APPROACHES TO THE ESTIMATION OF MICROBIAL VIABILITY USING FLOW CYTOMETRY

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Mini-Abstract

For microorganisms in particular, "viability" is a term that is difficult to define and a state consequently difficult to measure. The traditional (and 'gold-standard') usage equates viability and culturability (i.e. the ability to multiply), but determining culturability is often too slow for our needs. Flow cytometry provides us with the opportunity to make rapid and quantitative measurements of dye uptake in large numbers of cells, and we can therefore exploit the flow cytometric approach to evaluate so-called viability stains and to develop protocols for more routine assessments of microbial viability.

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Introduction

"*At present one must accept that the death of a microbe can only be discovered retrospectively: a population is exposed to a recovery medium, incubated, and those individuals which do not divide to form progeny are taken to be dead........there exist at present no short cuts which would permit assessment of the moment of death: vital staining, optical effects, leakage of indicator substances and so on are not of general validity*"......*The term 'viability' applies to populations, not individuals (except in an all-or-none sense: an individual is either viable or nonviable*) (p.5)

(Postgate, 1976)

"*An organism is considered living or viable if it is capable of continued multiplication; if it is not so capable it is called dead or non-viable.*" (p.31)

(Greenwood *et al.*, 1992)

One of the most basic questions that a microbiologist might ask of a microorganism is whether it is alive or not, and in microbiology, it is often necessary to determine the number of living (often termed 'viable') cells in a sample or culture of interest. However, perhaps surprisingly, this is a question that is not always easily answered (Kaprelyants *et al.*, 1993; Kaprelyants *et al.*, 1999; Kell *et al.*, 1998), even for *macro*organisms (Watson, 1987). The "gold-standard" determination of the number of viable microbial cells in a sample is usually achieved by plating a 0.1-1 ml sample of cells (diluted as required) onto an agar plate (Hattori, 1988; Postgate, 1969), and scoring as viable (*a posteriori*) those that were able to form visible colonies. The *culture* viability is then the ratio of these to the total cell count in the original sample determined microscopically. However, there are several problems associated with this technique, not least the length of time required to

obtain the results. For some slowly-growing organisms (e.g. mycobacteria) it may take several weeks to determine how many cells were "viable" in the original sample, and even when the sample contains fast-growing organisms and the plates are incubated under optimal growth conditions, a minimum of overnight growth is usually required before the resulting colonies can be counted. In clinical situations and for economic reasons, such a delay is unacceptable, and many so-called "rapid" methods have been developed to allow a speedier assessment of the "viable" microbial load in a sample (e.g. Adams and Hope, 1989; Fung, 1994; Harris and Kell, 1985; Jones, 1987)

These alternative and so-called "rapid methods" of viability measurement include a variety of stain-based methods. Such so-called "vital" stains which have been used in attempts to estimate microbial viability fall into three broad categories:

- Some dyes (e.g. propidium iodide) are excluded by the intact membranes of viable cells and therefore the presence of the dye within the cell indicates disruption of the cell membrane and thus may be expected to be correlated with cell death.

- Other dyes (e.g. rhodamine 123 (Rh123)) are actively accumulated by viable cells; thus the number of brightly stained cells will reflect the viability of the sample (although in some cases more active cells can actually pump such dyes out (Jernaes and Steen, 1994)!). Equivalently, some dyes are less tightly bound by energised membranes and so the more active cells are *less* brightly stained.

- In the case of dyes such as fluorescein diacetate a membrane-permeant non-fluorescent pre-cursor is converted to a membrane-impermeant fluorescent molecule by the activity of intracellular enzymes and thus is an indicator of metabolically active cells. These approaches will be discussed in more detail below.

A further problem with the plate count approach is that although it is usually considered to be the gold-standard measure of viability, a plate count actually only indicates how many of the cells can replicate under the conditions provided for growth. In the case of environmental samples the laboratory media, temperature etc may differ substantially from those in the original sample (Roszak and Colwell, 1987) and thus the

proportion of cells that can divide and form colonies may be expected to be (and indeed is (Amann *et al.*, 1995)) much lower than the number of cells that would score as "viable" using the dye-based rapid methods.

Although the rapid stain-based methods would therefore seem to offer the possibility of a major improvement over the plate count method, the plate count method has remained the gold standard. In part this may be due to the fact that traditional microscopic analyses of stained cells are still time consuming for the scientist and can lead to operator fatigue; thus conclusions are normally drawn from the analysis of at best a few hundred cells. Equally, microscopic examination is largely a qualitative technique and thus a judgement of "alive" or "dead" is all that is possible, and the interpretation of the extent of staining of the cells in the sample may vary between operators.

Flow cytometry offers an alternative method of determining the amount of fluorescent dye taken up by each cell in a population. Since *quantitative* measurements can be made very rapidly on a large number of *individual* cells an accurate picture of the distribution of dye uptake by many thousands of cells is possible within a few minutes of obtaining a sample. The flow cytometric approach will be discussed in more detail below but first we must try to define what is meant by the term 'viability'.

The Problem of Determining "Viability"

A microbial cell is generally considered "viable" if it possesses all the components and mechanisms necessary for sustained proliferation. Viability is evidently best determined by assessing cellular proliferation directly, and scoring only those cells that have visibly multiplied, but the underpinning assumption of rapid microbiology is that we can estimate something which might correlate with culturability just by assessing the presence and functionality of *individual* "vital" factors and processes.

The first ("classical") method of viability determination involves the provision of "suitable" nutrients and media and a "suitable" method of detecting growth, while the second ("cytochemical") approach gives an insight into the physiology of individual cells by providing data on parameters such as membrane energisation, enzyme activity etc. Both approaches have their advantages and problems, and studies in which the same cells are compared using each approach are regrettably rare indeed. The classical approach requires *a priori* knowledge of the "suitable" growth media and conditions for the organism or organisms present in the sample, and a "suitable" method of growth detection for the organisms must also be established. In practice, because of limitations of time, materials and prior knowledge, all too often the most convenient method is chosen. This usually involves the aerobic incubation of the sample at a temperature that is high relative to those from which the sample was collected (e.g. $\geq 30^{\circ}C$ may be used even in the case of environmental samples). High-nutrient complex media such as Luria broth and TSB (tryptic soy broth) are often used for these procedures although they are very different from conditions found in nature. Growth detection is usually measured either as colony-forming units on a solid agar plate or as turbidity in liquid media. There are several problems with this kind of approach:

- Many microorganisms have growth requirements that are very different from the "standard" conditions often applied - in fact, several medically important bacteria (such as *Mycobacterium leprae*) and the vast majority of bacteria in the environment have not yet been cultured axenically by any method devised

so far (Amann *et al.*, 1995), and in many cases where we have eventually succeeded (a well-known example is *Legionella* (Meyer, 1983)), organisms have defied our efforts to culture them until the critical component had been added to the medium.

- Microorganisms with known growth requirements may reside in a physiological state in which the (otherwise appropriate) standard culture conditions do not support growth, or do so only for a small fraction of the population, or only after long lag phases. Physiological states that can be difficult or impossible to detect include injury ("stress"), starvation (or "stationary phase"), and dormancy ("latency" or "cryptobiosis") (Kell *et al.*, 1998), and, in some cases at least, normally copiotrophic bacteria can be recovered only after incubation in comparatively oligotrophic conditions (MacDonell and Hood, 1982; Mukamolova *et al.*, 1998b).

- In some cases, growth of viable cells can remain undetected due to the constraints of the growth-determination method employed. Organisms displaying slow growth rates or long lag phases may not be capable of producing enough biomass to form visible colonies or detectable turbidity during the period of incubation allowed. In some cases cessation of growth may occur after a limited number of divisions (Kell *et al.*, 1998), or the organism may be unable to form colonies on solid media. These factors, alone or in combination, may lead to false-negative results.

