4–10^5 copies of 5S, 16S and 23S rRNA genes during the exponential phase of growth (Bauman & Bentvelzen, 1988). This number may be different from species to species and is additionally strongly dependent on the state of the cell in the cell cycle and the growth rate (DeLong *et al.*, 1989). Additionally, this number can be influenced by certain stress conditions. Under nutrient and substrate limitation or depletion, the signal may not be detectable because of the low cellular numbers of the rRNA. This is the usual state in natural environments where the cells do not have continuous access to carbon and energy resources. Therefore, the 16S rRNA gene signal can be reliably used to quantify desired groups of organisms growing under well-supplied microenvironments. In established artificial systems, it is even possible to obtain information on activity states (Herrmann *et al.*, 1997). Gray *et al.* (2000) used the FISH approach to label *Achromatium* populations with a bright fluorescence, which was verified by simultaneous monitoring of selectively metabolized radiolabelled substrates. Ecological and niche evolution studies can be performed using such approaches, which, however, are still limited to microscopic observations.

These limitations led to the development of a set of new labelling techniques to increase the detection level of bound probes. The start was led by Amann *et al.* (1990), who applied several probes of the same specificity and carrying the same fluorochrome, but binding to different regions of the target rRNAs that increased the signal. Wallner *et al.* (1993) introduced dual-label approaches into microbial FCM, which allowed differentiation of distinct populations as well as the combination of universal and specific rRNA-targeted probes. The universal probe was thought to function as a positive control to quantify the cell's contents of accessible ribosomes. In this way, loss of bacterial identification because of low ribosome content or limited cell permeability can be determined and excluded when supplied together with a general cell stain such as Syto® 9 or DAPI. Wallner and colleagues also reported that labelling of one oligonucleotide probe with several fluorochromes showed elevated levels of unspecific binding, a problem that seems to be solved now by a new microscopic approach from Stoecker *et al.* (2010). Another technique to enhance the signal-to-noise ratio slightly was achieved by application of digoxigenin-labelled, rRNA-targeted probes (Zarda *et al.*, 1991). Application of the catalysed fluorescent reporter deposition (CARD)-FISH or the TSA® techniques, both combined with horseradish peroxidase-labelled oligonucleotides, was described to increase the sensitivity at least a further order of magnitude (Schönhuber *et al.*, 1997; Sekar *et al.*, 2004). It should be mentioned that for whole-cell hybridization, the penetration of these large marker molecules through the cell wall is required, which may affect quantitative cell analyses. Sometimes, Cy3-labelled probes have been shown to mark higher percentages of labelled cells compared with TSA® methods (Schönhuber *et al.*, 1997). Nevertheless, penetration of the cell wall may also be a problem for the dye-labelled probes. In the case of the Gram-positive filamentous bacterium *Microthrix parvicella*, which frequently causes activated sludge bulking and foaming, an enzymatic treatment was necessary (Erhart *et al.*, 1997). The FISH techniques can be combined with further single-cell parameters to differentiate bacteria from debris that originates from natural soil or water sources. Clear discrimination of fixed and FISH-labelled cells from soil debris was described to be achieved down to 3×10^4 cells g^{-1} soil by additional application of EB (Thomas *et al.*, 1997). Another TSA®-FISH-based technique for cell separation from natural communities is Magneto-FISH, which, however, is not yet flow cytometrically applicable. Here, antibodies directed against the TSA®-metabolite and magnetobeads are used for small bacterial aggregate separation (Pernthaler *et al.*, 2008).

The three-dimensional structure of the ribosome may hinder the access of the oligonucleotides to their target sites and lower the resulting fluorescence signal to a high degree. The first systematic study with > 200 overlapping adjacent probes targeted to an *E. coli* strain was performed by Fuchs *et al.* (1998). The authors generated a map of accessibility of

the 16S rRNA gene for fluorescently labelled probes, applying standardized conditions and determining the intensity of the fluorescence of the bound probes by involving FCM. Also, eukaryotic microorganisms were identified using *in situ* hybridization techniques. For a small phytoplankton, with a small size down to 0.2–2 μm, 18S rRNA gene-targeted probes were developed and their specific binding was verified by FCM (Simon *et al.*, 1995). The authors described the sensitivity of the probes to be influenced by the cell size and the state of growth. They also reported a high fragility of the cells dependent on sample handling and found that ethanol, detergent and formamid treatment affected the red (chlorophyll) autofluorescence.

Key functions within communities

The diversity of the metabolic capacities of bacteria is astonishing. However, analysis of functionality on the single-cell level is problematic, as the small microbial cells contain only low amounts of metabolites or proteins active in a distinct metabolic or protection process. To overcome such limits, there are attempts to describe microbial diversity in nature by systems biology on the single-cell level (Slonim *et al.*, 2006). Although potential functional information is already available through increasing number of databases of sequenced genomes of microorganisms, it is essential to quantify these coded functions and to analyse the expression of their products in single cells.

Functionality by structural probes

In community studies, FCM is unparalleled in providing knowledge regarding bacterial activity states that lie hidden behind individual bacterial nucleic acid quantities. Staining of nucleic acid or DNA quantities results in cluster distributions with changing cell quantities in response to changing environmental conditions. The resolution of a community is higher for DNA-directed staining in comparison with nucleic acid targeting. Nucleic acid dyes such as Syto® 13 are frequently used to follow community dynamics. Most of the time, two different subcommunities appeared that are called 'low nucleic acid' (LNA) and 'high nucleic acid' (HNA) bacteria and are differentiated according to their respective low and high nucleic acid contents (Gasol *et al.*, 1999). There are also studies available that described the sorting of such subcommunity clusters determining the phylogenetic affiliation of the contained individuals after the sorting procedure (Sekar *et al.*, 2004). Another approach sorted LNA and HNA cells on filters and applied CARD-FISH afterwards to determine the phylogenetic affiliation for microscopic observation (M. Schattenhofer, pers. commun., MPI Bremen, Germany). DNA-related staining, especially in the case of DAPI labelling, produces high-resolution subcommunity patterns. Up to 20 different subcommunities were differentiated by FSC- vs. DNA-based clustering (Günther *et al.*, 2009). Such clusters can also be sorted and phylogenetically investigated for communities originating both from aerobic and from anaerobic environments (Kleinsteuber *et al.*, 2006; Müller *et al.*, 2010). DNA-based clustering can also be achieved by analysing the molar percentage of guanine-plus-cytosine (GC) contents using Hoechst 33258 (AT specific) and Chromomycin A3 (GC specific; Sanders *et al.*, 1990). The application of this technique requires knowledge of the GC content of the species involved and two-laser excitation (UV for the Hoechst dye and blue for Chromomycin A3).

The DNA clustering can be combined with the analysis of further intracellular contents, which provides information on specific abilities of bacteria and also about their physiological states. Such an investigation is the labelling of the polyphosphate-accumulating microorganisms (PAOs) by labelling the polyphosphate content with tetracycline additional to the DNA contents. By doing this, the authors were able to cluster active tetracycline-labelled PAOs within the DNA community distribution patterns as well as determine their phylogenetic affiliation after cell sorting and follow their dynamics quantitatively (Günther *et al.*, 2009). DNA patterns can also provide information on the composition and stability of microbial consortia if low species numbers are involved and their proliferation patterns are known. In such a case, DNA-based patterns are useful to specifically address mutualistic, commensalistic or parasitic interactions (Vogt *et al.*, 2005).

Functionality by vital probes

Flow cytometric characterization of living microorganisms have been successfully applied to environments such as the oral cavity (Nebe-von-Caron, 1996) or enrichment cultures from lake samples (Johnson *et al.*, 2009) using PI as an integrity marker. Nevertheless, the application of any functional probe is a challenge. Although FCM is able to identify cells within microbial communities that share distinct physiological characteristics visualized by specific fluorescence labelling, the technique cannot reliably link these properties with the metabolic state of the individual cell. Such linkages, however, increase knowledge of microbial community functioning. Combining FCM with cell sorting to separate physiologically distinct subsets of cells, followed by phylogenetic or proteomic approaches is a powerful tool to gain an insight into community functioning.

Cell sorting and cytomics

Successful cell sorting of bacteria was probably first described by Paau *et al.* (1979), who separated algae from bacteria. Years later, the technique was used to separate and

identify individuals by cell sorting and subsequent phylogenetic analyses, as reported (Porter *et al.*, 1993). This concept was, at the time, intensively used in some areas of microbiology (Wallner *et al.*, 1997; Nielsen *et al.*, 2004; Park *et al.*, 2004; Mou *et al.*, 2005), and was successfully shown for the diversity of faecal microbiota in a cross-sectional study (Mueller *et al.*, 2006), for preselection of key subcommunities for phylogenetic affiliating (Kleinsteuber *et al.*, 2006; Müller *et al.*, 2009) and metagenomic sequencing (Palenik *et al.*, 2009). However, diversity investigations provide only a limited idea regarding what may happen in a bacterial community and why. This is different when further 'omics' technologies are involved.

