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Quantifying heterogeneity: flow cytometry of bacterial cultures

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'In contrast to standard microbiological, genetic or biochemical techniques, this method provides information on individual cells, and not just average values for the population. This ability to analyze individual cells is invaluable in studying the distribution of cell parameters in a polydisperse population, and gives access to information that cannot be obtained in any other way.' Boye & Løbner-Olesen 1990

'Flow cytometry has revolutionized the study of the cell cycle of eukaryotes. It is also possible to apply the flow cytometry principles to bacteria. . . . The importance of the flow cytometry results should not be underestimated. They provide a crucial link in the analysis of the division cycle. . . . While other experiments have substantially supported the initial membrane-elution results, the flow cytometry results determine the pattern of DNA replication without any perturbations of the cell.' Cooper 1991

Key words: analysis, bacteria, cytofluorometry, flow cytometry, heterogeneity

Abstract

Flow cytometry is a technique which permits the characterisation of *individual* cells in populations, in terms of distributions in their properties such as DNA content, protein content, viability, enzyme activities and so on. We review the technique, and some of its recent applications to microbiological problems. It is concluded that cellular heterogeneity, in both batch and continuous axenic cultures, is far greater than is normally assumed. This has important implications for the quantitative analysis of microbial processes.

Introduction and scope

Based on work by Maxwell and Boltzmann, Gibbs developed the concept of an *ensemble*, as a collection of particles possessed of the same energy (mean and time-averaged distribution).Since that time, the treatment of macroscopic systems as ensembles of microscopic particles that, averaged over time, are identical has underpinned most of even modern thermodynamics (see e.g. Welch & Kell 1986; Westerhoff & van Dam 1987). Implicitly, microbial physiologists have normally followed the same path: we describe our cultures as having a certain growth yield or respiratory rate or internal pH or rate of glucose catabolism or whatever, with the implicit supposition that this represents a full description of these variables. However, this would be true only if our cells were not only identical but *at equilibrium*, constituting what thermodynamicists call an ergodic system. Since we know that growing cells are certainly non-equilibrium in character, it is usual, even within the framework on non-equilibrium thermodynamics, to ascribe a 'local' equilibrium to the macroscopic parameters and variables (forces and fluxes) in which we are interested, thus permitting us to refer to them as possessing a 'sharp' value. This approach is generally thought acceptable (but cf. Welch & Kell 1986;

Kamp et al. 1988) since the numbers of molecules participating in say glycolysis, or the ATP 'pool', even in a single cell, means that spontaneous thermodynamic fluctuations in their 'instantaneous' value will normally be negligible in the steady state.

The greater problem, which is the focus of the present article, is that the distribution of properties of *cells* in a culture is much more heterogeneous than we normally credit or assume. Whilst of course one appreciates that for reasons connected with the cell cycle alone (see e.g. Mitchison 1971; Donachie et al. 1973; Lloyd et al. 1982, Cooper 1991) there will be a distribution of properties such as cell size and macromolecular content, our problem is more acute than this: a culture with a respiratory rate of 100 nmol.(min.mg dry weight)$^{-1}$might be made up of an ensemble of cells which all possessed this property or of a mixture in which half of the cells respired at 200 nmol.(min.mg dry weight)$^{-1}$ and half were metabolically inert (or of course a myriad of other possibilities encompassed by these extremes (Kell 1988)). Similar statements may be made for all possible parameters and variables! Such differences, in cultures which appear macroscopically identical, probably underlie the so-called 'problem of scale-up' (Kell 1987). In general, then, a full(er) description of the quantitative behaviour of a microbial culture, the topic of this Special Issue, would require that we describe our cultures not only in terms of the mean or macroscopic values of its parameters and (especially) variables but also in terms of their *distribution between individual cells*. Whilst it is not yet possible to do this for all parameters and variables of interest, it *is* now possible to *begin* this task. This is primarily due to technical advances, especially (but not exclusively) in the area of flow cytometry and its application to bacterial cultures.

Thus the purpose of this article is to outline the principles of flow cytometry, to illustrate existing and potential applications in quantitative microbial physiology, and to point out some of the conceptual and practical difficulties accompanying the analysis of heterogeneity.

Principles of flow cytometry

In flow analysis generally, perhaps best known to microbiologists via its implementation in the Coulter counter (see Harris & Kell 1985), cells are constrained (usually hydrodynamically) in a path or flowing stream and pass, *one at a time* to a sensor which analyses the property of interest at the single-cell level. The most straightforward output of the instrument is then a plot of the number of cells possessing a certain property at a certain magnitude as a function of that magnitude. This certainly qualifies as quantitative microbiology. In the Coulter counter the property is the cell volume (or more strictly the volume surrounded by the cytoplasmic membrane), and this instrument is widely exploited by those studying the cell cycle (see e.g. Lloyd et al. 1982). However, we would stress again that although the concept of heterogeneity underpins studies of the cell cycle there has been but little attempt to integrate such measurements with those more conventionally employed by microbial physiologists (but cf. Neidhardt et al. 1990; Cooper 1991).