- In addition, it is of course entirely plausible (and even likely (Kaprelyants and Kell, 1996; Kaprelyants *et al.*, 1999; Mukamolova *et al.*, 1998a)) that dormant and "uncultured" microorganisms actually need autocrine or paracrine growth factors for their cultivation *in vitro*.

Thus the main drawback of the classical, growth-based viability assays is the possibility of false-negative results; false-positives can be excluded by correct sterile technique. But if we equate viability with culturability, only growth-based viability assays make any true sense, and thus proliferation-based viability assays may be considered to be both necessary and sufficient criteria for cellular viability.

This said, there are occasions in which it is the metabolic activity of the cells which may concern us, whether they are capable of multiplication or not. Clearly a cell whose DNA had been damaged at the origin of replication could not multiply, but the rest of its activities would probably be unaffected. If these activities included the production of a toxin we should be much more interested in a method which detected metabolic activity than one which required proliferation to score for cellular presence and activity.

Cytochemical assays can have several advantages over proliferation-based assays. They are generally less time-consuming, in some cases delivering instantaneous results, and facilitate (at least potentially) a method of measuring something which one might hope to be able to correlate with other measures of viability (such as culturability) in organisms for which "suitable" growth conditions have not been established. For organisms which display extremely slow growth rates, long lag phases or low growth yields, proliferation-based methods are often impossible or impractical, and thus the cytochemical approach offers an attractive alternative. In some cases (e.g. flow cytometry), the cytochemical approach allows simultaneous analysis of the taxonomic traits by specific antibodies or ribosomal RNA probes (Amann *et al.*, 1995; Vesey *et al.*, 1995; Wallner *et al.*, 1993). Thus "multidimensional" snapshots of mixed populations can be generated, providing information on species composition and physiological status of cell populations.

These "rapid" assays, however, often have their own drawbacks. They can be difficult to interpret, and their utility may be hampered by the following problems:

- So far, no viability assay has been developed that selectively and reliably detects viable cells, without, under any circumstances, giving a signal with 'dead' cells (*operationally* defined (Barer *et al.*, 1998; Kell *et al.*, 1998) as cells unable to form a colony on a plate under any condition tested (Kaprelyants *et al.*, 1993)). This is due to the fact that assays are normally based on single parameters such as membrane energisation (often referred to, despite the absence of evidence for it in bacteria (Kell, 1988; Kell, 1992), as membrane *potential*), enzyme activity, or uptake of substrate. Some of these criteria might be considered

necessary to define viability in most cases, but none of them is sufficient to exclude non-viable cells. Thus these assays can give rise to false positive results. For example, a cell could display some enzyme activity but may have lost its ability to divide by lethal lesions in the chromosome. Thus it is of the utmost importance to ascertain the reliability of any "viability assay" by negative control experiments, preferably involving samples of cells killed by a range of treatments (heat, ethanol, chlorine etc.).

- Commercial "viability assays" (see later) can also produce false negative results, even if the suppliers of assay kits include seemingly convincing data supporting their reliability (albeit under rather restricted conditions). In general, the test populations employed to demonstrate detection of viable microbes are either growing cells or cells subjected to rather short periods of stress (heat, cold etc). In natural environments starvation and/or stress may be long-term, and the activity of cells may be reduced to extremely low levels (especially in the case of dormant cells), such that positive results might be below the limits of detection of the assay. Similarly, injured cells may have damaged membranes and would score as 'non-viable' in these kits, but repair of the damage during cultivation on a rich medium would allow them subsequently to grow (such that they should have been scored as 'viable'). The apparent paradox is avoided by the use of operational definitions (Barer *et al.*, 1998; Kell *et al.*, 1998) in which 'viability' is not in fact an *innate* property of a cell but is scored as a result of our experimental measurements. Using these definitions however, we should not be suprised if different experiments lead to different results!

Taken together, the viability assays which are not based on proliferation might be less time-consuming and more convenient in a lot of cases, but they do not necessarily provide reliable data, as the principles on which they are based are not sufficient (or sometimes even necessary) criteria for viability. Thus, before use, each method for viability assessment must be validated for each organism and for each type of sample in order to avoid false-positive or false-negative results.

Fluorescent Stains for Microbial Viability Determinations by Flow Cytometry

Flow cytometry offers a rapid method for the quantitative determination of stain uptake within a sample of cells (Davey and Kell, 1996; Kell *et al.*, 1991; Lloyd, 1993; Shapiro, 1995). There are several classes of molecules whose uptake and/or fluorescence may be expected to reflect the viability of the cells (see Table 11.7.1) and these are discussed below.

i) Dye Exclusion

Fluorescent stains normally excluded by living cells have been used to assess viability on the grounds that dead cells will have leaky membranes that are permeable to the stains. Nucleic acid stains such as propidium iodide or ethidium bromide are indeed generally excluded by intact plasma membranes and their uptake is often used to indicate cell death (Aeschbacher *et al.*, 1986; Böhmer, 1985; Green *et al.*, 1994; Grogan and Collins, 1990; Jones, 1987; Lapinsky *et al.*, 1991; López-Amorós *et al.*, 1995; Schmid *et al.*, 1992).

Propidium iodide is often the dye of choice for viability determinations in animal cells, whether the assay is done using flow cytometry or fluorescence microscopy (see e.g. Garner *et al.*, 1997; Maxwell and Johnson, 1997; Ronot *et al.*, 1996). There is however, an inherent danger in blindly transferring protocols developed for one cell type to another - particularly when, of the two cell types in question, one is a eukaryotic cell and the other is a prokaryote. Thus, the applicability of such dyes for microbial viability determinations needs to be carefully considered for each type of organism, not least because efficient efflux pumps capable of removing ethidium bromide from *Escherichia coli* have been demonstrated by Jernaes and Steen (1994), and many other such pumps are known (Lewis, 1994).

ii) Dye uptake

It is well documented that the mitochondria of eukaryotic cells have the ability to accumulate "lipophilic" cations such as Rh123 concentratively (Chen, 1988; Chen *et al.*, 1982; Grogan and Collins, 1990; Johnson *et*

al., 1981; Johnson *et al.*, 1980), in an uncoupler-sensitive fashion, and the staining of mitochondria with Rh123, in conjunction with flow cytometry, has been used to study their activity (Darzynkiewicz *et al.*, 1981; Iwagaki *et al.*, 1990; Lizard *et al.*, 1990). 'Viable' bacteria also accumulate Rh123, but 'non-viable' ones do not (Diaper *et al.*, 1992), and under certain conditions the extent to which individual bacteria take up Rh123 quantitatively reflects the *extent* of their viability (Kaprelyants and Kell, 1992), i.e. whether they are immediately culturable, non-culturable or dormant.

On average, larger cells may be expected to accumulate more molecules of Rh123 than do smaller cells, but since flow cytometry allows collection of both fluorescence (Rh123 uptake) and forward light scattering (cell size) from each cell, the data can be plotted as a dual-parameter histogram, enabling one to take size differences between cells into account when interpreting the data.

In contrast to some of the other "viability" stains (e.g. acridine orange (Back and Kroll, 1991)), the uptake of Rh123 not only does not require the use of fixatives to permeabilise the cell, but the concentrative uptake is dependent on an intact and energised cytoplasmic membrane; thus living cells can be used for staining purposes. This has the great advantage that, following staining of the cells, further physiological studies may be conducted if required (Davey *et al.*, 1993).