The concept of cytomics (Valet, 2005; Bernas *et al.*, 2006) combines single-cell characterization on the basis of structural as well as functional information and the cells' contribution to the communities' state (or in the case of organism integrated eukaryotic cells: the study of complex and dynamic cellular systems starting with the single cell as its reference point; for a review, see Smith *et al.*, 2009). The acquisition, combining and interpreting of multiparameter data from individual cells can provide an understanding of the behaviour and state of a cell in a system using this information to develop predictive models in systems biology. In the above chapters, structure- and function-related markers were discussed, which focused on the characteristics and physiological states of microbial cells. To date, the main advantage of bacterial flow sorting implies separation of either single cells or large cell numbers of uncultivable bacteria out of a community and address them afterwards in various ways (see Fig. 5, flow chart). Applications are described with regard to the screening of pathogenicity factors using GFP-marked sensor strains in human tissue (Becker *et al.*, 2006; Bumann & Valdivia, 2007) and to detection of metabolically active species within communities (Kalyuzhnaya *et al.*, 2008; Günther *et al.*, 2009). Cell sorting of individual uncultivable microbial cells in combination with single-cell DNA sequencing was described to generate reference genomes of uncultured taxa from a complex microbial community and used to analyse biogeography and metabolic adaptation by comparing metagenomic data records (Stepanauskas & Sieracki, 2007; Woyke *et al.*, 2009). These techniques can provide considerable amounts of information; however, they may still miss certain knowledge. Individual-based molecular information can also be obtained by involving cell sorting, providing cell material for succeeding bulk techniques. In this view, bacterial flow sorting is to be seen just as a step between two or more bulk experiments to increase the resolution of the community that is practically disintegrated in this way. Microarray techniques are an approved tool in microbiology to study gene expression levels (Overton *et al.*, 2008). Recently, the so-called GeoChip has been developed containing 24 243 oligonucleotides (50-mer) probes covering genes involved in nitrogen, carbon, sulphur, phosphorus cycling, etc. (He *et al.*, 2007). However, analyses on the mRNA basis after cell sorting are still limited to higher organism levels (Achilles *et al.*, 2007). Transcriptome investigations of sorted subpopulations or subcommunities using low- or high-density microarrays or RT-PCR are hampered by the low half-time of the bacterial mRNA. This is different for proteomic approaches that proved to be successful, although large cell numbers needed to be sorted. One such application is the study of sorted subpopulations of *Cupriavidus necator* JMP 134 stressed by high-toxic phenol concentrations. This bacterium is a model organism for chloroaromatic biodegradation and its impressive versatility is well known (Perez-Pantoja *et al.*, 2008). Based on the DNA pattern, flow cytometric sorting in combination with proteome analysis of various upcoming subpopulations has been performed to analyse diverse protein expression levels in the sorted subpopulations. Wiacek *et al.* (2006) found down- as well as upregulated proteins that are involved in adaptation mechanisms against the stress trigger. The cell sorting for the proteomic approach was tedious due to the high number of bacterial cells (10^9) required in comparison with animal or blood cells (10^5–10^6, Bernas *et al.*, 2006) for reproducible and reliable 2-DE gel and the subsequent nano-LC tandem MS analyses. Recently, global gel-free proteomic analyses by shotgun MS have been widely used as an important tool for exploring biological bacterial systems (Bosch *et al.*, 2008; Mastroleo *et al.*, 2009). Modern MS instruments have, owing to recent development, the potential to analyse a huge number of peptides that are extremely sensitive, decreasing the number of cells necessary to 10^6 for bacteria. Using high-throughput MS approaches, it is possible to explore their specific physiological state in combination with sorted bacterial subpopulations and/or subcommunities (unpublished data). However, simultaneous analysis of a large number of proteins is a challenging task throughout all stages of gel-free proteomic analysis: experimental design, peptide/protein identification, computational data preprocessing, normalization and inferential analysis (Nie & Siebert, 2008). Cytomics and proteomics provide completely new prospects for precise insights into protein expression levels, their interactions and dynamic behaviours in subpopulations and/or subcommunities.

Standardization

Cytometric analyses are moving into the focus of many scientific areas. The direct indication for this is the spread of many national and international systems biology calls that inevitably contain single-cell analysis approaches. That means that scientists will start with single-cell analysis without having much experience on how to analyse, evaluate and interpret

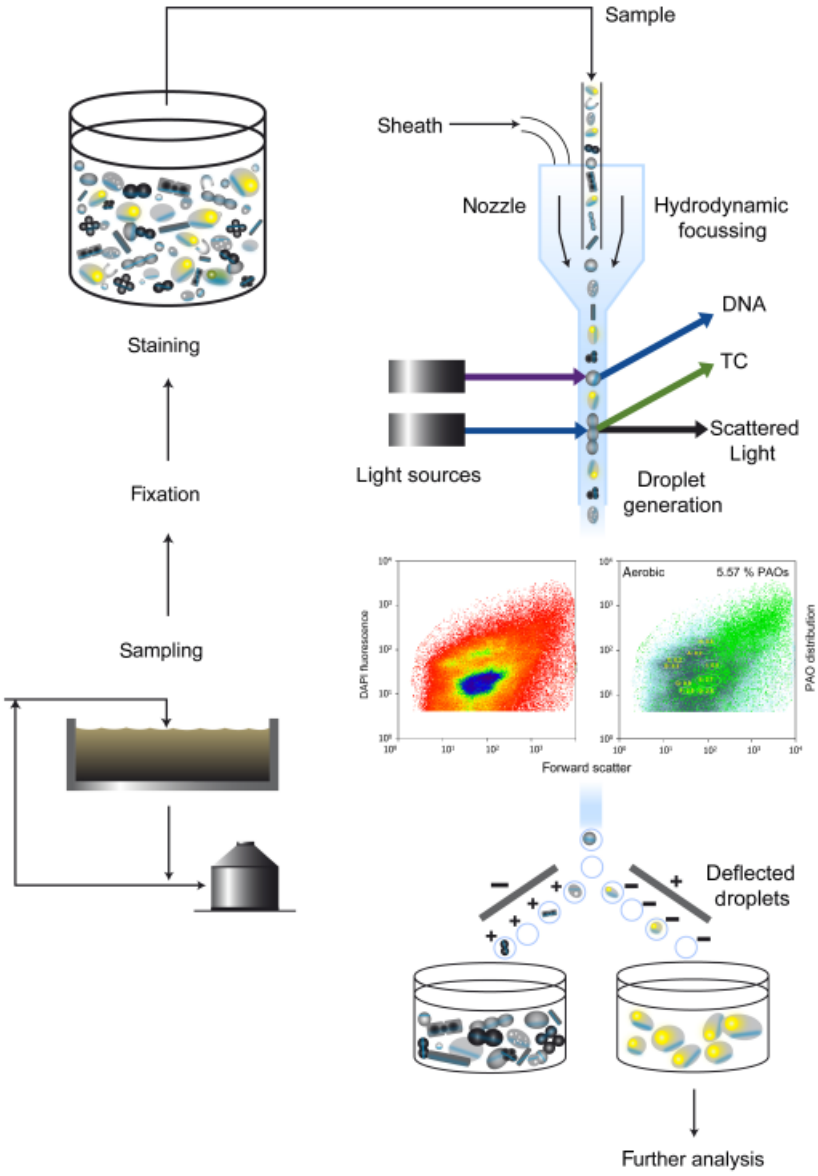

Fig. 5. Flow chart of a typical bacterial cell handling until separating them by cell sorting for further phylogenetic analysis. The samples were (e.g.) harvested from a wastewater treatment plant. Afterwards, the cells were treated according to the respective protocols and stained with the relevant probes. In this case, it was DAPI for DNA contents (blue marker) and tetracycline for polyphosphate contents (yellow marker). Then, all cells were analysed flow cytometrically. The scheme shows a cytometer equipped with a stream in an air flow chamber and a hydrodynamic focusing. The 2D plots show the patterns of DNA (with dominant subcommunities marked with white gates) and PAO distributions [green dots and numbers within the gates (%)] of cells double stained with DAPI and tetracycline. Cell sorting of the yellow fluorescent cells was performed using a two-way sort option in this case. The most accurate sort mode (single and one-drop-mode: highest purity 99%) was chosen for separating up to 5000 cells s^{-1}. The separated cells were centrifuged (12 000 ***g*** for 10 min at 4 °C) and deep-frozen until further phylogenetic analyses. The most frequent phylotype found belonged to the *Rhodocyclaceae* among them those affiliated to the '*Candidatus* Accumulibacter' lineage. The second most frequent phylotype was assigned to the genus *Pseudomonas*. A third group was a phylotype belonging to the *Gammaproteobacteria* (Günther *et al.*, 2009).

such data. The first scientists who came up with a proposal for experimental standardization were those dealing with microarray-based transcriptomics. Their consensus on the Minimal Information about a Microarray Experiment (MIAM) was the first to be published, back in 2001 (Brazma *et al.*, 2001). Since then, many groups started developing guidelines for their field. Presently, $>$ 20 such guidelines are either under development or have already been published (Taylor *et al.*, 2008). This includes MIs for cellular assays (MIACA), genome sequence (MIGS), RNA interference (MIARE) and, finally, for FCM (MIFlowCyt) (Lee *et al.*, 2008).

Additional complications might arise if cells that are on the limits of cytometric resolution such as bacteria are analysed. A long time ago, scientists involved in single-cell analysis attempted to establish rules for reliable analyses and data assessments. However, only a few authors were really interested in strictly utilizing guidelines on how to perform single-cell analysis. Now, with the spread of interdisciplinary research topics, the pressure for obligatory guiding principle has become vital again. Setting up the instrument to obtain stable results free of interference and with sufficient sensitivity to ensure all particles are detected is perhaps the first aim of a standardization effort. Sample preparation, measurement conditions and the data analysis are additional targets. The correlation of fluorescent signals with light scatter is one of the basic forms of data display revealing potential problems of aggregates, etc. (for an overview, see Nebe-von-Caron, 2009).

Conclusion

To understand the heterogeneity of populations and microbial-driven ecology in natural environments, new techniques are required for answering questions that could not be addressed by traditional methods alone (Lozupone & Knight, 2008; Durot *et al.*, 2009; Wilmes *et al.*, 2009).

Flow cytometric analysis and cell sorting of single cells, subpopulations or subcommunities, especially of ones not yet culturable or those that drive biotechnical facilities, are now increasingly recognized as valuable tools for microbiologists that provide unique opportunities to investigate and quantify various aspects of microenvironmental/biotechnical processes. The multitudinous operations of microorganisms can be followed in this way if combined with appropriate and sophisticated cell handling protocols, which are essential for the acquisition of both qualitative and quantitative information. Mining genes and transcriptomic as well as proteomic approaches are important additions to the concept of cytomics on the microbial scale and will allow an in-depth analysis of pathway regulation or the concurrence of the microbial cells within populations or complex consortia to be carried out. Of paramount importance now is the creation of comprehensive and capacious genomic and proteomic databases that also contain, besides information on well-known biotechnologically (Martinez *et al.*, 2009a, b) used strains or intensively investigated pathogenic germs (Ambur *et al.*, 2009), information on the massive amount of uncultivable bacteria. This implies the need to develop approaches for administering the huge single-cell data profiles in a form that can be understood and efficiently accessed by the user community. Here, the application of new biological and mathematical concepts should provide valuable contributions towards the understanding of microbial life. Last but not the least, high-resolution, but easy-to-handle and cheap cytometers (or imaging systems) and cell sorters that sort high cell numbers with high purity very quickly will be the precondition to the admission of these powerful techniques in the daily routine of a microbiological laboratory.