Though an important (and not at all recent) development, the microbiological variables which may be measured by the Coulter counter are really limited to the cell volume, and it is with *optically-based* instruments that the power of flow analysis is revealed. To begin with, the extent of low-angle light scattering by a cell depends largely (though not always linearly; Salzman 1982, Davey et al. 1990a) on the mass or volume of the cell. In a generalised flow cytometer (Fig. 1) (Melamed et al. 1979; Shapiro 1988), individual particles pass through an illumination zone, typically at a rate of some 1000 cells.s^{-1}, and appropriate detectors, gated electronically, measure the magnitude of a pulse representing the extent of light scattered. The magnitudes of these pulses are sorted electronically into 'bins' or 'channels', permitting the display of histograms of number of cells *vs* channel number. The angular-dependence of scattered light provides further information on the nature of the scattering particles, and in favourable cases may be selective towards different organisms (Steen 1990). In addition, and more importantly,

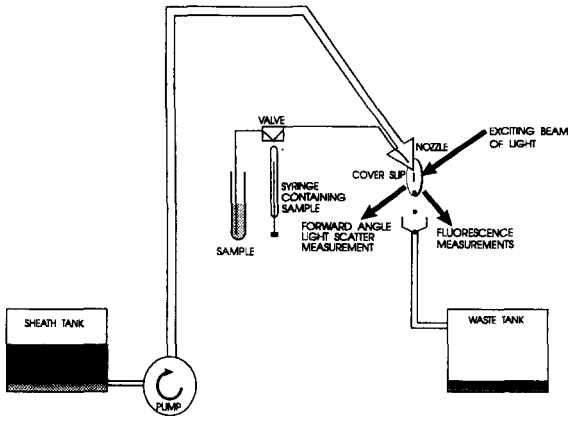

Fig. 1. The principle of flow cytometry. A pump passes fluid through a narrow tube, into which a slower-moving sample is injected as appropriate. Hydrodynamic focussing causes the sample to be constrained to the middle of the sheath fluid. In the type of system illustrated, based on the design of Steen et al. (1989) and optimised for work with microorganisms, the sample impinges on a microscope slide, and illumination is provided by a mercury arc lamp. Forward and right-angle light-scattering events are detected using photomultipliers, as (*via* suitable filters) is fluorescence, and are stored on a computer. In more traditional (and expensive) instruments, the source of illumination is a laser, and there is no cover slip; the particles pass through the zone of illumination in a jet, and may subsequently be sorted electrostatically. In either case, the computer may be used to gate measurements so that only particles scattering light or fluorescing above and/or below a critical amount are counted.

appropriate fluorophores may be added to the cell suspension. These may be stains which bind to (or react with) particular molecules such as DNA, RNA or protein, fluorogenic substrates which reveal distributions in enzymatic activity, indicators which change their property as a function of pH_{in} or which are taken up in response to membrane energisation, or, increasingly, antibodies (or oligonucleotides) tagged with a fluorescent probe. Clearly the possibilities are limited only by the ingenuity of the experimenter, and to avoid simply cataloguing these in the body of the text, we tabulate some of the better-known ones in Table 1. Whilst details should be sought in the references cited, it is worth mentioning that some, but not all, of these reagents require that the cells be fixed or permeabilised with ethanol (70 %), formaldehyde or glutaraldehyde. Several very useful overviews of technical aspects of flow cytometry (Melamed et al. 1979, Muirhead et al. 1985; van Dilla et al. 1985; Shapiro 1988; Ormerod 1990; Darzynkiewicz & Crissman 1990; Melamed et al. 1990), and its application to microorganisms (Olson et al. 1986; Scheper et al. 1987; Frelat et al. 1989; Robertson & Button 1989; Steen et al. 1989; Boye & Løbner-Olesen 1990; Patchett et al. 1990; Pinder et al. 1990; Shapiro 1990; Steen 1990), are also available.

Is a culture ever in a steady state?

From a thermodynamic point of view, it is usually desirable to study (biological) systems in the steady state (Caplan & Essig 1983; Westerhoff & van Dam 1987). Under these conditions, all transients have died down, and all variables such as metabolite concentrations or the fluxes through pathways of interest are unchanging in time. These are the types of conditions normally treated by metabolic control analysis (see Kell et al. 1989: Westerhoff et al. this issue), and those usually assumed to hold, for instance, in the mid-exponential phase of batch cultures and in any chemostat culture whose dilution rate has not altered for some 5 or more volume changes. There is, of course, some arbitrariness about the definition of a steady state, since a true or global steady state implies that, after normalisation, *no* variable is changing (by a measurable amount) in time, and it is evident that the more variables one measures *on individual cells* the less chance will there be of ever persuading a culture to come to an *observable* (let alone true) steady state.