There are however experimental problems with the use of lipophilic cations for microbial viability determinations. These include the fact that they may be pumped out of viable cells by microbial drug efflux pumps, and thus both viable and non-viable cells may appear to be non-fluorescent. In addition, while the stain is readily concentrated by Gram-positive bacteria such as *Micrococcus luteus*, the permeability of the stain in Gram-negative organisms is low unless the cells are pre-treated with EDTA (Kaprelyants and Kell, 1992). However one should bear in mind that such pretreatment is practically impossible to standardise, and thus the extent of lipophilic cation accumulation may vary from experiment to experiment. In addition, in a protocol for viability determination, it is generally desirable that the number of pre-processing steps be kept to a minimum in order to avoid the possibility of affecting the viability of the sample.

An alternative approach is the use of lipophilic anions, which, in contrast to cations bind preferentially to non-viable cells. The lipophilic anion bis-(1,3- dibutylbarbituric acid) trimethine oxonol (DiBAC$_4$(3)) has been shown to enter eukaryotic membranes only if their membranes had become de-energised (Wilson and Chused, 1985). The same stain has been used for the rapid assessment of microbial responses to antibiotics (Jepras *et al.*, 1997; Mason *et al.*, 1994; Mason *et al.*, 1995b; Suller *et al.*, 1997), allowing the analysis of heterogeneity within a microbial population in terms of susceptibility to an antibiotic.

iii) Metabolic Activity

A third class of viability stains are used in mammalian cell biology, often as a positive viability marker in a dual-staining protocol with ethidium bromide or propidium iodide (Aeschbacher *et al.*, 1986). One example is fluorescein diacetate which itself is non-fluorescent but membrane permeant. It is cleaved by intracellular esterases to produce a product (in this case fluorescein) that is fluorescent but under ideal conditions non-membrane permeant. Dead cells with leaky membranes do not stain because they lack enzyme activity and/or fluorescein diffuses freely through the membrane. Flow cytometric analyses of mammalian cells with this class of dyes is well established (e.g. Aeschbacher *et al.*, 1986; Frey, 1997). Diaper and Edwards (1994a; 1994b) used flow cytometry to detect a variety of viable bacteria after staining with fluorescein diacetate and its derivatives and with ChemChrome B. Importantly, none of the dyes tested was found to be universal for the detection of viable bacteria. However, ChemChrome B was found to stain the widest number of Gram-positive and Gram-negative species, whereas the FDA derivatives preferentially stained Gram-positive bacteria. Breeuwer and colleagues showed that FDA and carboxy-FDA penetrated yeast rapidly, and that esterase activity was probably most limiting (Breeuwer *et al.*, 1995); an energy-dependent efflux from viable cells of carboxyfluorescein was also observed (Breeuwer *et al.*, 1994; Ueckert *et al.*, 1995). It is probable that fluorescein can be pumped out of or leak rapidly from viable bacteria, thus giving the appearance of lack of metabolic activity in cells that are nonetheless viable.

iv) Commercial kits for microbial viability assessment

A variety of kits have been produced specifically for the measurement of viability of specific types of organisms. For example, the LIVE/DEAD BacLight Bacterial Viability Kit from Molecular Probes (Oregon, USA) gives a two-colour (SYTO 9 (green=live) / propidium iodide (red=dead)) viability assessment of both Gram-negative and Gram-positive bacteria. However, as freely admitted by the company, the kit equates the presence of intact plasma membranes with viability. Thus, "bacteria rendered nonviable by exposure to agents that do not necessarily compromise the integrity of the plasma membrane, such as formaldehyde, usually appear viable by this criterion" (Haugland, 1996). Despite this limitation the kit is becoming widely used in microbial flow cytometry (Braux *et al.*, 1997; Buchmeier and Libby, 1997; Decamp *et al.*, 1997; Duffy and Sheridan, 1998; Jacobsen *et al.*, 1997; Joux *et al.*, 1997; Korber *et al.*, 1997; Langsrud and Sundheim, 1996; Rigsbee *et al.*, 1997; Swarts *et al.*, 1998; Taghikilani *et al.*, 1996; Terzieva *et al.*, 1996; Virta *et al.*, 1998; Weir *et al.*, 1996). The growing use of such kits reflects, at least in part, their ease of use. In the case of the BacLight kit the reagents are simultaneously added to the bacterial suspension, which is then incubated for 5 - 10 minutes. The sample is then analysed without washing so "live" and "dead" bacteria can be distinguished and quantitated in a few minutes. There is a great danger that because of the name the uninitiated may use these tests blindly, despite the manufacturer's warnings, without checking the reliability of the dyes with the organisms and conditions used in their experiments.

Despite the problems associated with fluorescent staining protocols for viability measurements, providing one carefully selects and tests an appropriate protocol for the problem under investigation, useful information can still be obtained. To illustrate this, we present a case study of the use of flow cytometric viability testing in the Gram-positive bacterium *Micrococcus luteus*.

Estimations of Viability of *Micrococcus luteus*

In nature and under conditions of stress, bacterial cultures display significant heterogeneity in terms of the percentage of viable (culturable) cells, and with respect to cellular metabolic activities (Kaprelyants *et al.*, 1993). An important task is therefore to find reliable and rapid methods for estimating the number of cells with different characteristics in the whole bacterial population. To this end, the application of flow cytometric methods seems very promising, since they allow one to distinguish the properties of *individual* cells in the population. Since fluorescence and light scattering are measured on a quasi-continuous scale it is possible to quantify the heterogeneity of a sample fully rather than a simple classification into one of two classes (live or dead). The following experiments with *Micrococcus luteus* illustrate the application of flow cytometry for discrimination between cells in different physiological states.

A variety of flow cytometric approaches have been investigated for the determination of viability in the Gram-positive, non-spore forming bacterium *Micrococcus luteus* NCIMB 13267 (Kell *et al.*, 1995). The bacteria are grown aerobically at 30°C in shake flasks in a lactate minimal medium containing L-lactate (see e.g. Kaprelyants and Kell, 1992 for full details). In order to obtain samples of low viability (as judged by plate counts) the cells may then be subjected to a starvation stress by allowing them to reach stationary phase before holding them at 30°C aerobically for up to 1 month, followed by a further incubation of up to 3 months at room temperature without agitation. As a result of these procedures cell populations of low viability can be obtained (less than 0.01% of the cells may grow on agar plates (solidified Nutrient Broth E) that would normally support growth). However the total cell count, estimated microscopically using a counting chamber, remains close to 100% of the initial pre-starvation value (Kaprelyants and Kell, 1993a). In fact, as illustrated below, starved populations consist of different subpopulations which can be visualised flow cytometrically.

i) Estimation of numbers of active versus inactive cells

The proportion of active cells in a population of *M. luteus* can be estimated using the membrane energisation-sensitive probe Rh123. Figure 11.7.1 shows the typical distribution of the fluorescence of non-starved *M.*

luteus cells, stained with Rh123 and analysed by flow cytometry. The regions (A, B and C) were demarcated following the analysis of a freshly-harvested sample of viable cells stained with Rh123, which in the absence of uncoupler, exhibited a level of fluorescence between channels 80 and 136 (Figure 11.7.1a), which was fully uncoupler-sensitive (Figure 11.7.1b).