Acknowledgements

We would like to thank Nico Jehmlich and Dagmar Fiedler and the members of the labs for comments and suggestions on the manuscript. The research programme 'Biodiversity and bioprocess control' from the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany, is acknowledged for financial support.

References

Achilles J, Müller S, Bley T & Babel W (2004) Affinity of single *S. cerevisiae* cells to 2-NBD glucose under changing substrate concentrations. *Cytom Part A* **61A**: 88–98.

Achilles J, Harms H & Müller S (2006) Analysis of living *S. cerevisiae* cell states: a three color approach. *Cytom Part A* **69A**: 173–177.

Achilles J, Stahl F, Harms H & Müller S (2007) Isolation of intact RNA from cytometrically sorted *Saccharomyces cerevisiae* for the analysis of intrapopulation diversity of gene expression. *Nature Prot* **2**: 2203–2211.

Ackermann JU, Müller S, Lösche A, Bley T & Babel W (1995) *Methylobacterium rhodesianum* cells tend to double the DNA content under growth limitations and accumulate PHB. *J Biotechnol* **39**: 9–20.

Akin D, Sturgis J, Ragheb K, Sherman D, Burkholder K, Robinson JP, Bhunia AK, Mohammed S & Bashir R (2006) Bacteria-mediated delivery of nanoparticles and cargo into cells. *Nat Nanotechnol* **2**: 441–449.

Albertini MC, Accorsi A, Teodori L, Pierfelici L, Uguccioni F, Rocchi BL, Burattini S & Citterio B (2006) Use of multiparameter analysis for *Vibrio alginolyticus* viable but nonculturable state determination. *Cytom Part A* **69A**: 260–265.

Allemand J-F & Maier B (2009) Bacterial translocation motors investigated by single molecule techniques. *FEMS Microbiol Rev* **33**: 593–610.

Alvarez AM, Ibanez M & Rotger R (1993) β-Galactosidase activity in bacteria measured by flow-cytometry. *Biotechniques* **15**: 974–976.

Álvarez-Barrientos A, Arroyo J, Cantón R, Nombela C & Sánchez-Pérez M (2000) Applications of flow cytometry to clinical microbiology. *Clin Microbiol Rev* **13**: 167–195.

Amann R & Kühl M (1998) *In situ* methods for assessment of microorganisms and their activities. *Curr Opin Microbiol* **1**: 352–358.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microb* **56**: 1919–1925.

Ambur OH, Davidsen T, Frye SA & Balasingham SV (2009) Genome dynamics in major bacterial pathogens. *FEMS Microbiol Rev* **33** (Special Issue): 453–470.

Amor KB, Breeuwer P, Verbaarschot P, Rombouts FM, Akkermans ADL, De Vos WM & Abee T (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Appl Environ Microb* **68**: 5209–5216.

Andreatta S, Wallinger MM, Piera J, Catalan J, Psenner R, Hofer JS & Sommaruga R (2004) Tools for discrimination and analysis of lake bacterioplankton subgroups measured by flow cytometry in a high-resolution depth profile. *Aquat Microb Ecol* **36**: 107–115.

Avery SV (2006) Microbial cell individuality and the underlying sources of heterogeneity. *Nat Rev Microbiol* **4**: 577–587.

Bailey JE, Fazel-Madjlessi J, McQuitty DN, Lee LY, Allred JC & Oro JA (1977) Characterization of bacterial growth by means of flow microfluorometry. *Science* **198**: 1175–1176.

Banks CJ & Walker I (1977) Sonication of activated sludge flocs and the recovery of their bacteria on solid media. *J Gen Microbiol* **98**: 363–368.

Baquero F & Lemonnier M (2009) Generational coexistence and ancestor's inhibition in bacterial populations. *FEMS Microbiol Rev* **33**: 958–967.

Barbesti S, Citterio S, Labra M, Baroni MD, Neri MG & Sgorbati S (2000) Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. *Cytometry* **40**: 214–218.

Barnett JN, Cuchens MA & Buchanan W (1984) Automated immunofluorescent speciation of oral bacteria using flow cytometry. *J Dent Res* **63**: 1040–1042.

Bauman JG & Bentvelzen P (1988) Flow cytometric detection of ribosomal RNA in suspended cells by fluorescent *in situ* hybridization. *Cytometry* **9**: 517–524.

Becker CL, Parker JW & Hechinger MK (2002) *Is Forward Scatter Monotonic on Commercial Flow Cytometers?* ISAC, XXI Congress, San Diego, CA.

Becker D, Selbach M, Rollenhagen C, Ballmaier M, Meyer TF, Mann M & Bumann D (2006) Robust *Salmonella* metabolism limits possibilities for new antimicrobials. *Nature* **440**: 303–307.

Bernas T, Grégori G, Asem EK & Robinson JP (2006) Integrating cytomics and proteomics. *Mol Cell Proteomics* **5**: 2–13.

Berney M, Vital M, Hülshoff I, Weilenmann HU, Egli T & Hammes F (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Res* **42**: 4010–4018.

Betz W, Aretz W & Härtel W (1984) Use of flow cytometry in industrial microbiology for stain improvement programs. *Cytometry* **5**: 145–150.

Bhupathiraju VK, Hernandez M, Landfear D & Alvarez-Cohen L (1999) Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria. *J Microbiol Meth* **37**: 231–243.

Boenigk J (2004) A disintegration method for direct counting of bacteria in clay-dominated sediments: dissolving silicates and subsequent fluorescent staining of bacteria. *J Microbiol Meth* **56**: 151–159.

Bongaerts R, Hautefort I, Sidebotham J & Hinton J (2002) Green fluorescent protein as a marker for conditional gene expression in bacterial cells. *Method Enzymol* **358**: 43–66.

Borges-Walmsley MI, McKeegan KS & Walmsley AR (2003) Structure and function of efflux pumps that confer resistance to drugs. *Biochem J* **376**: 313–338.

Borth N, Mitterbauer R, Mattanovich D, Kramer W, Bayer K & Katinger H (1998) Flow cytometric analysis of bacterial physiology during induction of foreign protein synthesis in recombinant *Escherichia coli* cells. *Cytometry* **31**: 125–129.

Bosch G, Skovran E, Xia Q, Wang T, Taub F, Miller JA, Lidstrom ME & Hackett M (2008) Comprehensive proteomics of *Methylobacterium extorquens* AM1 metabolism under single carbon and nonmethylotrophic conditions. *Proteomics* **8**: 3494–3505.

Bouzakri K, Zachrisson A, Al-Khalili L, Zhang BB, Koistinen HA, Krook A & Zierath JR (2006) siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. *Cell Metab* **4**: 89–96.

Braga PC, Bovio C, Culici M & Dal Sasso M (2003) Flow cytometric assessment of susceptibilities of *Streptococcus pyogenes* to erythromycin and rokitamycin. *Antimicrob Agents Ch* **47**: 408–412.

Brazma A, Hingamp P, Quackenbush J *et al.* (2001) Minimum information about a microarray experiment (MIAME) – toward standards for microarray data. *Nat Genet* **29**: 365–371.

Buesing N & Gessner MO (2002) Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. *Aquat Microb Ecol* **27**: 29–36.

Bumann D & Valdivia RH (2007) Identification of host-induced pathogen genes by differential fluorescence induction reporter systems. *Nature Prot* **2**: 770–777.

Button DK & Robertson BR (2001) Determination of DNA content of aquatic bacteria by flow cytometry. *Appl Environ Microb* **67**: 1636–1645.

Button DK, Schut F, Quang P, Martin R & Robertson BR (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl Environ Microb* **59**: 881–891.

Cabeen MT & Jacobs-Wagner C (2005) Bacterial cell shape. *Nat Rev Microbiol* **3**: 601–610.

Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA & Tsien RY (2002) A monomeric red fluorescent protein. *P Natl Acad Sci USA* **11**: 7877–7882.

Chan SM, Olson JA & Utz PJ (2006) Single-cell analysis of siRNA-mediated gene silencing using multiparameter flow cytometry. *Cytom Part A* **69A**: 59–65.

Christensen H, Garton NJ, Horobin RW, Minnikin DE & Barer MR (1999) Lipid domains of mycobacteria studied with fluorescent molecular probes. *Mol Microbiol* **31**: 1561–1572.

Chung JD, Conner S & Stephanopoulos G (1995) Flow cytometric study of differentiating cultures of *Bacillus subtilis*. *Cytometry* **20**: 324–333.

Comas J & Vives-Rego J (1997) Assessment of the effects of gramicidin, formaldehyde, and surfactants on *Escherichia coli* by flow cytometry using nucleic acid and membrane potential dyes. *Cytometry* **29**: 58–64.

Comas J & Vives-Rego J (1998) Enumeration, viability and heterogeneity in *Staphylococus aureus* cultures by flow cytometry. *J Microbiol Meth* **32**: 45–53.

Comas-Riu J & Vives-Rego J (1999) Use of calcein and SYTO-13 to assess cell cycle phases and osmotic shock effects on *E. coli* and *Staphylococcus aureus* by flow cytometry. *J Microbiol Meth* **34**: 215–221.

Conners SB, Mongodin EF, Johnson MR, Montero CI, Nelson KE & Kelly RM (2006) Microbial biochemistry, physiology, and biotechnology of hyperthermophilic *Thermotoga* species. *FEMS Microbiol Rev* **30**: 872–905.