With a growing appreciation of the complexity of the dynamics of nonlinear systems (e.g. Glansdorff & Prigogine 1971; Gleick 1987; Moon 1987; Glass & Mackey 1988; Wolpert & Miall 1990) naturally comes the realization that quite small changes in a parameter that might normally be considered insignificant can have far-reaching consequences for the (time-)evolution of the system. In what follows, we wish to illustrate some of the unexpectedly complex dynamics of microbial cultures that have been observed, and to indicate the possibility, and means, of exploiting flow cytometry for their analysis.

Growth in batch cultures may be exponential but not balanced

When a batch culture is inoculated with a small inoculum that has been grown on the same medium, it is generally observed that after a short lag period the culture enters an exponential phase of growth (at its μ_{max} for the medium and other parameters such as temperature) that will continue until medium constituents are exhausted or toxic end-products accumulate to inhibit cell growth and division. The usual criterion for assessing the exponentiality of growth is to take measurements of the optical density or of the cell numbers in the culture; if a straight line occurs when these are plotted semi-logarithmically against time the culture is deemed to be exponential, and the growth taken to be balanced (in that μ_{max} represents a boundary value, and it would be a remarkable coincidence if major changes in the metabolic make-up of the cell were unaccompanied by changes in μ_{max}). At least two recent studies show that this is a highly dangerous practice.

Skarstad et al. (1983) studied the growth of *Escherichia coli* in batch culture, and measured both light scattering and DNA content of individual cells (the latter using a combination of ethidium bromide and mithramycin) via flow cytometry. Even when the culture growth was strictly exponential as judged by cell numbers, however, the

Table 1. Some determinands amenable to analysis by flow cytometry.

Determinand	Stain or reagent	Selected reference(s)
DNA	Hoechst 33258, Hoechst 33342, Ethidium bromide, propidium iodide, DAPI, Acridine orange., Chromomycin A_3, Mithramycin, Olivomycin	Darzynkiewicz 1979; Steen et al. 1982; Muirhead et al. 1985; Seo et al. 1985; Seo & Bailey 1987; Frelat et al. 1989; Sanders et al. 1990; Steen et al. 1990
RNA	Ethidium Bromide, Propidium iodide, Acridine Orange, Pyronin Y, Thioflavin T.	Darzynkiewicz 1979; Tanke 1990; Waggoner 1990
Protein	Fluorescein isothiocyanate, Rhodamine 101 isothiocyanate (Texas Red)	Hutter & Eipel 1978; Steen et al. 1982; Miller & Quarles 1990
Chlorophyll Phycoerythrin Carotenoids	Autofluorescent	Olson et al. 1986; Robertson & Button 1989; Cunnigham 1990 An et al. 1991
Enzyme activities	Substrates linked with: naphthoyl-, fluorescein-, umbelliferyl-, coumaryl- and rhodamine groups β-galactosidase	Dolbeare & Smith 1979; Kruth 1982 Srienc et al. 1986; Wittrup & Bailey 1988
Antigens	Fluorescently-labelled antibodies	Ingram et al. 1982; Steen et al. 1982; Frelat et al. 1989; Srour et al. 1991
Nucleotide sequences	Fluorescently-labelled oligonucleotides	Amman et al. 1990; Bertin et al. 1990
Internal pH	Numerous	Rabinovitch & June 1990b; Waggoner et al. 1990
Membrane fluidity	Anthroyloxy-labelled fatty acids	Collins & Grogan 1991
Inclusion bodies poly-β-hydroxy-butyrate	Changes in light-scattering behaviour	Wittrup et al. 1988 Srienc et al. 1984
Cellular Morphology		Betz et al. 1984; Allman et al. 1990 Hunter & Asenjo 1990
pCa	Aequorin, Indo-1, Fluo-3	Rabinovitch & June 1990a
Membrane energisation	Oxonols, cyanine, rhodamine 123	Ronot et al. 1986; Rabinovitch & June 1990a,b; Shapiro 1990; Kaprelyants & Kell 1991

distributions of both DNA and light scattering were highly inconstant. They concluded (correctly) that this was the likely cause of the variability in the reported cell cycle parameters of slowly-growing batch cultures of this organism (see also Jepras 1991). Steen (1990) extended this study to include optical density measurements, and found that whilst both the OD and the cell number increased in a strictly exponential manner, *they did so with different doubling times*, that based on cell counts being the shorter (18 vs. 23 min). It is difficult to implicate changes in the nutritional status of the medium, since the highest optical density considered was only 1 % of that attained in stationary phase. The sins of the parents, one might say, extend even unto the third and fourth generations.

This issue of heterogeneity within cultures of what is notionally a single clone comes on top of that which one might expect to find between different strains (and one might comment that flow cytometry could be of taxonomic utility). Even within a supposedly tightly defined taxon, however, Allman et al. (1991) found, using similar methodology to that described in the previous paragraph, that strains of *Escherichia coli* K-12 as a group are rather dissimilar to each other with respect to the pattern of their DNA replication, a finding consistent with the rapid changes in populations which are evidenced by the polymorphisms observable in this organism (Krawiec & Riley 1990).