A good correlation is observed between the percentage of viable *M. luteus* cells in the population and the percentage of cells with CCCP-sensitive accumulation of Rh123, judged flow cytometrically (Kaprelyants and Kell, 1992). Similar results were obtained by the flow cytometric study of *M. luteus* cells stained with CTC (5-cyano-2,3-ditolyl tetrazolium chloride), the reduced form of which (a fluorescent formazan) allows one to monitor the respiratory activity of individual cells (Kaprelyants and Kell, 1993b; Rodriguez *et al.*, 1992).

ii) Estimation of dormant and dead cells

It has been shown that 10-50% of *M. luteus* cells in 3-month-old populations can be resuscitated to normal, colony-forming bacteria under conditions which exclude any significant regrowth of initially viable cells (Kaprelyants *et al.*, 1993; Kaprelyants and Kell, 1992; Kaprelyants and Kell, 1993a; Kaprelyants and Kell, 1996; Kaprelyants *et al.*, 1996; Kaprelyants *et al.*, 1994; Kaprelyants *et al.*, 1999; Mukamolova *et al.*, 1998a; Mukamolova *et al.*, 1998b; Votyakova *et al.*, 1994; Votyakova *et al.*, 1998). This indicates the persistence of a significant percentage of cells in the dormant state. This hypothesis was confirmed using the Most Probable Number (MPN) method by the resuscitation of cells from samples which, statistically, contained no "initially-viable" cells (Kaprelyants *et al.*, 1994). It was found that when the medium also contained spent growth medium from a culture in late log phase, a substantial increase (1000- to 100,000-fold) in the number of viable bacteria was observed when compared with those estimated with the agar plate method (Table 11.7.2). These experiments were the first that served conclusively to exclude regrowth as a contributor to the observed resuscitation - an enormous problem that is rarely tackled satisfactorily in this context (Kell *et al.*, 1998) (and one that is also highly significant for the isolation of slowly-growing strains from natural ecosystems (Button *et al.*, 1993; Schut *et al.*, 1993)). It was also concluded that viable cells of *M. luteus* can secrete a pheromone-like substance, which is apparently necessary (though not on its own sufficient in all

cases) for the resuscitation of starved, dormant cells of the same organism (Kaprelyants *et al.*, 1994). This substance (Resuscitation Promoting Factor (RPF-factor)) is a secreted small protein with a MW of ca 17-18 kDa (Kaprelyants *et al.*, 1999; Mukamolova *et al.*, 1998a).

As shown in Figure 11.7.1, the proportion of dormant cells can also be determined using flow cytometry. Figure 11.7.1c shows a typical distribution of the fluorescence of *M. luteus* cells that had been starved for 5 months, stained with Rh123 and analysed by flow cytometry. A bimodal fluorescence distribution is evident. Region A (channel 0 to channel 80) represents cells which bind Rh123 nonspecifically: 98 % of fresh late logarithmic phase *M. luteus* cells stained with the same concentration of Rh123 followed by treatment with a suitable concentration of the uncoupler CCCP exhibited a fluorescence in this region (Figure 11.7.1b). Although starved cells also occurred in region B (between channels 80 and 136) their sensitivity to CCCP was very low (only 2-5% of the cells in region B exhibiting a decrease in fluorescence after CCCP treatment). This phenomenon was not due to any inability of the uncoupler to act *per se*, since octanol treatment also failed to decrease the extent of staining of such cells (not shown). It has been suggested that cells accumulated in region A and B represent bacteria in different physiological states (Kaprelyants *et al.*, 1996). To determine whether this is indeed the case a cell sorting approach was used (see the section on the use of cell sorting, below).

iii) Estimation of injured cells

The presence of injured bacteria in starved populations or in populations subjected to stress (freezing, drying etc) is very likely. Commonly, such cells have an impaired membrane permeability barrier which has been tested by the following flow cytometric approaches:

a) Membrane-impermeant probes

The use of dye exclusion for monitoring viability by flow cytometry was discussed above, but in the case of bacteria a damaged or leaky membrane may not be a sufficient criterion for defining a cell as non-viable but it can be used as an indication of stress-induced injury. The permeability barrier of the cells that were starved

for 5 months was monitored by staining with PI (Mukamolova *et al.*, 1998b). It was shown that PI does not penetrate the cytoplasmic membrane of intact *M. luteus*, while the administration of 0.5% v/v octanol to the cell suspension resulted in 100% of the cells being stained with PI (see Figure 11.7.2). Observation of different starved cultures of *M. luteus* revealed that in some cultures where the percentage of PI-positive cells is close to 100% the resuscitation of cells was not successful (even in the presence of RPF-factor). This indicates a correlation between the state of the permeability barrier and the ability of starved cells to recover and this may allow the use of PI staining for discrimination between dormant and dead cells in a population.

b) NADH-induced respiration

The ability to monitor the respiratory activity of individual cells allows the design of experiments for the quantitative determination of injured cells in a population following stress such as freezing. It is well known that some bacteria in stressed populations become injured, as reflected for example in their elevated sensitivity to surface active agents (Ray and Speck, 1973). This effect has been used for enumerating injured bacteria by plating them on selective media containing detergents (Ray and Speck, 1973). However this approach can reflect only injuries connected with damage to the outer portion of the cell envelope of Gram-negative bacteria (Ignatov *et al.*, *1981;* Ray and Speck, 1973), whilst it is damage to the cytoplasmic membrane that is more important in determining the *viability* of bacteria after freezing (Ignatov *et al.*, *1981*). Thus, it has been shown that an increase in the permeability of the cytoplasmic membrane to NADH after freezing (which in contrast to normal cells resulted in the stimulation of endogenous respiration by NADH), was well correlated with a decrease in the viability of *E. coli* (Ignatov *et al.*, *1981*). The flow cytometric behaviour of frozen/thawed *M. luteus* cells after the addition of CTC and in the presence or absence of exogenous NADH revealed that after the first 5 minutes of incubation in the presence of CTC approximately 25% of the population gave a significant fluorescence when NADH was also present but only 1 % when it was not. After 17 min of incubation with CTC the *percentage* of cells fluorescing above a channel number of 20 had increased in both cases (± NADH), whilst their difference had not. Further incubation of the cells resulted in a decreasing difference in the distribution pattern for the two types of sample. The kinetics of CTC reduction in the two samples are summarised in Figure 11.7.3.

The reduction of CTC within the first few minutes of incubation in the presence of NADH indicates the existence of cells with an injured permeability barrier but with an intact respiratory chain (Ignatov *et al.*, 1982; Ignatov *et al.*, *1981*). These cells very rapidly reduce CTC to formazan, to a concentration comparable with that in intact cells (as judged by the channel number of the fluorescence), whilst some endogenous substrates left in the cells after freezing and thawing permit a slower reduction of CTC in the samples without NADH. We can conclude that at least 25 -30 % of the cells in a frozen population of *M. luteus* are injured (although the final viability of this sample, as judged by plating on a rich medium which permitted repair processes to take place (Ignatov *et al.*, 1982; Ray and Speck, *1972*), was 90-95 %).

Designing a Protocol for Microbial Viability Assessment

When selecting a stain for a particular application there are several factors that need consideration. Some of these such as the extinction coefficient, quantum yield, photostability etc are of general applicability to flow cytometric fluorescence measurements and are discussed in detail elsewhere (Davey and Kell, 1996; Shapiro, 1995). The wavelengths available for excitation must also be considered and a list of viability stains that are compatible with common flow cytometric light sources is shown in Table 11.7.1.

Factors that are of particular relevance in the measurement of viability include the toxicity of the stain. When one is making such measurements it is desirable that the ptotocols used do not perturb the viability of the cells under study. This becomes essential when one wishes to perform further physiological studies on the cells - for example by exploiting flow cytometric cell sorting to isolate subpopulations with different fluorescence properties. In this case the toxicity of the stain (and indeed of any other chemicals used) must be assessed at the concentrations used in the protocol to ensure that they do not have any unwanted effects.

Even where further physiological study is not required, it is generally desirable to use cells that have not been fixed to avoid any possible perturbation of what one is trying to measure. This approach does however, have the disadvantage that levels of cellular autofluorescence are generally higher for unfixed cells than for e.g. ethanol-fixed cells. For the majority of microorganisms (chlorophyll-containing organisms are the most notable exception) cellular autofluorescence tends to decrease substantially towards the red end of the optical spectrum, and this is driving the development of red-excited fluorophores (Fabian *et al.*, 1992; Patonay and Antoine, 1991; Shealy *et al.*, 1995a; Shealy *et al.*, 1995b). Such dyes can be exploited in flow cytometry using a 633 nm Helium-Neon laser or a 635 nm laser diode.