Cooper S (1969) Cell division and DNA replication following a shift to a richer medium. *J Mol Biol* **43**: 1–11.
Cooper S (1979) Unifying model for the G1 period in prokaryotes and eukaryotes. *Nature* **280**: 17–19.
Cooper S (1990) The *Escherichia coli* cell-cycle. *Res Microbiol* **141**: 17–29.
Cooper S (1991) *Bacterial Growth and Division*. Academic Press, San Diego, CA.
Cooper S (2006) Regulation of DNA synthesis in bacteria: analysis of the Bates/Kleckner licensing/initiation-mass model for cell cycle control. *Mol Microbiol* **62**: 303–307.
Couzin J (2004) Molecular biology: RNAi shows cracks in its armor. *Science* **306**: 1124–1125.
Czechowska K, Johnson DR & van der Meer JR (2008) Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Curr Opin Microbiol* **11**: 205–212.
Davey HM & Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol Rev* **60**: 641–696.
Degelau A, Freitag R, Linz F, Middendorf C, Scheper T, Bley T, Müller S, Stoll P & Reardon KF (1992) Immuno- and flow cytometric analytical methods for biotechnological research and process monitoring. *J Biotechnol* **25**: 115–144.
DeLong EF, Wickham GS & Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* **243**: 1360–1363.
Devyatyarova-Johnson M, Rees IH, Robertson BD, Turner MW, Klein NJ & Jack DL (2000) The lipopolysaccharide structures of *Salmonella enterica* serovar *Typhimurium* and *Neisseria gonorrhoeae* determine the attachment of human mannose-binding lectin to intact organisms. *Infect Immun* **68**: 3894–3899.
Dittrich W & Göhde W (1969) Impulsfluorometrie bei Einzelzellen in Suspensionen. *Z Naturforsch* **24b**: 221–228.
Donachie WD (1968) Relationship between cell size and time of initiation of DNA replication. *Nature* **219**: 1077–1079.
Dubarry N, Pasta F & Lane D (2006) ParABS systems of the four replicons of *Burkholderia cenocepacia*: new chromosome centromeres confer partition specificity. *J Bacteriol* **188**: 1489–1496.
Duhamel S, Gregori G, Van Wambeke F & Nedoma J (2009) Detection of extracellular phosphatase activity at the single-cell level by enzyme-labeled fluorescence and flow cytometry: the importance of time kinetics in ELFA labelling. *Cytometry* **75A**: 163–168.
Dühnen J, Stegemann J, Wiezorek C & Mertens H (1983) A new fluid switching flow sorter. *Histochem Cell Biol* **77**: 117–121.
Durot M, Bourguignon PY & Schachter V (2009) Genome-scale models of bacterial metabolism: reconstruction and applications. *FEMS Microbiol Rev* **33**: 164–190.
Endo H, Nakayama J, Ushio H, Hayashi T & Watanabe E (1998) Application of flow cytometry for rapid detection of *Lactococcus garvieae*. *Appl Biochem Biotech* **75**: 295–306.
Epand RM & Kraayenhof R (1999) Fluorescent probes used to monitor membrane interfacial polarity. *Chem Phys Lipids* **101**: 57–64.
Erhart R, Bradford D, Seviour EM, Seviour RJ, Amann R & Blackall LL (1997) Development and use of fluorescent *in situ* hybridisation probes for the identification and enumeration of *Microthrix parvicella* in activated sludge. *Syst Appl Microbiol* **20**: 310–318.
Fernandez C, Thyssen M & Denis M (2008) Microbial community structure along 18°W (39°N -44.5°N) in the NE Atlantic in late summer 2001 (POMME programme). *J Marine Systems* **71**: 46–62.
Ferrari BC, Binnerup SJ & Gillings M (2005) Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. *Appl Environ Microb* **71**: 8714–8720.
Ferry RM, Farr LE Jr & Hartman MG (1949) The preparation and measurement of the concentration of dilute bacterial aerosols. *Chem Rev* **44**: 389–417.
Foladori P, Quaranta A & Ziglio G (2008) Use of silica microspheres having refractive index similar to bacteria for conversion of flow cytometric forward light scatter into biovolume. *Water Res* **42**: 3757–3766.
Fouchet P, Jayat C, Héchard Y, Ratinaud MH & Frelat G (1993) Recent advances of flow cytometry in fundamental and applied microbiology. *Biol Cell* **78**: 95–109.
Francisco JA, Campbell R, Iversont BL & Georgiou G (1993) Production and fluorescence-activated cell sorting of *Escherichia coli* expressing a functional antibody fragment on the external surface. *P Natl Acad Sci USA* **90**: 10444–10448.
Fu AY, Chou H-P, Spence C, Arnold FH & Quake SR (2002) An integrated microfabricated cell sorter. *Anal Chem* **74**: 2451–2457.
Fuchs BM, Wallner G, Beisker W, Schwippl I, Ludwig W & Amann R (1998) Flow cytometric analysis of the *in-situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. *Appl Environ Microb* **64**: 4973–4982.
Fuchs BM, Spring S, Teeling H *et al.* (2007) Characterization of a marine gammaproteobacterium capable of aerobic anoxygenic photosynthesis. *P Natl Acad Sci USA* **104**: 2891–2896.
Fulwyler MJ (1965) Electronic separation of biological cells by volume. *Science* **150**: 910–911.
Gant VA, Warnes G, Phillips I & Savidge GF (1993) The application of flow cytometry to the study of bacterial responses to antibiotics. *J Med Microbiol* **39**: 147–154.
Garcia D, Gomez N, Manas P, Raso J & Pagan R (2007) Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. *Int J Food Microbiol* **113**: 219–227.
García-Ochoa F, Santos VE & Alcón A (1998) Intracellular compounds quantification by means of flow cytometry in

bacteria: application to xanthan production by *Xanthomonas campestris*. *Biotechnol Bioeng* **57**: 87–94.

Gasol JM, Zweifel UL, Peters F, Fuhrman JA & Hagström A (1999) Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Appl Environ Microb* **65**: 4475–4483.

Gauthier C, St-Pierre Y & Villemur R (2002) Rapid antimicrobial susceptibility testing of urinary tract isolates and samples by flow cytometry. *J Med Microbiol* **51**: 192–200.

Gefen O & Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiol Rev* **33**: 704–717.

Germ M, Yoshihara E, Yoneyama H & Nakae T (1999) Interplay between the efflux pump and the outer membrane permeability barrier in fluorescent dye accumulation in *Pseudomonas aeruginosa*. *Biochem Bioph Res Co* **261**: 452–455.

Gill SR, Pop M, DeBoy RT *et al.* (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**: 1355–1359.

Gray ND, Howarth R, Pickup RW, Jones JG & Head IM (2000) Use of combined microautoradiography and fluorescence *in situ* hybridization to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus *Achromatium*. *Appl Environ Microb* **66**: 4518–4522.

Gucker FT & O'Konski C (1949) Electronic methods of counting aerosol particles. *Chem Rev* **44**: 373–388.

Gucker FT, O'Konski C, Pickard HB & Pitts JN (1947) A photoelectric counter for colloidal particles. *J Am Chem Soc* **69**: 2422–2431.

Guindulain T, Comas J & Vives-Rego J (1997) Use of nucleic acid dyes SYTO-13, TOTO-1, and YOYO-1 in the study of *Escherichia coli* and marine prokaryotic populations by flow cytometry. **63**: 4608–4611.

Günther S, Geyer W, Harms H & Müller S (2007) Fluorogenic surrogate substrates for toluene-degrading bacteria: are they useful for activity analysis? *J Microbiol Meth* **70**: 272–283.

Günther S, Hübschmann T, Rudolf M, Eschenhagen M, Röske I, Harms H & Müller S (2008) Fixation procedures for flow cytometric analysis of environmental bacteria. *J Microbiol Meth* **75**: 127–134.

Günther S, Trutnau M, Kleinsteuber S, Hause G, Bley T, Röske I, Harms H & Müller S (2009) Dynamics of polyphosphate-accumulating bacteria in wastewater treatment plant microbial communities detected via DAPI (4′,6′-diamidino-2-phenylindole) and tetracycline labeling. *Appl Environ Microb* **75**: 2111–2121.

Gupta RS & Griffiths E (2002) Critical issues in bacterial phylogeny. *Theor Popul Biol* **61**: 423–434.

Haidinger W, Mayr UB, Szostak MP, Resch S & Lubitz W (2003) *Escherichia coli* ghost production by expression of lysis gene E and staphylococcal nuclease. *Appl Environ Microb* **69**: 6106–6113.

Hakkila K, Maksimow M, Rosengren A, Karp M & Virta M (2003) Monitoring promoter activity in a single bacterial cell by using green and red fluorescent proteins. *J Microbiol Meth* **54**: 75–79.

Harold FM & Baarda JR (1967) Gramicidin, valinomycin, and cation permeability of *Streptococcus faecalis*. *J Bacteriol* **94**: 53–60.

Haugland RP (ed) (2005) *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*. 10th edn. Invitrogen/Molecular Probes, Eugene, OR. Available at http://probes.invitrogen.com/handbook.

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C & Zhou J (2007) GeoChip: a comprehensive microarray for investigating biochemical, ecological and environmental processes. *ISME J* **1**: 67–77.

Hechard Y, Jayat C, Letellier F, Julien R, Cenatiempo Y & Ratinaud MH (1992) Online visualization of the competitive behavior of antagonistic bacteria. *Appl Environ Microb* **58**: 3784–3786.

Hedhammar M, Stenvall M, Lonneborg R, Nord O, Sjölin O, Brismar H, Uhlén M, Ottosson J & Hober S (2005) A novel flow cytometry-based method for analysis of expression levels in *Escherichia coli*, giving information about precipitated and soluble protein. *J Biotechnol* **119**: 133–146.

Heine F, Stahl F, Sträuber H *et al.* (2009) Prediction of flocculation ability of brewing yeast inoculates by flow cytometry, proteome analysis, and mRNA profiling. *Cytom Part A* **75A**: 140–147.

Helmstetter CE (1967) Rate of DNA synthesis during division cycle of *Escherichia coli* B/r. *J Mol Biol* **24**: 417–427.