Due in particular to their possession of a number of cofactors, cells are autofluorescent when excited with light of appropriate wavelengths. Fig. 2 shows the distribution in both light-scattering and autofluorescence of cells of *Micrococcus luteus* grown in batch culture. At the exciting and emission wavelengths used, the autofluorescence is ascribable predominantly to reduced flavin and pyridine nucleotides. Whilst one might have expected that larger cells would have a greater autofluorescence, the data observed (Fig. 2) show that there are functionally two populations of cells, with 'low' and 'high' autofluorescences that are not correlated with cell size, in what is by normal microbiological criteria an axenic culture multiplying exponentially under conditions of balanced growth. The protocol of the experiment (Fig. 2) suggests that the heterogeneity observed is not due to *genotypic* differences between the cells, whilst the fact that the accumulation of the flow cytometric data takes time does not permit one to exclude the existence of *oscillations* in the pyridine nucleotide concentrations in individual cells.

The steady state in chemostat cultures

Based on the kinetics of exponential washout, it is usually assumed that after a change in dilution rate, a culture attains the steady state characteristic of the new dilution rate after some 5 volume changes. Certainly this is a longer period than that considered in the usual batch culture, but one may doubt that even this is sufficient if *distributions* of properties are considered. To date, we are not aware of any studies that have looked carefully at this question, and flow cytometry obviously opens up many possibilities in this area. A strong pointer is given by the work of Rutgers et al. (1987), who found that the steady-state glucose concentration in glucose-limited chemostat cultures continued to decrease for as much as 50 generations after a change in dilution rate, long after the steady-state biomass level (and hence Y_{glu}) had been reached. Cells taken from the culture during this period showed a continuous increase in μ_{max} and cell size (measured with a Coulter counter), and a decrease in K_s. Based on the relevant kinetics, it was argued that the changes were likely to be genotypic in nature, and one may certainly state that cells which have high rates of 'spontaneous' mutation will eventually outcompete those which do not, since the former will eventually acquire beneficial mutations (Chao & McBroom 1985). Broadly similar data were obtained by Höfle (1983).

The above analysis is but one example of the long-term dynamics of continuous cultures; those in which the growing organism harbours a plasmid are of course notoriously complex (e.g. Caulcott et al. 1987; Weber & San 1990), and the selection pressures easy to construe (Westerhoff et al. 1983). However, conventional selection pressures can hardly explain the *oscillatory* behaviour of continuous cultures (Heinzle et al. 1982; Koizumi & Aiba