Laser-diodes can be used to construct smaller, cheaper and more robust flow cytometers e.g. the Microcyte (see Internet Resources section for further information). The Microcyte was developed by Gjelsnes and

Tangen (1994) primarily for the analysis of microorganisms. Using this instrument it is possible to obtain both a total count and a viable count in absolute terms very rapidly (Davey and Kell, 1997). Flow cytometric analyses of samples of *M. luteus* stained with the exclusion dye TO-PRO-3 are shown in Figure 11.7.4, see the legend for details.

When the appropriate stain and excitation source have been selected it is important to perform a series of experiments to determine the optimum concentration of the stain and the optimum length of time between addition of the stain and the subsequent analysis. The optimum concentration will inevitably be a compromise between choosing a high concentration to give maximum signal, and a low concentration to give specificity. It may be necessary to measure and adjust the cell concentration to ensure that stain uptake is not limiting. In this case the use of a flow cytometer that allows determination of absolute cell numbers is an ideal approach.

In some situations it may be desirable to exploit the multiparametric nature of flow cytometry to use two different viability stain that rely on measuring different cellular parameters (e.g. Yurkow and McKenzie, 1993). Alternatively, one may wish to combine the viability assay with measurements of other cellular properties. In either case, careful selection of all of the stains involved is required to ensure that there is minimal overlap in the emission spectra.

The Use of Cell Sorting in Viability Studies

While flow cytometric analysis allows the investigator to perform a rapid and quantitative version of the experiments that could otherwise be performed by fluorescent microscopy, flow cytometric *cell sorting* allows the process to be taken one very important step further. With flow cytometric analysis one can simply say that the distribution of dye uptake is correlated with a plate count of the same sample. However, providing the staining protocol does not affect the viability of the cells (which may be determined by plate counts of stained and unstained samples) one can exploit the sorting capability of appropriate instruments to separate cells from the purported viable and non-viable fractions of the histogram. This allows determination of the culturability of exactly those cells whose cytological properties had already been determined directly.

To this end we sorted cultures whose fluorescence was of the type displayed in Figure 11.7.1c into two populations: (i) cells of which the rhodamine staining was sensitive or partially sensitive to CCCP (regions B+C) and (ii) cells whose rhodamine-dependent fluorescence was not sensitive to CCCP (region A). After sorting, cells were plated on nutrient agar and examined in an MPN assay for viable count determinations, while the total count of sorted samples was also examined. These experiments revealed that the resuscitation of cells as judged by the MPN assay was successful for cells in regions B+C but not for cells in region A. This constitutes direct evidence that dormant cells are concentrated in regions B+C (Kaprelyants *et al.*, 1996).

Some flow sorters such as the Coulter Epics Elite have a motorised stage (the Autoclone module) for the collection of single sorted cells. While this is primarily designed for the collection of cells into the wells of microtitre plates, it is also possible to modify the stage and collection protocol (see Figure 11.7.5) to allow microbial cells to be collected directly onto agar plates. Thus an event from a tightly defined region on a histogram can be correlated directly to growth (or failure of growth) of a colony on an agar plate. This approach has been pioneered by Nebe-von Caron and colleagues (see e.g. Nebe-von Caron and Anderson, 1996; Nebe-von Caron and Badley, 1996; see also

http://www.cyto.purdue.edu/flowcyt/research/micrflow/gerhard/genmic10.htm) and has great potential for many microbiological applications.

While the sorting approach offers many advantages, one must be aware of potential pitfalls of the method. Microbial cells often grow in clumps or form aggregates during sample preparation. If one cell in a clump has a leaky membrane it will take up an exclusion dye such as propidium iodide and thus the clump will be fluorescent and will score as "dead" in the viability assay. However, if this clump also contains one or more live cells, a colony will result when the clump is sorted onto an agar plate. This problem can be overcome to some extent by using the forward scatter signal as an indicator of size. However, since the size of microbial cells, even within a single species, can vary greatly with growth conditions, a more robust approach may be to use two or more viability stains to give a broader picture of the physiological status of the cells. An additional problem may arise with damaged cells. The process of flow cytometric analysis, followed by sorting onto an agar plate may in its own right be considered as an additional stress which may convert an injured cell into a non-viable one. Such stresses can be quantified to some extent by plating injured cells before and after sample preparation. The effect, if any, of the sheath fluid on *injured* cells should also be determined.

Conclusions

The rapid cytological estimation of true microbial viability is extremely difficult (if not impossible in principle), not least because of the problems of defining viability in microbial cells. Despite the difficulties mentioned above, the view to which we subscribe (Kell *et al.*, 1998) is that only culturability can provide a gold standard for positive viability. Although the flow cytometric approach has much to offer for the determination of microbial viability it must be emphasised that no single stain nor even cocktail is likely to be a universal indicator of viability, especially if we require that its interpretation reflects our ability to induce the cells to divide (see Table 11.7.3).

A cell that is killed by exposure to environmental extremes such as heat, pH etc is likely to be very different from a cell that is killed by exposure to an antibiotic or other chemical, and different again from a cell that dies due to a lack of nutrients in its environment. Thus the flow cytometric properties of a cell and the distribution of dye uptake within a population will depend on how the cells die, and more generally on their entire physiological state and its history.

While most instruments designed specifically for the analysis of microorganisms (e.g. the Biorad Bryte or the Aber Instruments Microcyte) do not permit cells to be sorted, microbes may be sorted using the standard commercial instruments. The exploitation of the sorting capability of flow cytometers permits the design of experiments that carefully evaluate the applicability of so-called viability stains, and we strongly recommend authors to adopt this approach.

We conclude that although there are currently no perfect stains, careful protocol development currently allows valuable information to be obtained regarding specific problems. In the case of organisms that have not been exposed to excessive stress (e.g. laboratory cultures under normal conditions and in some cases clinical samples) substantial progress is being made towards the rapid and routine flow cytometric assessment of microbial viability or vitality.

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Internet Resources

http://pcfcij.dbs.aber.ac.uk/home.htm

The Aberystwyth flow cytometry site has information on microbial flow cytometry including viability determinations.

http://www.cyto.purdue.edu/flowcyt/research/micrflow/index.htm

Microbial flow cytometry section with contributions from several authors on viability measurements.

http://www.aber-instruments.co.uk/microcyt.htm

A flow cytometer designed for the analysis of microbial samples employing a 635 nm laser diode as the excitation source.

http://www.probes.com/handbook/ch16-2.html

Source for various viability stains including viability kits.

Figure Legends

Figure 11.7.1 : Distribution of the fluorescence of *M. luteus* following staining with 0.3 μM Rh123 (Sigma). Flow cytometry was performed using a Skatron Argus 100 instrument which was set up as described in the manufacturers manual. The PMT voltage for the fluorescence channel was 700V, and the full scale of the abscissa represents 3.5 decades in fluorescence intensity. **a)** The cells were grown in lactate minimal medium until late logarithmic phase, harvested, washed and resuspended in lactate minimal medium lacking lactate (Kaprelyants *et al.*, 1996) prior to 20-fold dilution and staining. **b)** The cells were prepared as in **a)** but 15 μM CCCP was also added prior to analysis. **c)** Cells were grown in lactate minimal medium and then starved for 5 months before dilution and staining.

Figure 11.7.2 : Distribution of the fluorescence of cells of *M. luteus* cells that have been starved for 5 months, stained with propidium iodide and assessed by flow cytometry. Octanol was added to the cells as indicated to give a final concentration of 0.5%.

Figure 11.7.3 : Flow cytometric fluorescence behaviour of a sample of *M. luteus* that had been frozen and thawed. Cells were diluted 10-fold in 50 mM phosphate buffer and incubated with 4 mM CTC for the times indicated, either with or without 1 mM NADH. The ordinate gives the difference, between the samples with or without added NADH, in the percentage of cells whose fluorescence was in a channel number greater than 20.