Helmstetter CE (1968) Origin and sequence of chromosome replication in *Escherichia coli* B/r. *J Bacteriol* **95**: 1634–1641.

Helmstetter CE (1969) Sequence of bacterial reproduction. *Annu Rev Microbiol* **23**: 223–238.

Helmstetter CE & Cooper S (1968) DNA synthesis during division cycle of rapidly growing *Escherichia coli* B/r. *J Mol Biol* **31**: 507–518.

Henningson EW, Krocova Z, Sandström G & Forsman M (1998) Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. *FEMS Microbiol Ecol* **25**: 241–249.

Hercher M, Mueller W & Shapiro HM (1979) Detection and discrimination of individual viruses by flow cytometry. *J Histochem Cytochem* **27**: 350–352.

Herman P, Konopasek I, Plasek J & Svobodova J (1994) Time-resolved polarized fluorescence studies the temperature adaptation in *Bacillus subtilis* using DPH and TMA-DPH fluorescent-probes. *Biochim Biophys Acta* **1190**: 1–8.

Herrmann C, Lösche A, Müller S, Bley T & Babel W (1997) Flow cytometric discrimination between *Acinetobacter calcoaceticus* 69-V and *Alcaligenes eutrophus* JMP 134 by fluorescently labelled rRNA-targeted oligonucleotide probes and DNA staining. *Acta Biotechnol* **17**: 19–38.

Hewitt CJ & Nebe-von-Caron G (2001) An industrial application of multiparameter flow cytometry: assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry* **44**: 179–187.

Hewitt CJ & Nebe-von-Caron G (2004) The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. *Adv Biochem Eng Biot* **89**: 197–223.

Hewitt CJ & Nienow AW (2007) The scale-up of microbial batch and fed-batch fermentation processes. *Adv Appl Microbiol* **62**: 105–135.

Hewitt CJ, Nebe-von-Caron G, Axellsson B, McFarlane CM & Nienow AW (2000) Studies related to the scale-up of high cell density *Escherichia coli* fed batch fermentations using multi-parameter flow cytometry: effect of a changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotechnol Bioeng* **70**: 381–390.

Hiraoka Y & Kimbara K (2002) Rapid assessment of the physiological status of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 by flow cytometry. *Appl Environ Microb* **68**: 2031–2035.

Ho HY, Cheng ML, Wang YH & Chiu DTY (2006) Flow cytometry for assessment of the efficacy of siRNA. *Cytom Part A* **69A**: 1054–1061.

Hodson RE, Dustman WA, Garg RP & Moran MA (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl Environ Microb* **61**: 4074–4082.

Hoefel D, Monis PT, Grooby WL, Andrews S & Saint CP (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *J Appl Microbiol* **99**: 175–186.

Hsieh PC, Siegel SA, Rogers B, Davis D & Lewis K (1998) Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. *P Natl Acad Sci USA* **95**: 6602–6606.

Hübschmann T, Vogt C, Till S, Rohwerder T, Sand W, Harms H & Müller S (2007) Detection of sulfur microparticles in bacterial cultures by flow cytometry. *Eng Life Sci* **7**: 403–407.

Hughes EE, Gilleland HE Jr & Matthews-Greer JM (1996) Analysis by flow cytometry of surface-exposed epitopes of outer membrane protein F of *Pseudomonas aeruginosa*. *Can J Microbiol* **42**: 859–862.

Huijbregts RPH, deKroon A & deKruijff B (1996) Rapid transmembrane movement of C-6-NBD-labeled phospholipids across the inner membrane of *Escherichia coli*. *Biochim Biophys Acta* **1280**: 41–50.

Hulett HR, Bonner WA, Barrett J & Herzenberg LA (1969) Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. *Science* **166**: 747–749.

Hutter KJ (1974) Untersuchungen über die DNS-, RNS- und Proteinsynthese von Hefezellen der Gattung *Sacharomyces* mit Hilfe neuer fluorometrischer Methoden. 19/FB13, Berlin.

Hutter KJ & Eipel HE (1978) Flow cytometric determination of cellular substances in algae, bacteria, moulds and yeasts. *Antonie van Leeuwenhoek* **44**: 269–282.

Hutter KJ & Eipel HE (1979) Simultaneous measurements of DNA and protein content of microorganisms by flow cytometry. *Eur J Appl Microbiol* **6**: 223–231.

Hutter KJ, Göhde W & Emeis C (1975) Investigations about the synthesis of DNA, RNA and proteins of selected populations of microorganisms by cytophotometry and pulse-cytophotometry. 1. Methodical investigations about appropriate fluorescence dyes and staining procedures. *Chem Mikrobiol Technol Lebensm* **4**: 29–32.

Ingram M, Cleary TJ, Price BJ & Price RL (1982) Rapid detection of *Legionella pneumophila* by flow cytometry. *Cytometry* **3**: 134–137.

Jacobson MR, Cao LG, Wang YL & Pederson T (1995) Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. *J Cell Biol* **131**: 1649–1658.

Jernaes MW & Steen HB (1994) Staining of *Escherichia coli* for flow-cytometry: influx and efflux of ethidium bromide. *Cytometry* **17**: 302–309.

Johnson DR, Czechowska K, Chèvre N & van der Meer JR (2009) Toxicity of triclosan, penconazole, and metalaxyl on *Caulobacter crescentus* and a freshwater microbial community as assessed by flow cytometry. *Environ Microbiol* **11**: 1682–1691.

Joux F, Lebaron P & Troussellier M (1997) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. *FEMS Microbiol Ecol* **22**: 65–76.

Kalyuzhnaya MG, Lidstrom ME & Chistoserdova L (2008) Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *ISME J* **2**: 696–706.

Kamentsky LA & Melamed MR (1969) Rapid multiple mass constituent analysis of biological cells. *Ann NY Acad Sci* **157**: 310–323.

Kamentsky LA, Melamed MR & Derman H (1965) Spectrophotometer: new instrument for ultrarapid cell analysis. *Science* **150**: 630–631.

Kaprelyants AS & Kell DB (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. *J Appl Bacteriol* **72**: 410–422.

Kawai M, Yamaguchi N & Nasu M (1999) Rapid enumeration of physiologically active bacteria in purified water used in the pharmaceutical manufacturing process. *J Appl Microbiol* **86**: 496–504.

Keller M & Zengler K (2004) Tapping into microbial diversity. *Nat Rev Microbiol* **2**: 141–150.

Klauth P, Pallerla SR, Vidaurre D, Ralfs C, Wendisch VF & Schoberth SM (2006) Determination of soluble and granular inorganic polyphosphate in *Corynebacterium glutamicum*. *Appl Microbiol Biot* **72**: 1099–1106.

Kleinsteuber S, Riis V, Fetzer I, Harms H & Müller S (2006) Population dynamics within a microbial consortium during growth on diesel fuel in saline environments. *Appl Environ Microb* **72**: 3531–3542.

Knemeyer JP, Herten DP & Sauer M (2003) Detection and identification of single molecules in living cells using spectrally resolved fluorescence lifetime imaging microscopy. *Anal Chem* **75**: 2147–2153.

Konings WN, Lolkema JS, Bolhuis H, van Veen HW, Poolman B & Driessen AJ (1997) The role of transport processes in survival of lactic acid bacteria: energy transduction and multidrug resistance. *Antonie van Leeuwenhoek* **71**: 117–128.

Krishan A (1987) Effect of drug efflux blockers on vital staining of cellular DNA with HOECHST 33342. *Cytometry* **8**: 642–645.

Kulaev I & Kulakovskaya T (2000) Polyphosphate and phosphate pump. *Annu Rev Microbiol* **54**: 709–734.

Lebaron P & Joux F (1994) Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater. *Appl Environ Microb* **60**: 4345–4350.

Lebaron P, Catala P & Parthuisot N (1998a) Effectiveness of SYTOX green stain for bacterial viability assessment. *Appl Environ Microb* **64**: 2697–2700.

Lebaron P, Parthuisot N & Catala P (1998b) Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Appl Environ Microb* **64**: 1725–1730.

Lee JA, Spidlen J, Boyce K *et al.* (2008) MIFlowCyt: the minimum information about a flow cytometry experiment. *Cytom Part A* **73A**: 926–930.

Lee TC, Mohsin S, Taylor D, Parkesh R, Gunnlaugsson T, O'Brien FJ, Giehl M & Gowin W (2003) Detecting microdamage in bone. *J Anat* **203**: 161–172.

Levanon A, Inbar M & Kohn A (1979) Fluorescence polarization of DPH-labeled cells adsorbing viruses and its diagnostic potential. *Arch Virol* **59**: 223–230.

Libertin CR, Woloschak GE, Wilson WR & Smith TF (1984) Analysis of *Pneumocystis carinii* cysts with a fluorescence-activated cell sorter. *J Clin Microbiol* **20**: 877–880.

Lloyd D & Hayes AJ (1995) Vigor, vitality and viability of microorganisms. *FEMS Microbiol Lett* **133**: 1–7.

Lopez D, Vlamakis H & Kolter R (2009) Generation of multiple cell types in *Bacillus subtilis*. *FEMS Microbiol Rev* **33**: 152–163.

Lotz W, Acker G & Schmitt R (1977) Bacteriophage 7-7-1 adsorbs to the complex flagella of *Rhizobium lupini* H13-3. *J Gen Virol* **34**: 9–17.

Lozupone CA & Knight R (2008) Species divergence and the measurement of microbial diversity. *FEMS Microbiol Rev* **32**: 557–578.

Mailaender C, Reiling N, Engelhardt H, Bossmann S, Ehlers S & Niederweis M (2004) The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Microbiology* **150**: 853–864.

Manafi M & Kneifel W (1991) Fluorogenic and chromogenic substrates: a promising tool in microbiology. *Acta Microbiol Hung* **38**: 293–304.

Marie D, Vaulot D & Partensky F (1996) Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. *Appl Environ Microb* **62**: 1649–1655.