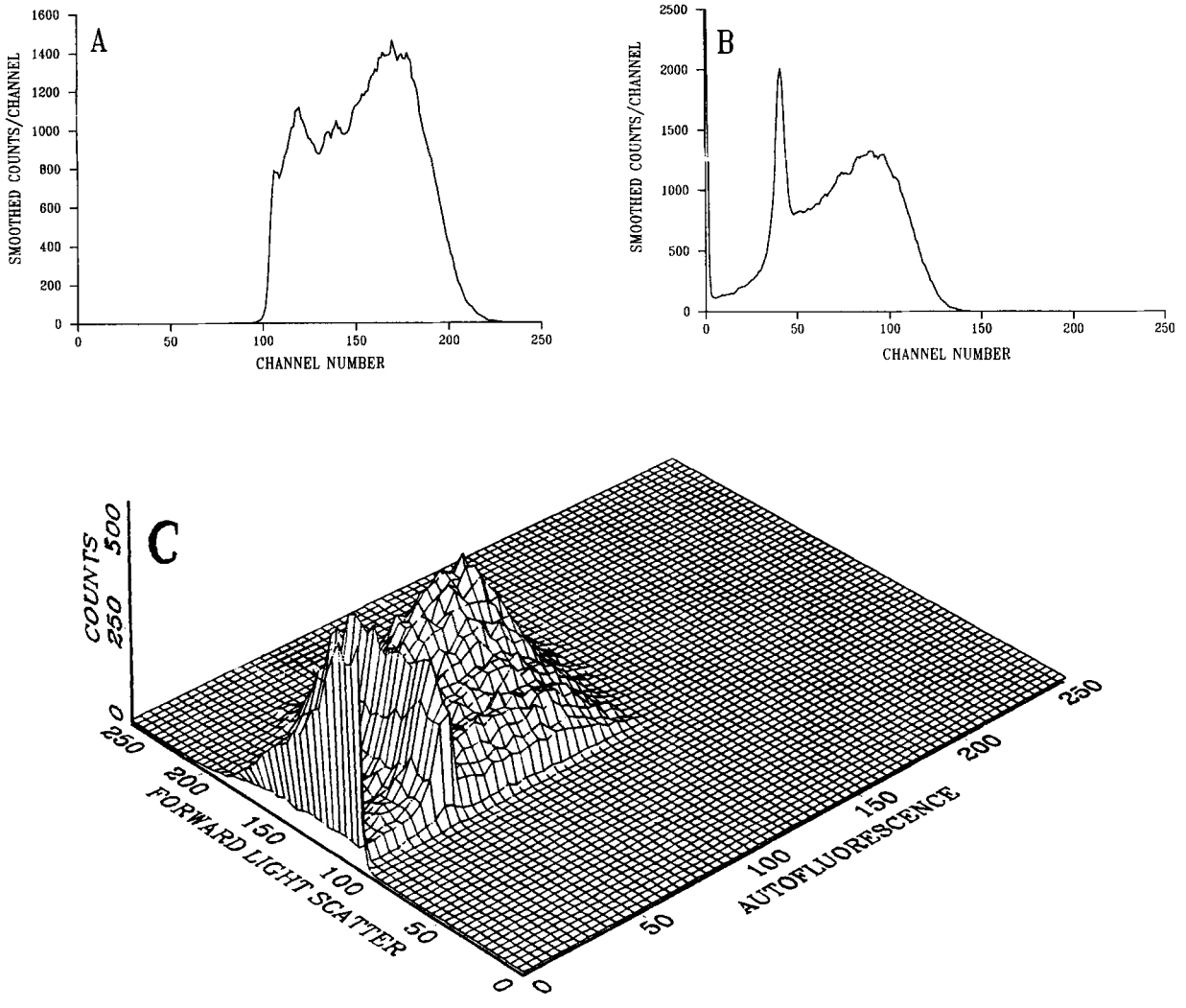

Fig. 2. Flow cytometric behaviour of the light-scattering *(A)* and autofluorescence *(B)* of a batch culture of *Micrococcus luteus*, and of their 2-dimensional distribution *(C)*. A single colony was selected following repeated streaking onto agar and growth in broth. Cells were grown in batch culture on 1.3 % Nutrient E broth, to stationary phase, and inoculated to give an optical density (680 nm) of 0.11. The data illustrated are from a sample taken when the OD_{680} of the culture was 1.93 (i.e. after more than 4 doublings had taken place). Flow cytometry was performed as described (Davey et al. 1990a,b, Kaprelyants & Kell 1991, Markx et al. 1991a,b), save that fluorescence was measured using a filter block with the following characteristics: excitation 395–440 nm, bandstop 460 nm, emission 470 nm and above. In *(A)* and *(B)* data were passed through a 3-point smoothing filter prior to plotting (Davey et al. 1990a). The photomultipler voltages for the light-scattering and fluorescence channels were respectively 450V and 950V, and the scales are logarithmic. Very similar flow cytometric data were obtained in samples taken at all stages of the growth of the culture. In addition, samples were taken periodically throughout the culture and streaked out to ensure that the culture remained axenic.

1989), and it is evident that we have a long way to go before we can claim a good understanding of these highly nonlinear processes. Experimentally tiny fluctuations in what are ostensibly parameters, such as oxygen tension and the concentrations of inhibitory molecules, may effect substantial changes in steady-state variables such as dry weights, and whilst the importance of 'perfect' mixing has been known for many years (e.g. Sinclair & Brown 1970), recent studies show that the relevant microscale (50–300 mm) below which turbulence is not manifest, even in highly-agitated fermentors, is far

greater than that of the dimensions of typical microorgansims (Fowler & Dunlop 1989; Dunlop & Ye 1990), providing a substantial contribution to heterogeneity in CSTRs. Indeed, although the mathematical analysis of bacterial size distributions is rather highly developed (e.g. Harvey 1983), the size of a microorganism is actually something that is only rarely considered a contributor to fitness or selection. Indeed, the analysis of selection in chemostats (see Kubitschek 1974; Dykhuizen & Hartl 1983), and in more complex ecosystems (e.g. Robertson & Button 1989), constitutes a fundamental topic, which is undoubtedly of biotechnological importance and which is conveniently carried out using flow cytometric procedures.

Life, death and other states

It may be taken that the most fundamental question which a microbial physiologist might ask about a cell is whether it is alive or not. This turns out to be far from simple to answer. It is well known, especially in Nature, that the number of cells observable by direct counts greatly exceeds the number capable of forming colonies (and thereby considered 'viable') (see Postgate 1976; Poindexter 1981; Morita 1982; Mason et al. 1986; Kjellberg et al. 1987; Roszak & Colwell 1987; Morita 1988; Matin et al. 1989; Gottschal 1990). However, not all the non-'viable' cells are 'dead', since many of these 'non-culturable' cells may be resuscitated by preincubation in a suitable nutrient broth prior to plating out. We may refer to such cells as 'vital'. The question then arises, for instance in slowly-growing cultures, as to what causes a cell to pass from the status of viable through vital to dead, and whether cells of each type in such cultures, though nominally homogeneous, coexist.