Figure 11.7.4 : Flow cytometric analysis of *M. luteus*. Cells were grown overnight in Nutrient Broth E in a shaking water bath at 30°C. Samples were removed from the culture and stained with 0.1 μM TO-PRO-3 (Molecular Probes). The samples were then analysed on a Microcyte flow cytometer. Under these conditions unstained samples did not give any detectable fluorescence. The thick line represents cells freshly harvested from the culture. The thin line represents cells that had been freeze-thaw treated prior to staining. The sample was frozen (-20°C) for 30 minutes, rapidly defrosted by plunging the tube into a 50°C water bath, then re-

frozen and then re-thawed in the same manner. The dotted line represents cells that had been permeabilised by fixing in 70% ethanol. The fixative was removed by centrifugation prior to staining. In all cases the total cell count (based on forward light scatter data - not shown) has been normalised to 1 million cells.

Figure 11.7.5 : a) Flow cytometric cell sorting using the Autoclone module of the Coulter Elite can be used to place individual cells onto approximately 60-65 discreet locations on a standard 90 mm agar plate. **b)** An agar plate with *M. luteus* colonies following sorting.

Table 11.7.1 : Fluorescent dyes whose use has been considered for the flow cytometric measurement of viability.

Stain	Mode of Action	Excitation Source	Reference(s)
BacLight Kit (Molecular Probes)	Exclusion of PI and staining with SYTO9	Argon (488 nm)	Joux *et al.*, 1997; Langsrud and Sundheim, 1996; Swarts *et al.*, 1998; Virta *et al.*, 1998
bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3))	Uptake by dead cells	Argon (488 nm)	Beck and Huber, 1997; Deere *et al.*, 1995; Jepras *et al.*, 1995; López-Amorós *et al.*, 1997; Mason *et al.*, 1994; Mason *et al.*, 1997; Suller *et al.*, 1997
Calcofluor White	Uptake by dead cells	Helium-Cadmium (325 nm)	Berglund *et al.*, 1987; Mason *et al.*, 1995a
Carboxy-naphtho-fluorescein-diacetate	Enzymic activity	Helium-Neon (633 nm) / Laser diode (635 nm)	Presented by Bergersen *et al.* at the NORDFOOD conference, Turku (Åbo) Finland, 1995. See also Davey and Kell, 1996
Chemchrome B/Y	Proprietary information	Argon (488 nm)	Clarke and Pinder, 1998; Deere *et al.*, 1998; Diaper and Edwards, 1994b
5-cyano-2,3-ditolyltetrazolium chloride (CTC)	Respiratory activity	Argon (488 nm)	Joux *et al.*, 1997; Kaprelyants and Kell, 1993b
Ethidium bromide	Exclusion	Argon (488 nm)	Aeschbacher *et al.*, 1986)
Fluorescein diacetate	Enzymic activity	Argon (488 nm)	(Aeschbacher *et al.*, 1986; Berglund *et al.*, 1987; Diaper and Edwards, 1994b; Norden *et al.*, 1995
Fluorescein-di-β-D-galactopyranoside	Enzymic activity	Argon (488 nm)	Plovins *et al.*, 1994
FUN-1 kit (Molecular probes)	Metabolic activity		Millard *et al.*, 1997; Prudencio *et al.*, 1998; Wenisch *et al.*, 1997
Propidium Iodide	Excluion	Argon (488 nm) or Helium-Neon (544 nm)	Auger *et al.*, 1993; Berglund *et al.*, 1987; Deere *et al.*, 1998; Gant *et al.*, 1993; Niven and Mulholland, 1998)
Rhodamine 123	Uptake by live cells	Argon (488 nm)	(Auger *et al.*, 1993; Comas and VivesRego, 1998; Davey *et al.*, 1993; Diaper *et al.*, 1992; Kaprelyants and Kell, 1992; Porro *et al.*, 1994
Sytox Green	Exclusion	Argon (488 nm)	Langsrud and Sundheim, 1996; Roth *et al.*, 1997
TO-PRO-3	Exclusion	Helium-Neon (633 nm) / Laser diode (635 nm)	Davey and Kell, 1997

Table 11.7.2 : Resuscitation of dormant *M. luteus* cells in liquid medium.

Culture	Time of starvation	Total count (cells.ml^{-1})	Viable count by cfu (cells.ml^{-1})	Viable count by MPN (cells.ml^{-1})*
1	2 months	$5.3 \ 10^9$	$5 \ 10^6$	$3.5 \ 10^9$
2	4.5 months	10^{10}	$1.3 \ 10^6$	$9.2 \ 10^9$
3	6 months	$1.2 \ 10^{10}$	$3.6 \ 10^4$	$9.2 \ 10^9$
4	9 months	$6.2 \ 10^9$	$5.2 \ 10^5$	$5.4 \ 10^9$

*- estimation was performed in the presence of RPF factor. For the details of these experiments - see

Mukamolova *et al.*, (1998a).

Table 11.7.3 : Summary of advantages and disadvantages of viability determination methods

	Advantages	**Disadvantages**
Multiplication assay	Provides sufficient proof that cell was alive Generally straightforward to interpret	Slow Requires knowledge of growth requirements Underestimates viable cell numbers
Cytological assay	Rapid Can be used without knowledge of growth requirements Total count can be determined simultaneously	Can be difficult to interpret Viability is not measured directly False positives and false negatives may occur