Marisol F, Andreatta S, Sommaruga R, Straskrábová V & Catalan J (2007) Suitability of flow cytometry for estimating bacterial biovolume in natural plankton samples: comparison with microscopy data. *Appl Environ Microb* **73**: 4508–4514.

Martinez JL, Fajardo A, Garmendia L, Hernandez A, Linares JF, Martínez-Solano L & Sánchez MB (2009a) A global view of antibiotic resistance. *FEMS Microbiol Rev* **33**: 66–97.

Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A & Alvarez-Ortega C (2009b) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* **33**: 430–449.

Masami Y, Daniel G & Denis M (2003) Monitoring the uptake of protein-derived peptides by *Porphyromonas gingivalis* with fluorophore-labeled substrates. *Curr Microbiol* **47**: 1–4.

Mason DJ, Lopez-Amoros R, Allman R, Stark JM & Lloyd D (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *J Appl Bacteriol* **78**: 309–315.

Mastroleo F, Leroy B, Van Houdt R, S' Heeren C, Mergeay M, Hendrickx L & Wattiez R (2009) Shotgun proteome analysis of *Rhodospirillum rubrum* S1H: integrating data from gel-free and gel-based peptides fractionation methods. *J Proteome Res* **8**: 2530–2541.

McFeters GA, Singh A, Byun S, Callis PR & Williams S (1991) Acridine-orange staining reaction as an index of physiological activity in *Escherichia coli*. *J Microbiol Meth* **13**: 87–97.

Miao F, Todd P & Komapala DS (1993) A single-cell assay of β-galactosidase in recombinant *Escherichia coli* using flow cytometry. *Biotechnol Bioeng* **42**: 708–715.

Mileykovskaya E & Dowhan W (2000) Visualization of phospholipid domains in *Escherichia coli* by using cardiolipin specific fluorescent dye 10-*N*-nonyl-acridine orange. *J Bacteriol* **182**: 1172–1175.

Minas W, Sahar E & Gutnick D (1988) Flow cytometric screening and isolation of *Escherichia coli* clones which express surface antigens of the oil-degrading microorganism *Acinetobacter calcoaceticus* rag-1. *Arch Microbiol* **150**: 432–437.

Molenaar D, Bolhuis H, Abee T, Poolman B & Konings WN (1992) The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in *Lactococcus lactis*. *J Bacteriol* **174**: 3118–3124.

Monfort P & Baleux B (1992) Comparison of flow-cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems. *Cytometry* **13**: 188–192.

Monfort P & Baleux B (1996) Cell cycle characteristics and changes in membrane potential during growth of *Escherichia coli* as determined by a cyanine fluorescent dye and flow cytometry. *J Microbiol Meth* **25**: 79–86.

Mou X, Moran MA, Stepanauskas R, González JM & Hodson RE (2005) Flow-cytometric cell sorting and subsequent molecular analyses for culture-independent identification of bacterioplankton involved in dimethylsulfoniopropionate transformations. *Appl Environ Microb* **71**: 1405–1416.

Mueller S, Saunier K, Hanisch C *et al.* (2006) Differences in fecal microbiota in different European study populations in relation

to age, gender, and country: a cross-sectional study. *Appl Environ Microb* **72**: 1027–1033.
Müller S (2007) Modes of cytometric bacterial DNA pattern: a tool for pursuing growth. *Cell Proliferat* **40**: 621–639.
Müller S & Babel W (2003) Analysis of multiplication activity: an approach for controlling biotechnological processes. *J Microbiol Meth* **55**: 851–858.
Müller S, Lösche A & Bley Th (1992) Flow cytometric investigation of sterol content and proliferation activity of yeast. *Acta Biotechnol* **12**: 365–375.
Müller S, Lösche A, Bley T & Scheper T (1995) A flow cytometric approach for characterization and differentiation of bacteria during microbial processes. *Appl Microbiol Biot* **43**: 93–101.
Müller S, Loffhagen N, Bley T & Babel W (1996) Membrane-potential-related fluorescence intensity indicates membrane injury. *Microbiol Res* **151**: 127–131.
Müller S, Bley T & Babel W (1999) Adaptive responses of *Ralstonia eutropha* to master feast and famine conditions analysed by flow cytometry. *J Biotechnol* **75**: 81–97.
Müller S, Lösche A, Mertingk H, Beisker W & Babel W (2000a) Flow cytometric monitoring of *Rhodococcus erythropolis* and *Ochrobactrum anthropi* in a mixed culture. *Acta Biotechnol* **20**: 219–233.
Müller S, Ullrich S, Lösche A, Loffhagen N & Babel W (2000b) Flow cytometric techniques to characterise physiological states of *Acinetobacter calcoaceticus*. *J Microbiol Meth* **40**: 67–77.
Müller S, Kiesel B & Berthe-Corti L (2001a) *Muricauda ruestringensis* has an asymmetric cell cycle. *Acta Biotechnol* **21**: 343–357.
Müller S, Lösche A, Schmidt M & Babel W (2001b) Optimisation of high gravity and diet beer production in a German brewery by flow cytometry. *J I Brewing* **107**: 373–382.
Müller S, Sträuber H, Lösche A & Babel W (2002) Population analysis of a binary bacterial culture by multi-parametric flow cytometry. *J Biotechnol* **97**: 163–176.
Müller S, Vogt C, Laube M, Harms H & Kleinsteuber S (2009) Community dynamics within a bacterial consortium during growth on toluene under sulfate-reducing conditions. *FEMS Microbiol Ecol* **70**: 586–596.
Müller S, Harms H & Bley T (2010) Origin and analysis of microbial population heterogeneity in bioprocesses. *Curr Opin Biotech* **21**: doi: 10.1016/j.copbio.2010.01.002.
Muthian S, Nithipatikom K, Campbell WB & Hillard CJ (2000) Synthesis and characterization of a fluorescent substrate for the *N*-arachidonoylethanolamine (anandamide) transmembrane carrier. *J Pharmacol Exp Ther* **293**: 289–295.
Nadell CD, Xavier JB & Foster KR (2009) The sociobiology of biofilms. *FEMS Microbiol Rev* **33**: 206–224.
Nader WF, Nebe CT, Nebe G, Dastani A & Birr C (1991) Analysis of bacteria in environmental and medical microbiology by flow cytometry. *Rapid Methods and Automation in Microbiology and Immunology* (Veheri A, Tilton RC & Barlow A, eds), pp. 131–140. Springer Verlag, Berlin, Germany.
Natarajan A & Srienc F (1999) Dynamics of glucose uptake by single *Escherichia coli* cells. *Metab Eng* **1**: 320–333.
Natarajan A & Srienc F (2000) Glucose uptake rates of single *E. coli* cells grown in glucose-limited chemostat cultures. *J Microbiol Meth* **42**: 87–96.
Nebe-von-Caron G (1996) Purdue Cytometry CDRom 2, Section 4, ISSN #1091-2037.
Nebe-von-Caron G (2009) Standardization in microbial cytometry. *Cytom Part A* **75A**: 86–89.
Nebe-von-Caron G & Badley RA (1995) Viability assessment of bacteria in mixed populations using flow cytometry. *J Microscopy Oxford* **179**: 55–66.
Nebe-von-Caron G, Stephens P & Badley RA (1998) Assessment of bacterial viability status by flow cytometry and single sorting. *J Appl Microbiol* **84**: 988–998.
Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR & Badley RA (2000) Analysis of bacterial function by multi-colour and single cell sorting. *J Microbiol Meth* **42**: 97–114.
Nedoma J & Vrba J (2006) Specific activity of cell-surface acid phosphatase in different bacterioplankton morphotypes in an acidified mountain lake. *Environ Microbiol* **8**: 1271–1279.
Nelson ML (1998) Chemical and biological dynamics of tetracyclines. *Adv Dent Res* **12**: 5–11.
Nexmann Jacobsen C, Rasmussen J & Jakobsen M (1997) Viability staining and flow cytometric detection of *Listeria monocytogenes*. *J Microbiol Meth* **28**: 35–43.
Nie L & Siebert KJ (2008) Modeling physicochemical properties and activity of aspartyl proteinases based on amino acid composition. *Crit Rev Biotechnol* **28**: 297–307.
Nielsen JL, Schramm A, Bernhard AE, van den Engh GJ & Stahl DA (2004) Flow cytometry-assisted cloning of specific sequence motifs from complex 16S rRNA gene libraries. *Appl Environ Microb* **70**: 7550–7554.
Nir R, Yisraeli Y, Lamed R & Sahar E (1990) Flow cytometry sorting of viable bacteria and yeasts according to beta-galactosidase activity. *Appl Environ Microb* **56**: 3861–3866.
Nolan GP, Fiering S, Nicolas JF & Herzenberg LA (1988) Fluorescence-activated cell analysis and sorting of viable mammalian-cells based on β-D-galactosidase activity after transduction of *Escherichia coli* lacZ. *P Natl Acad Sci USA* **85**: 2603–2607.
Novo D, Perlmutter NG, Hunt RH & Shapiro HM (1999) Accurate flow cytometric membrane potential measurement in bacteria using diethylcarbocyanine and a ratiometric technique. *Cytometry* **35**: 55–63.
Ochman H, Lawrence JG & Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**: 299–304.
Ordóñez JV & Wehman NM (1993) Rapid flow cytometric antibiotic susceptibility assay for *Staphylococcus aureus*. *Cytometry* **14**: 811–818.
Overton TW, Whitehead R, Li Y, Griffiths L & Cole J (2008) Sense and nonsense from whole genome microarray data in the analysis of microbial physiology. *Biotechnologia* **80**: 15–30.
Ozanne V, Ortalo-Magne A, Vercellone A, Fournie JJ & Daffe M (1996) Cytometric detection of mycobacterial surface

antigens: exposure of mannosyl epitopes and of the arabinan segment of arabinomannans. *J Bacteriol* **178**: 7254–7259.

Paau AS, Cowles JR & Oro J (1977) Flow-microfluorometric analysis of *Escherichia coli*, *Rhizobium meliloti*, and *Rhizobium japonicum* at different stages of the growth cycle. *Can J Microbiol* **23**: 1165–1169.