Several workers have studied the decline in ATP, adenylate energy charge, and/or the ability to accumulate lipophilic cations in starving cells or in cells grown at low dilution rates (Horan et al. 1981; Jones & Rhodes-Roberts 1981; Zychlinski & Matin 1983; Otto et al. 1985; Poolman et al. 1987), generally finding that none of these bioenergetic parameters could be correlated with the loss of viability (as judged by plate counts). However, these types of experiments possess the following, insurmountable problem: they represent bulk or ensemble measurements and it is therefore not possible (given our ignorance about the 'critical' values of these, if any, for the individual cell) to distinguish whether a decrease in ATP levels or in the uptake of the tetraphenylphosphonium cation, say, is due to the irreversible death of a proportion of the cells or an identical decrease of these parameters, unaccompanied by death, in all cells (or of course any combination of these extreme possibilities (Kell 1988)). In recent work, we have shown by flow cytometry (Kaprelyants & Kell 1991) that *Micrococcus luteus* cells grown in a chemostat at a low dilution rate (and even those grown in batch culture at μ_{max} (Fig. 3)) are *extremely* heterogeneous with respect to their ability to accumulate the lipophilic cationic dye Rhodamine 123. In particular, and in contrast to earlier suggestions based on bulk measurements, we found (Kaprelyants & Kell 1991) that cell viability, and resuscitation, *could* be quite well correlated with the ability of individual cells to accumulate the dye. Indeed, it was possible in part to relate the degree to which individual cells accumulated rhodamine 123 and the distinguishable physiological states ('viable', 'non-viable' and 'non-viable but resuscitable') exhibited by cells in the culture. Thus flow cytometry of cells stained with Rh 123 (or other appropriate dyes) allows one rapidly to distinguish not only 'viable' and 'non-viable' cells but the *degree of viability of individual cells* reflecting the heterogeneity of a culture observable following sub-lethal starvation, stress or injury.

Implications of heterogeneity for the analysis of microbial behaviour

From the experiments just described, it is obvious that the flow cytometric approach gave an answer that was exactly opposite to that which had previously been opined by others on the basis of cognate macroscopic experiments on *cultures*. Since these other workers had expected a correlation to exist between (say) adenylate energy charge or the up-

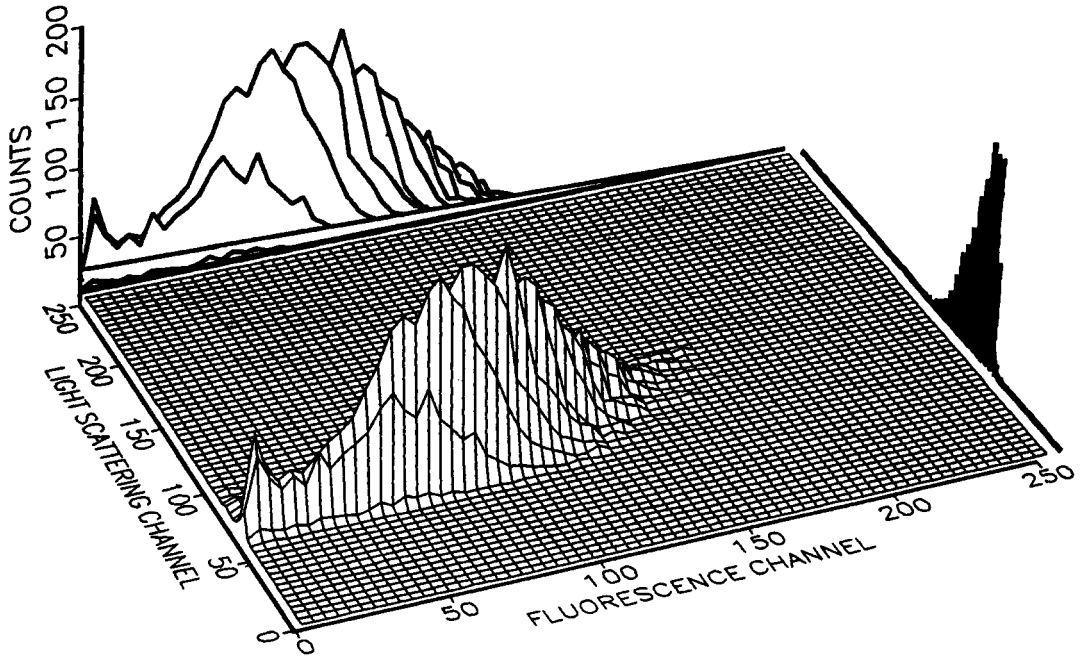

Fig. 3. Flow cytometric behaviour of the light-scattering and fluorescence of a batch culture of *Micrococcus luteus* stained with rhodamine 123. Cells were grown in batch culture on lactate minimal medium as described (Kaprelyants & Kell 1991), to an optical density (680 nm) of 1.8. Cells were stained with rhodamine 123 (final concentration 0.26 μM), incubated at room temperature 10 min, and flow cytometry was performed as described (Kaprelyants & Kell 1991). The optical characteristic of the relevant filter are: Excitation 470–495 nm, band-stop 510 nm, emission 520–550 nm. The photomultipler voltages for the light-scattering and fluorescence channels were respectively 500V and 650V, and the scales are logarithmic.

take of the tetraphenylphosphonium cation and viability on plates, it is important to understand the relevant points.