Davey et al 1998 Figure 11.7.1a
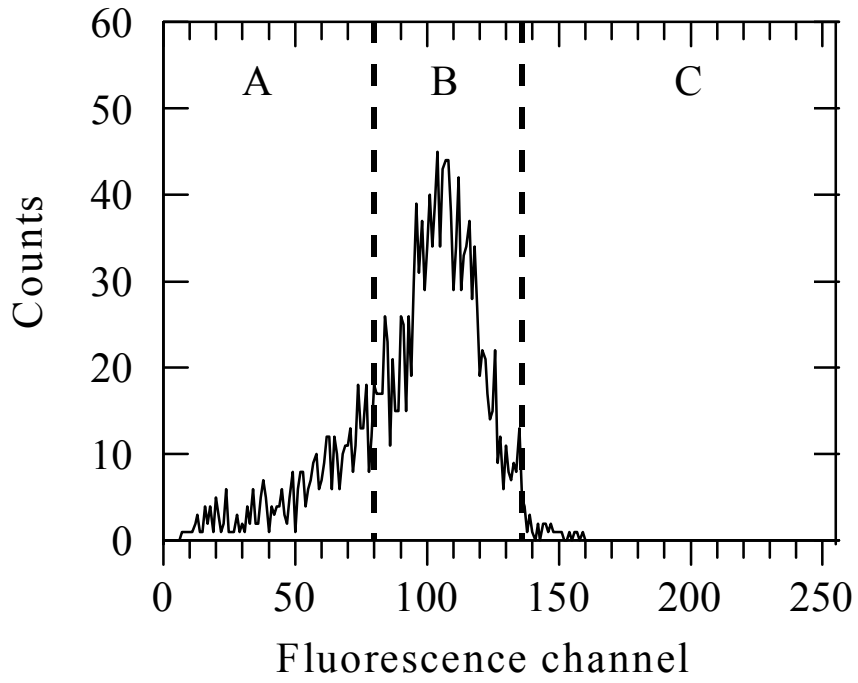
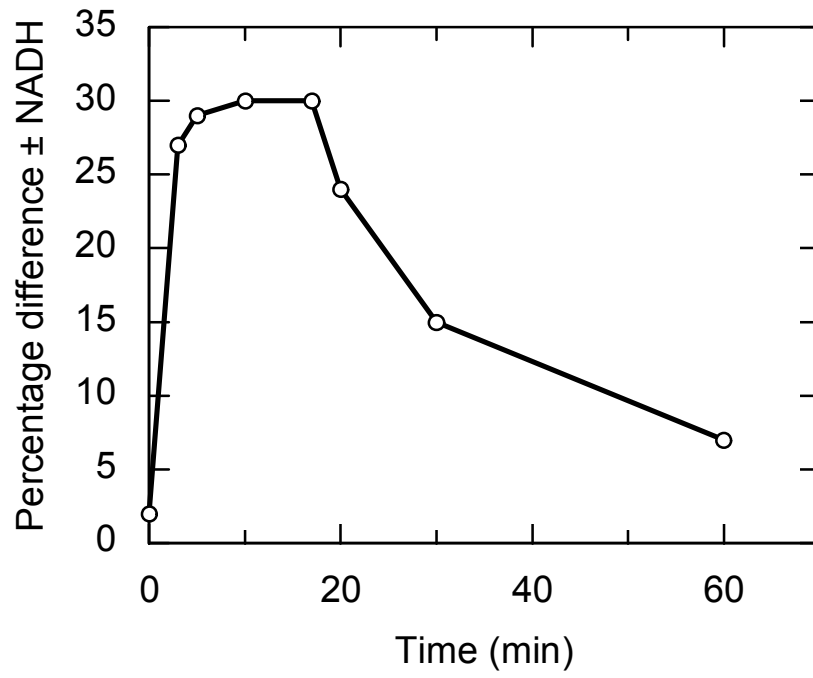Davey et al 1998 Figure 11.7.1b
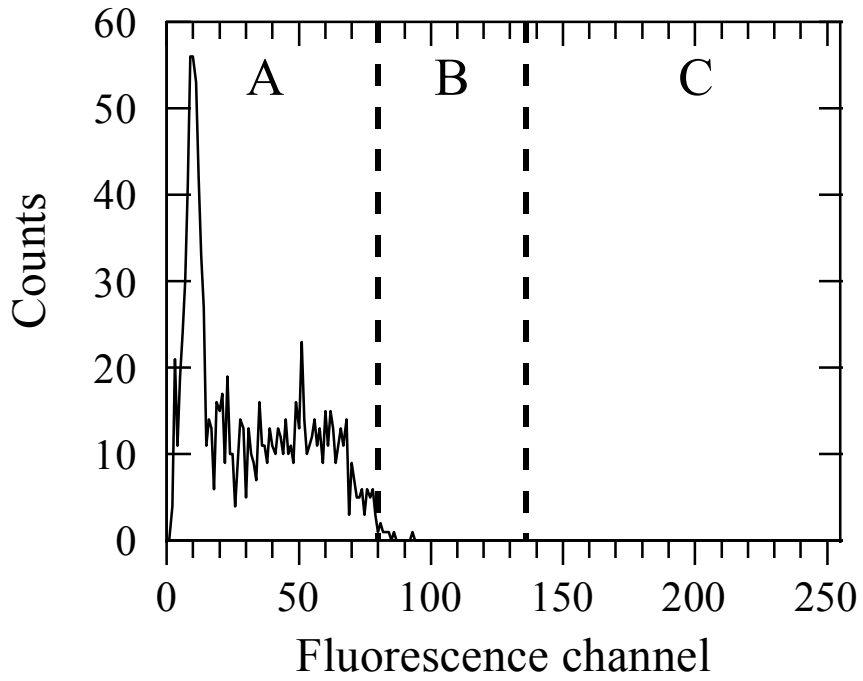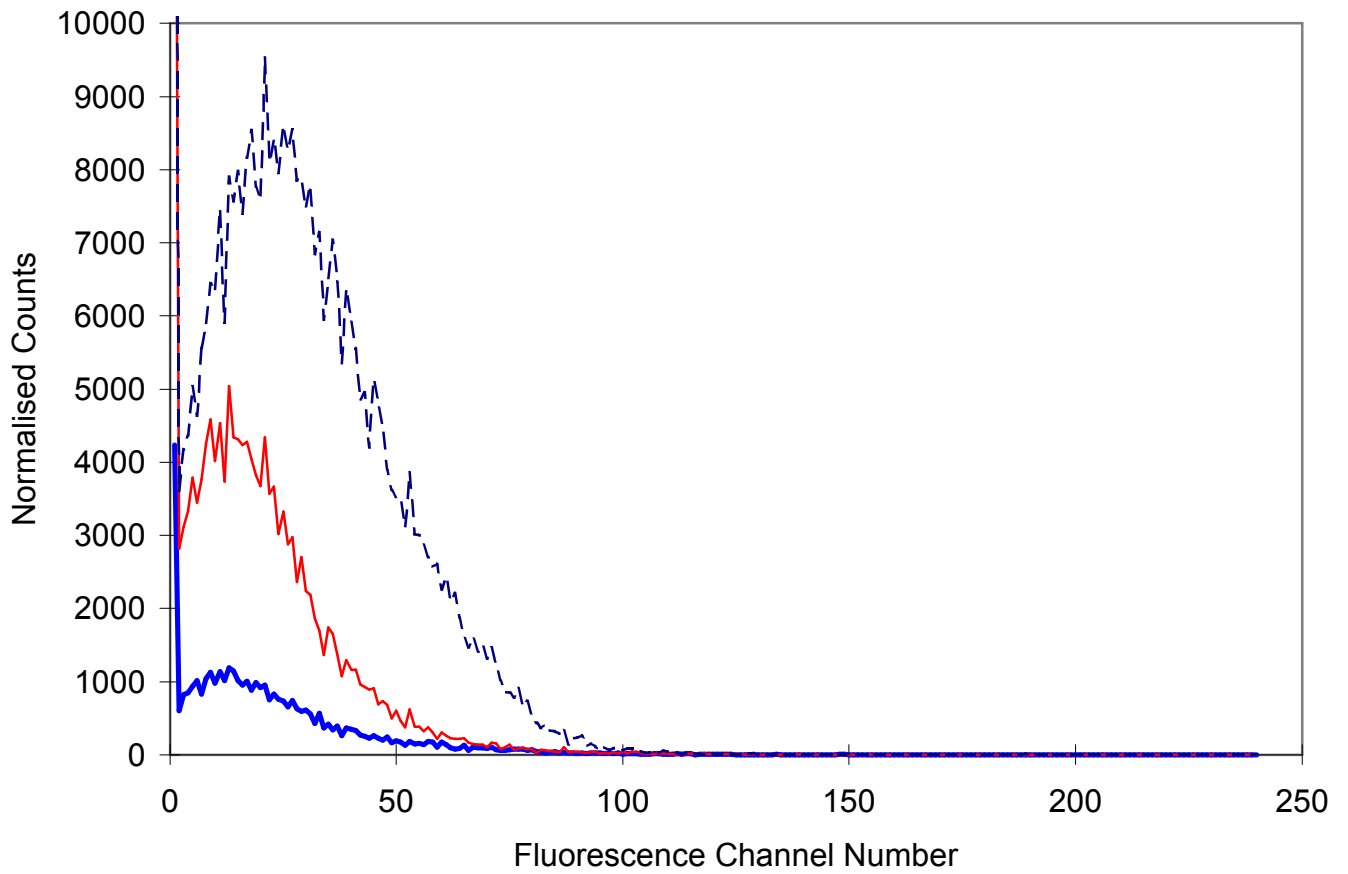
Davey et al 1998 Figure 11.7.1c
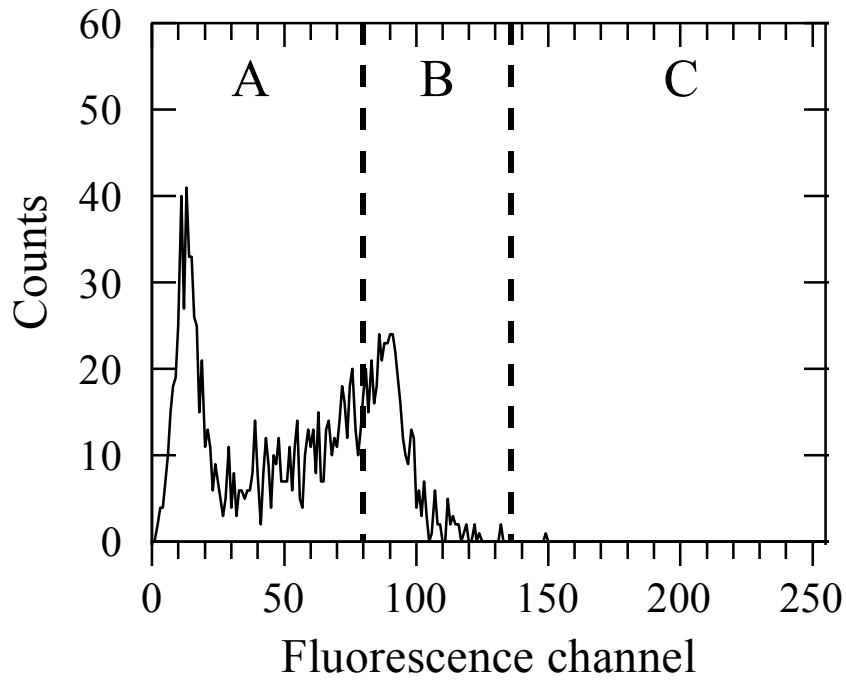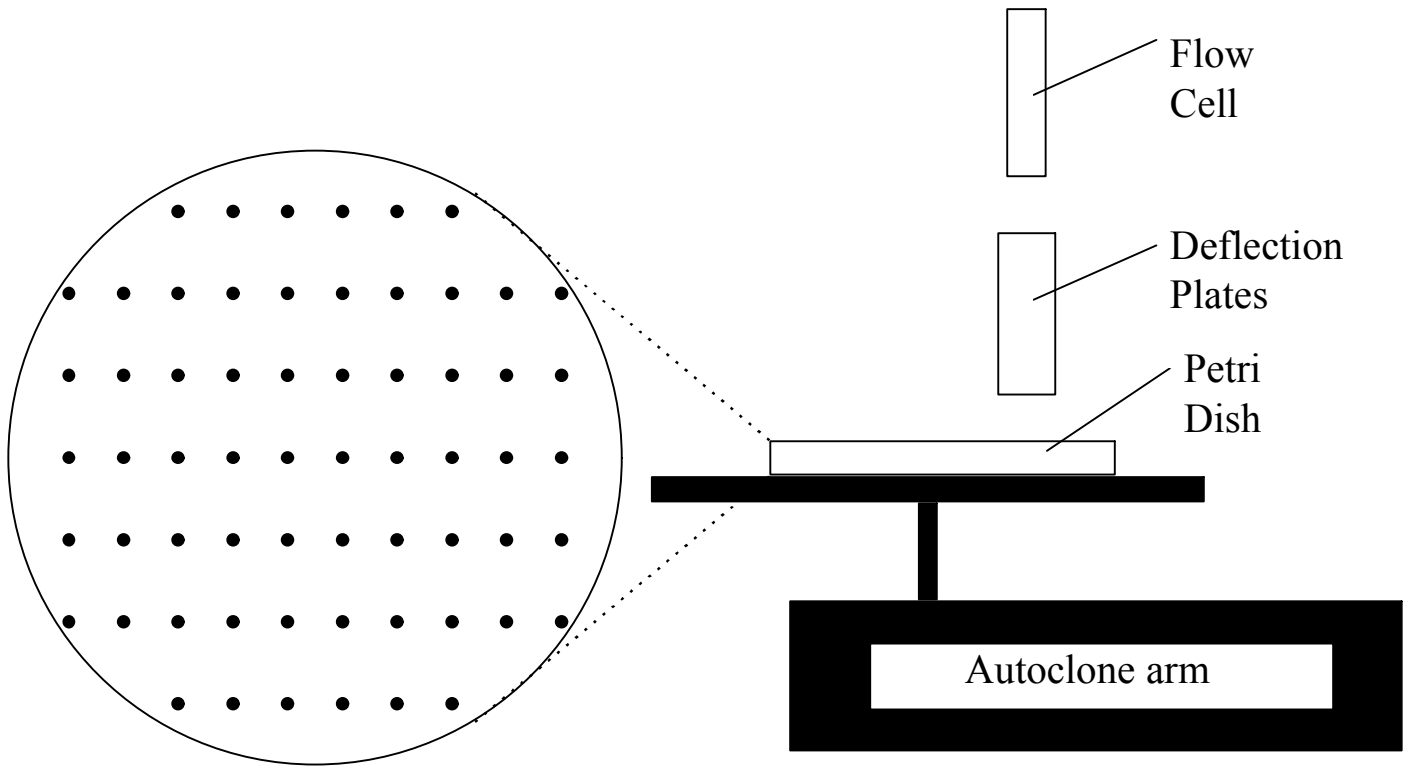