Paau AS, Cowles JR, Oro J, Bartel A & Hungerford E (1979) Separation of algal mixtures and bacterial mixtures with flow-microfluorometer using chlorophyll and ethidium bromide fluorescence. *Arch Microbiol* **120**: 271–273.

Pace NR (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.

Palenik B, Ren Q, Tai V & Paulsen IT (2009) Coastal *Synechococcus* metagenome reveals major roles for horizontal gene transfer and plasmids in population diversity. *Environ Microbiol* **11**: 349–359.

Pamp SJ, Sternberg C & Tolker-Nielsen T (2009) Insight into the microbial multicellular lifestyle via flow-cell technology and confocal microscopy. *Cytom Part A* **75A**: 90–103.

Pandey J, Chauhan A, Rakesh K & Jain RK (2009) Integrative approaches for assessing the ecological sustainability of *in situ* bioremediation. *FEMS Microbiol Rev* **33**: 324–375.

Papadimitriou K, Pratsinis H, Nebe-von-Caron G, Kletsas D & Tsakalidou E (2007) Acid tolerance of *Streptococcus macedonicus* as assessed by flow cytometry and single-cell sorting. *Appl Environ Microb* **73**: 465–476.

Park H-S, Schumacher R & Kilbane J (2004) New method to characterize microbial diversity using flow cytometry. *J Ind Microbiol Biot* **32**: 94–102.

Patankar AV & Gonzalez JE (2009) Orphan LuxR regulators of quorum sensing. *FEMS Microbiol Rev* **33**: 739–756.

Patkar A, Vijayasankaran N, Urry DW & Srienc F (2002) Flow cytometry as a useful tool for process development: rapid evaluation of expression systems. *J Biotechnol* **93**: 217–229.

Paulsen IT, Brown MH & Skurray RA (1996) Proton-dependent multidrug efflux systems. *Microbiol Rev* **60**: 575–608.

Pawelczyk S, Abraham W-R, Harms H & Müller S (2008) Community-based degradation of 4-chlorosalicylate tracked on the single cell level. *J Microbiol Meth* **75**: 117–126.

Perez-Pantoja D, De la Iglesia R, Pieper DH & Gonzalez B (2008) Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. *FEMS Microbiol Rev* **32**: 736–794.

Pernthaler A, Dekas AE, Brown CT, Goffredi SK, Embaye T & Orphan VJ (2008) Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. *P Natl Acad Sci USA* **105**: 7052–7057.

Pernthaler J & Amann R (2005) Fate of heterotrophic microbes in pelagic habitats: focus on populations. *Microbiol Mol Biol R* **69**: 440–461.

Petit JM, Maftah A, Ratinaud MH & Julien R (1992) 10*n*-nonylacridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur J Biochem* **209**: 267–273.

Phillips AP & Martin KL (1983) Immunofluorescence analysis of *Bacillus* spores and vegetative cells by flow cytometry. *Cytometry* **4**: 123–131.

Phillips AP & Martin KL (1985) Dual-parameter scatter-flow immunofluorescence analysis of *Bacillus* spores. *Cytometry* **6**: 124–129.

Pohlmann A, Fricke WF, Reinecke F *et al.* (2006) Genome sequence of the bioplastic-producing 'Knallgas' bacterium *Ralstonia eutropha* H16. *Nat Biotechnol* **24**: 1257–1262.

Poot M & Arttamangkul S (1997) Verapamil inhibition of enzymatic product efflux leads to improved detection of beta-galactosidase activity in lacZ-transfected cells. *Cytometry* **28**: 36–41.

Porro D, Vai M, Vanoni M, Alberghina L & Hatzis C (2009) Analysis and modeling of growing budding yeast populations at the single cell level. *Cytom Part A* **75**: 114–120.

Porter J, Edwards C, Morgan JA & Pickup RW (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Appl Environ Microb* **59**: 3327–3333.

Porter J, Edwards C & Pickup RW (1995a) Rapid assessment of physiological status in *Escherichia coli* using fluorescent-probes. *J Appl Bacteriol* **79**: 399–408.

Porter J, Pickup R & Edwards C (1995b) Flow cytometric detection of specific genes in genetically modified bacteria using *in situ* polymerase chain reaction. *FEMS Microbiol Lett* **134**: 51–56.

Posch T, Pernthaler J, Alfreider A & Psenner R (1997) Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. *Appl Environ Microb* **63**: 867–873.

Radajewski S, McDonald IR & Murrell JC (2003) Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr Opin Biotechnol* **14**: 296–302.

Ratinaud MH & Revidon S (1996) Flow cytometric method to assess functional state of the *Listeria* membrane. *J Microbiol Meth* **25**: 71–77.

Resina-Pelfort O, Comas-Riu J & Vives-Rego J (2001) Biological effects of deflected droplet electrostatic cell sorting on bacterial cultures and marine bacterioplankton. *Syst Appl Microbiol* **24**: 31–36.

Riis SB, Pedersen HM, Sorensen NK & Jakobsen M (1995) Flow-cytometry and acidification power test as rapid techniques for determination of the activity of starter cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Food Microbiol* **12**: 245–250.

Riis V, Lorbeer H & Babel W (1998) Extraction of microorganisms from soil: evaluation of the efficiency by counting methods and activity measurements. *Soil Biol Biochem* **30**: 1573–1581.

Robertson BR, Button DK & Koch AL (1998) Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. *Appl Environ Microb* **64**: 3900–3909.

Rockberg J, Löfblom J, Hjelm B, Uhlén M & Stefan Ståhl S (2008) Epitope mapping of antibodies using bacterial surface display. *Nat Methods* **5**: 1039–1045.

Roselló-Mora R, Lee N, Anton J & Wagner M (2003) Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence *in situ* hybridization. *Extremophiles* **7**: 409–413.

Roth BL, Poot M, Yue ST & Millard PJ (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Appl Environ Microb* **63**: 2421–2431.

Rutten TBA, Sandee B & Hofman AR (2005) Phytoplankton monitoring by high performance flow cytometry: a successful approach? *Cytom Part A* **64A**: 16–26.

Sachetelli S, Khalil H, Chen T, Beaulac C, Senechal S & Lagace J (2000) Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. *Biochim Biophys Acta* **1463**: 254–266.

Sahar E, Lamed R & Ofek I (1983) Rapid identification of *Streptococcus pyogenes* by flow cytometry. *Eur J Clin Microbiol* **2**: 192–195.

Saier MH (2000) A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol Mol Biol R* **64**: 354–411.

Sakamoto C, Yamaguchi N & Nasu M (2005) Rapid and simple quantification of bacterial cells by using a microfluidic device. *Appl Environ Microb* **71**: 1117–1121.

Sanders CA, Yajko DM, Hyun W, Langlois RG, Nassos PS, Fulwyler MJ & Hadley WK (1990) Determination of guanine-plus-cytosine content of bacterial DNA by dual-laser flow cytometry. *J Gen Microbiol* **136**: 359–365.

Schaechter M, Maaloe O & Kjeldgaard NO (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *J Gen Microbiol* **19**: 592–606.

Scheper T, Gebauer A, Sauerbrei A, Niehoff A & Schügerl K (1984) Measurement of biological parameters during fermentation processes. *Anal Chim Acta* **163**: 111–118.

Scheper T, Hitzmann B, Rinas U & Schügerl K (1987) Flow cytometry of *Escherichia coli* for process monitoring. *J Biotechnol* **5**: 139–148.

Schönhuber W, Fuchs BM, Juretschko S & Amann R (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Appl Environ Microb* **63**: 3268–3273.

Schut F, Gottschal JC & Prins RA (1997a) Isolation and characterisation of the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256. *FEMS Microbiol Rev* **20**: 363–369.

Schut F, Prins RA & Gottschall JC (1997b) Oligotrophy and pelagic marine bacteria: facts and fiction. *Aquat Microb Ecol* **12**: 177–202.

Scow KM & Hicks KA (2005) Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Curr Opin Biotech* **16**: 246–253.

Sekar R, Fuchs BM, Amann R & Pernthaler J (2004) Flow sorting of marine bacterioplankton after fluorescence *in situ* hybridization. *Appl Environ Microb* **70**: 6210–6219.

Seo JH & Bailey JE (1985) A segregated model for plasmid content and product synthesis in unstable binary fission recombinant organisms. *Biotechnol Bioeng* **27**: 156–165.

Seo JH, Srienc F & Bailey JE (1985) Flow-cytometry analysis of plasmid amplification in *Escherichia coli*. *Biotechnol Progr* **1**: 181–188.

Servais P, Courties C, Lebaron P & Troussellier M (1999) Coupling bacterial activity measurements with cell sorting by flow cytometry. *Microb Ecol* **38**: 180–189.

Shapiro HM (2000) Membrane potential estimation by flow cytometry. *Methods* **21**: 271–279.

Shapiro HM (2002) *Practical Flow Cytometry*. Wiley & Sons, New York.

Shi L, Günther S, Hübschmann T, Wick LY, Harms H & Müller S (2007) Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytom Part A* **71A**: 592–598.

Simon N, LeBot N, Marie D, Partensky F & Vaulot D (1995) Fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. *Appl Environ Microb* **61**: 2506–2513.

Slater ML, Sharrow SO & Gart JJ (1977) Cell cycle of *Saccharomyces cerevisiae* in populations growing at different stages. *P Natl Acad Sci USA* **74**: 3850–3854.

Slonim N, Elemento O & Tavazoie S (2006) *Ab initio* genotype–phenotype association reveals intrinsic modularity in genetic networks. *Mol Syst Biol* 2006; **2**: 2006.0005. Epub 2006 Jan 31.1-14.

Smith JP, Khan IA & Errington RJ (2009) Cytomics and the cellular informatics – coping with asymmetry and heterogeneity in biological systems. *Drug Discov Today* **14**: 271–277.

Sogin ML, Morrison HG, Huber JA *et al.* (2006) Microbial diversity in the deep sea and the underexplored 'rare biosphere'. *P Natl Acad Sci USA* **103**: 12115–12120.