An example may serve to illustrate this. Suppose one wishes to understand how a physiological flux or process in a microbe depends on the intracellular concentration of its substrate. We assume that there is a single reaction determining the rate of the process (i.e. a reaction with a control coefficient of 1 on the overall process), that this reaction is insensitive to the concentration of its product, and that it follows simple Michaelis-Menten kinetics. In order to examine whether regulation of the process occurs only through the concentration of the substrate of the reaction, or if there is additional allosteric regulation, the relationship between the intracellular substrate concentration and the process rate is determined experimentally in a population of cells for two series of physiological transitions in which the concentration of S is modulated by two separate means, and data concerning the flux and the (average) substrate concentration obtained. The idea is that, if the relationship between the rate of the process rate and concentration is not the same for the two series of experiments, the hypothesis that regulation is only through the substrate is falsified. We shall now show why, if the culture is heterogeneous, one would in fact obtain an *artefactual* falsification of the hypothesis.

Let us compare (i) a homogeneous population of cells in which the substrate concentration happens to be equal to the K_m of the flux-controlling enzyme (and the rate of the process therefore at $V_{max}/2$) to (ii) a heterogeneous population with the same average substrate concentration. An extreme case of heterogeneity would be constituted by a population in which half the cells contain the substrate at zero concentration, whereas the other half contain it at a concentration equal to twice the K_m. This gives rise to an average rate of the process for the heterogeneous population of $(0 + 2/(2+1))/2$ times $V_{max} = V_{max}/3$. Now, if in one of the series of

experiments, only 50% of the cells are affected by the physiological transition chosen, whereas in the other physiological transition, all cells are equally affected, but to the same average substrate concentration, the two sets of transitions will reveal different overall fluxes at the same average substrate concentration. If the possibility of heterogeneity were not considered, this could easily lead to the conclusion, that there is (normally) additional regulation, which differs between the two physiological transitions. Clearly, quantitative approaches (such as flow cytometry), which analyse the *distribution* of cell properties between members of a population, are essential for the analysis of microbial physiology whenever the possibility of sigificant heterogeneity exists.

The essence of the problem is that one is trying, typically, to correlate a rate of change (v) of a certain variable with respect to the value of a certain property (p), and that a correlation may be expected between the mean values v and p *only* if v is kinetically of first order with respect to p. Whilst the previous example used the relationship between an internal substrate concentration (a variable) and a certain flux, the problem also exists when p is a parameter. Indeed, when one studies the extent to which the activity of an enzyme determines growth rate of a cell, for instance, one has to consider the possibility that the activity of that enzyme may be distributed heterogeneously between individual cells. Similarly, the heterogeneity (in viability) of chemostat populations at low dilution rates can lead to substantial errors in the estimation of Monod coefficients (Sinclair & Topiwala 1970).

Other cases in which unsuspected heterogeneity may be expected to have signifcant effects upon the kinetic analyses of microbial processes include fluctuating systems (Westerhoff et al. 1986), membrane bioenergetics (see the experiments with rhodamine 123, above) and stochastic systems in which small numbers of repressor molecules (say) may control gene expression.

Given the general nonlinearity of biochemical reactions, and the existence of thresholds, one may anticipate that the discovery of important heterogeneities will be the rule, and not the exception, when cultures are investigated by flow analysis.

Future prospects: cataloguing complexity

In the above, we have concentrated mainly on illustrating heterogeneities in the distributions of but a few parameters in cultures, not least because they are easy to display graphically in a 2-D plot. However, the ability to discriminate (sub)populations of cells will increase as the number of the measured parameters increases. Even a modest extension of this philosophy (of looking at many independent parameters on each cell) is likely to end by showing us that our cultures consist, when viewed in multi-dimensional space, of many more populations than we normally consider, a fact which follows simply from the properties of normal distributions (Williams 1956)). This does not of itself seem to be an intellectually useful goal, and it is not possible, in a simple way, to visualise the distribution of populations in multi-dimensional space. We therefore wish to end by discussing qualitatively the types of analytical advance that *could* benefit the practising microbial physiologist.

Technically, it is now fairly straightforward to acquire several measurements on an individual cell during its passage through a flow cytometer, and Kachel et al. (1990), for instance, describe an 8-parameter system based on a simple personal computer. Robinson et al. (1991) go even further, and acquire a set of multicolour immunofluorescence data from a given sample incubated with 11 tubes containing multiple phenotypic markers. One may imagine that the exploitation of diode array detectors in flow cytometry will produce even more data which one might wish to exploit for the analysis of heterogeneity! Since preparing a table listing the magnitude of each of the many determinands for each of the cells studied does not convey the observations in a digestible form, how are we to extract the salient features of an n-parameter data set, manipulate them mathematically, and encapsulate them in 2 (or at most 3) dimensions? Clearly the major problem is that of reducing the dimensionality of the representation of the data (Sychra et al.