Davey et al 1998 Figure 11.7.2
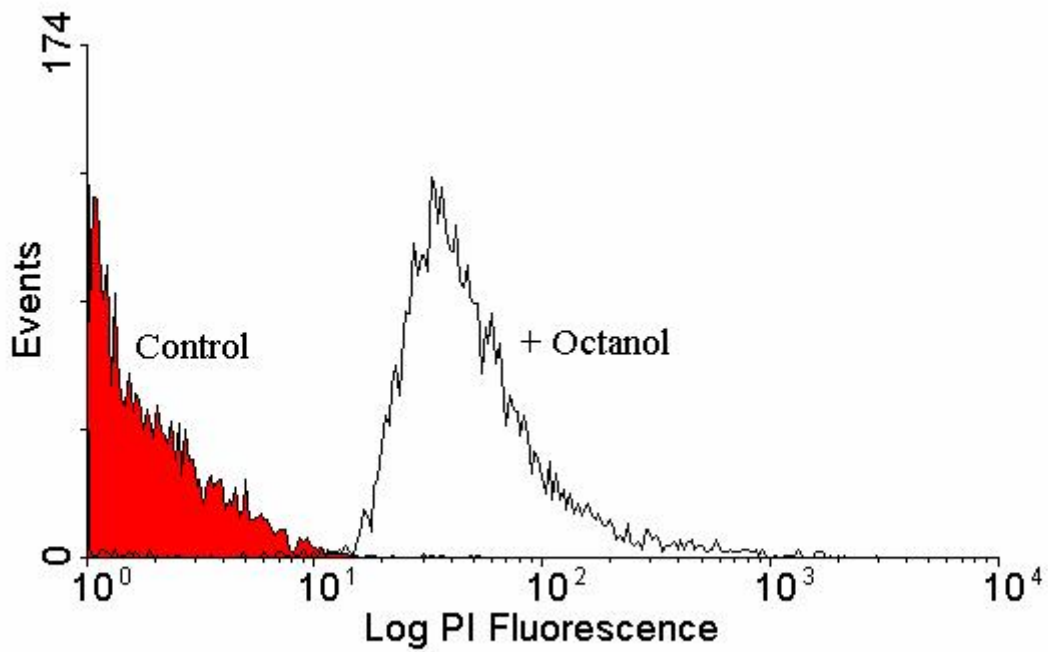
Davey et al 1998 Figure 11.7.3

Davey et al 1998 Figure 11.7.4

Davey et al 1998 Figure 11.7.5a

Top view of petri dish
showing sort grid

Davey et al 1998 Figure 11.7.5b