Sonnleitner B (1991) Quantitation of microbial metabolism. *Antonie van Leeuwenhoek* **60**: 133–143.

Srienc F, Arnold B & Bailey JE (1984) Characterisation of intracellular accumulation of poly-β-hydroxy-butyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow-cytometry. *Biotechnol Bioeng* **26**: 982–987.

Steen HB (1983) A microscope-based flow cytophotometer. *Histochem J* **15**: 147–160.

Steen HB (2000) Flow cytometry of bacteria: glimpses from the past with a view to the future. *J Microbiol Meth* **42**: 65–74.

Steen HB & Boye E (1980) Bacterial growth studied by flow cytometry. *Cytometry* **8**: 32–36.

Steen HB & Lindmo T (1979) Flow cytometry: a high-resolution instrument for everyone. *Science* **204**: 403–404.

Steen HB, Boye E & Skarstad K (1982) Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects, and quantitation of antibody binding. *Cytometry* **2**: 249–257.

Steen HB, Skarstad K & Boye E (1986) Flow cytometry of bacteria: cell cycle kinetics and effects of antibiotics. *Ann NY Acad Sci* **468**: 329–338.

Stepanauskas R & Sieracki ME (2007) Matching phylogeny and metabolism in the uncultured marine bacteria, one cell at a time. *P Natl Acad Sci USA* **104**: 9052–9057.

Stephens PJ, Druggan P & Nebe-von-Caron G (2000) Stressed *Salmonella* are exposed to reactive oxygen species from two independent sources during recovery in conventional culture media. *Int J Food Microbiol* **60**: 269–285.

Stoecker K, Dorninger C, Daims H, Wagner M (2010) Double labeling of oligonucleotide probes for in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. *Appl Environ Microbiol* **76**: 922–926.

Stöhr M & Petrova L (1975) The alkaline hydrolysis of nucleic acid for removal of RNA associated fluorescence in phenantridium related flow through cytofluorometry. *Histochemistry* **45**: 95–99.

Sträuber H, Hübschmann T, Jehmlich N, Schmidt F, von Bergen M, Harms H & Müller S (2010) NBDT (3-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-3-toluene) – a novel fluorescent dye for studying mechanisms of toluene uptake into bacteria. *Cytom Part A* **77**: 113–120.

Streichan M, Golecki JR & Schon G (1990) Polyphosphate-accumulating bacteria from sewage plants with different processes for biological phosphorus removal. *FEMS Microbiol Ecol* **73**: 113–124.

Suller MTE & Lloyd D (1998) Flow cytometric assessment of the postantibiotic effect of methicillin on *Staphylococcus aureus*. *Antimicrob Agents Ch* **42**: 1195–1199.

Suller MTE & Lloyd D (1999) Fluorescence monitoring of antibiotic-induced bacterial damage using flow cytometry. *Cytometry* **35**: 235–241.

Talbot G, Topp E, Palin MF & Masse DI (2008) Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Res* **42**: 513–537.

Taylor CF, Field D, Sansone SA *et al.* (2008) Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat Biotechnol* **26**: 889–896.

Thomas JC, Desrosiers M, St-Pierre Y, Lirette P, Bisaillon JG, Beaudet R & Villemur R (1997) Quantitative flow cytometric detection of specific microorganisms in soil samples using rRNA targeted fluorescent probes and ethidium bromide. *Cytometry* **27**: 224–232.

Tijssen JPF, Beekes HW & Vansteveninck J (1982) Localization of polyphosphates in *Saccharomyces fragilis*, as revealed by 4′,6-diamidino-2-phenylindole fluorescence. *Biochim Biophys Acta* **721**: 394–398.

Tolker-Nielsen T, Holmstrom K & Molin S (1997) Visualization of specific gene expression in individual *Salmonella typhimurium* cells by *in situ* PCR. *Appl Environ Microb* **63**: 4196–4203.

Trefault N, de la Iglesia R, Molina AM, Manzano M, Ledger T, Perez-Pantoja D, Sanchez MA, Stuardo M & Gonzalez B (2004) Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. *Environ Microbiol* **6**: 655–668.

Troussellier M, Courties C, Lebaron P & Servais P (1999) Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. *FEMS Microbiol Ecol* **29**: 319–330.

Tsien R & Prasher D (1998) Molecular biology and mutation of green fluorescent protein. *Green Fluorescent Protein: Properties, Applications, and Protocols* (Calfie M & Cain S, eds), pp. 97–118. John Wiley and Sons Ltd, Chichester, UK.

Ueckert JE, Nebe-von-Caron G, Bos AP & ter Steeg PF (1997) Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury. *Lett Appl Microbiol* **25**: 295–299.

Valet G (2005) Cytomics: an entry to biomedical cell systems biology. *Cytom Part A* **63A**: 67–68.

Van Dilla MA, Trujillo TT, Mullaney PF & Coulter JR (1969) Cell microfluorometry: a method for rapid fluorescence measurement. *Science* **163**: 1213–1214.

van Groenestijn JW, Vlekke G, Anink DME, Deinema MH & Zehnder AJB (1988) Role of cations in accumulation and release of phosphate by *Acinetobacter* strain 210A. *Appl Environ Microb* **54**: 2894–2901.

Vaulot D, Mane D, Olson RJ & Chisholm SW (1995) Growth of *Prochlorococcus*, a photosynthetic prokaryote, in the Equatorial Pacific Ocean. *Science* **268**: 1480–1482.

Veldhuis MJW, Cucci TL & Sieracki ME (1997) Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. *J Phycol* **33**: 527–541.

Versey G, Slade JS, Byrne M, Shepard K & Fricker CR (1993) A new method for the concentration of *Cryptosporidium* oocysts from water. *J Appl Bacteriol* **75**: 82–86.

Verthé K & Verstraete W (2006) Use of flow cytometry for analysis of phage-mediated killing of *Enterobacter aerogenes*. *Res Microbiol* **157**: 613–618.

Vital M, Hammes F & Egli T (2008) *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. *Environ Microbiol* **10**: 2387–2396.

Vives-Rego J, Guindulain T, Vazquez-Dominguez E, Gasol JM, Lopez-Amoros R, Vaque D & Comas J (1999) Assessment of the effects of nutrients and pollutants on coastal bacterioplankton by flow cytometry and SYTO-13 staining. *Microbios* **98**: 71–85.

Vives-Rego J, Lebaron P & Nebe-von-Caron G (2000) Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiol Rev* **24**: 429–448.

Vogt C, Lösche A, Kleinsteuber S & Müller S (2005) Population profiles of a stable, commensalistic bacterial culture grown with toluene under sulphate-reducing conditions. *Cytom Part A* **66A**: 91–102.

Völsch A, Nader WF, Geiss HK, Nebe G & Birr C (1990) Detection and analysis of two serotypes of ammonia-oxidizing bacteria in sewage plants by flow cytometry. *Appl Environ Microb* **56**: 2430–2435.

Walberg M, Gaustad P & Steen HB (1997) Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin susceptibility in exponentially growing *E. coli* cells by means of flow cytometry. *Cytometry* **27**: 169–178.

Wallner G, Amann R & Beisker W (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Wallner G, Fuchs BM, Spring S, Beisker W & Amann R (1997) Flow sorting of microorganisms for molecular analysis. *Appl Environ Microb* **63**: 4223–4231.

Walsh S, Lappin-Scott HM, Stockdale H & Herbert BN (1995) An assessment of the metabolic activity of starved and vegetative bacteria using two redox dyes. *J Microbiol Meth* **24**: 1–9.

Want A, Thomas ORT, Kara B, Liddell J & Hewitt CJ (2009) Studies related to antibody fragment (Fab) production in *Escherichia coli* W3110 fed-batch fermentation processes using multiparameter flow cytometry. *Cytom Part A* **75A**: 148–154.

Wiacek C, Müller S & Benndorf D (2006) A cytomic approach reveals population heterogeneity of *Cupriavidus necator* in response to harmful phenol concentrations. *Proteomics* **6**: 5983–5994.

Wickens HJ, Pinney RJ, Mason DJ & Gant VA (2000) Flow cytometric investigation of filamentation, membrane patency, and membrane potential in *Escherichia coli* following ciprofloxacin exposure. *Antimicrob Agents Ch* **44**: 682–687.

Williams SC, Hong Y, Danavall DCA, Howard-Jones MH, Gibson D, Frischer ME & Verity PG (1998) Distinguishing between living and nonliving bacteria: evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *J Microbiol Meth* **32**: 225–236.

Wilmes P, Simmons SL, Denef VJD & Banfield JF (2009) The dynamic genetic repertoire of microbial communities. *FEMS Microbiol Rev* **33**: 109–132.

Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *P Natl Acad Sci USA* **74**: 5088–5090.

Woyke T, Xie G, Copeland A *et al.* (2009) Assembling the marine metagenome, one cell at a time. *PLoS One* **4**: e5299.

Yagoda-Shagam J, Barton LL, Reed WP & Chiovetti R (1988) Fluorescein isothiocyanate-labeled lectin analysis of the surface of the nitrogen-fixing bacterium *Azospirillum brasilense* by flow cytometry. *Appl Environ Microb* **54**: 1831–1837.

Yamaguchi H, Osaki T, Taguchi H, Hanawa T, Yamamoto T & Kamiya S (1996) Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*. *J Med Microbiol* **45**: 270–277.

Yanagihara K, Tashiro M, Fukuda Y *et al.* (2006) Effects of short interfering RNA against methicillin-resistant *Staphylococcus aureus* coagulase *in vitro* and *in vivo*. *J Antimicrob Chemoth* **57**: 122–126.

Yang X, Li X, Prow TW *et al.* (2003) Immunofluorescence assay and flow-cytometry selection of bead-bound aptamers. *Nucleic Acids Res* **31**: e54.

Zarda B, Amann R, Wallner G & Schleifer K-H (1991) Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. *J Gen Microbiol* **137**: 2823–2830.

Zubkov MV & Burkill PH (2006) Syringe pumped high-speed flow cytometry of oceanic phytoplankton. *Cytom Part A* **69**: 1010–1019.