1978), a problem similar in essence to some of those that are being faced and solved by bacterial taxonomists (Goodfellow et al. 1985; Austin & Priest 1986) and analytical chemists (Massart et al. 1988; Levy et al. 1991).

Conventional approaches to reducing the dimensionality of multivariate data, such as principal components analysis (PCA) (see Chatfield & Collins 1980; Causton 1987; Flury & Riedwyl 1988) and the related canonical variate analysis, work by transforming correlated variables exhibited by the cell, organism or molecule of interest into uncorrelated ones, and projecting these transformed variables onto a two-dimensional plane. Different populations are thus separated to a greater or lesser extent, depending on the method used and the structure of the data. In PCA, the two largest principal components are usually plotted against each other, and can show the clustering or otherwise of individual cells in the population according to these principal components. Statistical analysis permits statements to be made concerning the extent (%) to which (say) the first two principal components account for the variance in the sample.

In PCA, which is in fact but a subset of the Universe of possible pattern recognition algorithms, we are trying to construct a relation between several 'input' properties (the measured determinands on each cell) and some output property (for instance the cell's taxonomic status, or even *if* it is a cell and not a piece of debris). The conceptual disadvantages with the above PCA approach, however, are that (i) it assumes that all variables of interest (inputs) are linearly related to each other, which is *a priori* unlikely, and (ii) as a linear mapping it is inevitably associated with a loss of information. Thus, nonlinear mappings may in general be expected, and are found (Aoyama & Ichikawa 1991; Rose et al. 1991), to give much better discrimination or classification.

Neural networks (in this context, more strictly, *artificial* neural networks) are collections of very simple 'computational units' which can take a numerical input and transform it into an output (see McClelland & Rumelhart 1988; Kohonen 1989; Pao 1989; Simpson, 1990). The inputs and outputs may be to and from the 'external world' or to other units within the network. The way in which each unit transforms its input depends on the so-called 'connection weight' (or 'connection strength') and 'bias' of the unit, which are modifiable. The output of each unit to another unit or to the external world then depends on both its strength and bias and on the weighted sum of all its inputs, which are transformed by a (normally) nonlinear weighting function referred to as its activation function. The great power of neural networks stems from the fact that it is possible to present ('train') them with known inputs (and outputs) and to provide them with some form of learning rule which may be used, iteratively, to modify the strengths and biases until the outputs of the network as a function of the inputs correspond to the desired ('known' or 'true') outputs. The trained network may then be exposed to 'unknown' inputs which it will then be able to relate to the appropriate outputs.

A neural network therefore consists of at least 3 layers, representing the inputs and outputs and one or more so-called 'hidden' layers. It is, in particular, the totality of weights and biases of the interactions between inputs and outputs and the hidden layer(s) which reflect the underlying structures of the system of interest, even if its actual (physical) structure is not known. By training up a neural network with known data, then, it is possible to obtain outputs that can accurately predict the behaviour of systems, such as the (continuing) evolution of a time series, even if it is (deterministically) chaotic (Wolpert & Miall 1990). Although the training may be lengthy, the great advantage is that, once trained, interrogating the network is practically instantaneous and no lengthy, iterative processes are required.

In the present context, it is clear that trained neural networks have the potential to reduce the dimensionality of a graphical display by arbitrary amounts, since one may have all the parameters that one measures on a cell as the input nodes to the network and two output nodes representing the X- and Y-coordinates of a 2D-plot. The only question then is how one trains the network. In fact, for this, one simply needs 'standards' which one may assign, arbitrarily, to specific classes (well-separated and appropriately-chosen (x,y) coordinates in one's re-

duced display), which one can then use to train the network using the dataset of inputs and arbitrarily-assigned outputs. (Alternatively, simply to discriminate *n* types of cells or subpopulations one would provide *n* outputs, which take the value of 1 if the cell is of the stated type, otherwise zero.) After the network has been trained, which may be a lengthy process, one may test it on samples used it in the training set (to check its performance against what was a *known* input) and then, of course, on unknown populations of interest. Thus, by combining flow cytometry with the abilities of trained neural networks, one may expect to be able to provide accurate classifications of cell populations that are easy both to visualise and to comprehend.

Concluding remarks

We have seen that the technique of flow cytometry, though a relatively recent development in microbiology, has allowed penetrating insights into hitherto unrecognised aspects of the physiology of microorganisms. The observations have shown that not all problems of microbial physiology are amenable to correct analysis by performing conventional macroscopic measurements on representative samples of whole cultures. Indeed, one might hazard that a reevaluation of some fundamental tenets may be forthcoming when cellular heterogeneity is taken properly into account. The quantification of heterogeneity therefore constitutes a crucial component of the quantitative analysis of microbial metabolism.

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