Estimation of Microbial Viability Using Flow Cytometry

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 (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, 1999; Kell et al., 1998), even for macroorganisms (Watson, 1987). The gold standard for determining the number of viable microbial cells in a sample is usually achieved by plating a 0.1- to 1-ml sample of cells (diluted as required) onto an agar plate (Hattori, 1988; Postgate, 1969) and scoring as viable (a posteriori) those cells that were able to form visible colonies. The culture viability is then the ratio of these cells to the total cell count in the original sample, which is determined microscopically. However, there are several problems associated with this technique, not the least of which is 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. 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 often unacceptable. Thus, 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 rapid viability measurements include a variety of stain-based methods. The so-called vital stains that have been used in attempts to estimate microbial viability fall into three broad categories. (1) Some dyes, such as propidium iodide (PI), are excluded by the intact membranes of viable cells. Therefore, the presence of the dye within the cell indicates disruption of the cell membrane and may be expected to be correlated with cell death. (2) Other dyes, such as rhodamine 123, are actively accumulated by viable cells; thus, the number of brightly stained cells reflects the viability of the sample. However, in some cases more active cells can actually pump such dyes out (Jernaes and Steen, 1994). Additionally, some dyes are less tightly bound by energized membranes, so that the more active cells are less brightly stained. (3) In the case of dyes such as fluorescein diacetate (FDA), a membrane-permeant nonfluorescent precursor is converted to a membrane-impermeant fluorescent molecule by the activity of intracellular enzymes, and thus is an indicator of metabolically active cells. Each of these dye-based approaches is discussed in more detail below.

Although 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, the temperature, and other factors may differ substantially from those in the original sample (Roszak and Colwell, 1987); thus, the proportion of cells that can divide and form colonies may be much lower than the number of cells that would score as viable using the dye-based rapid methods (Amann et al., 1995). Nevertheless, the plate count method has remained the gold standard, in part due to the fact that traditional microscopic analyses of stained cells are time consuming and can lead to operator fatigue; thus, conclusions are normally drawn from the analysis of at best a few hundred cells. Furthermore, microscopic examination is largely a qualitative technique, wherein a judgment of alive or dead is all that is possible, and the interpretation of the extent of a cell’s staining may vary among operators.

Flow cytometry offers an alternative method of determining the amount of fluorescent dye taken up by each cell in a population (Davey and Kell, 1996; Kell et al., 1991; Lloyd, 1993; Shapiro, 1995). 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.

This unit begins with a discussion of the various advantages and disadvantages of classical (proliferative) versus cytochemical (dye-based) viability assays. It discusses the three classes of cytochemical methods in greater detail, and provides instructions for three simple protocols. Finally, it discusses the use of cell
THE PROBLEM OF DETERMINING VIABILITY

In classical terms, a microbial cell is generally considered viable if it possesses all the components and mechanisms necessary for sustained proliferation (Greenwood and Peutherer, 1992). According to Postgate (1976): “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 shortcuts 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).”

Viability is evidently best determined by the classical method of assessing cellular proliferation directly, and scoring only those cells that have visibly multiplied. However, the underpinning assumption of rapid microbiology is that one can estimate something that might correlate with culturability by assessing the presence and functionality of individual vital factors and processes. The cytochemical approach gives an insight into the physiology of individual cells by providing data on parameters such as membrane energization and enzyme activity. Both approaches have advantages and disadvantages, as discussed in this section. Regrettably, studies in which the same cells are compared using each approach are rare indeed.

The Growth-Based Approach

The classical approach requires an a priori knowledge of the suitable growth media and conditions for the organism or organisms present in the sample, as well as the use of a suitable method of growth detection for the organisms. In practice, because of limited time, materials, and prior knowledge, the most convenient method is all too often chosen. High-nutrient, complex media such as Luria broth and trypticase soy broth (TSB) are often used for these procedures to ensure growth. Growth detection is usually measured either as colony-forming units (cfu) on a solid agar plate or as turbidity in liquid media. There are several problems associated with this kind of approach.

Standard growth conditions. Many microorganisms have growth requirements that are very different from the standard conditions applied. For instance, standard conditions usually involve the aerobic incubation of a sample at a higher temperature than that at which it was collected (e.g., ≥30°C may be used even in the case of environmental samples). 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 to date (Amann et al., 1995). In many cases where success has eventually been obtained, organisms defied efforts to culture them until some critical component was added to the medium. A well-known example is Legionella (Meyer, 1983).

Changes in physiological state. 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 (stationary phase), and dormancy (latency or cryptobiosis; Kell et al., 1998). Additionally, 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., 1998).

Growth determination method. 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.

Growth factor requirements. It is of course entirely plausible and even likely that dormant and uncultured microorganisms actually need autocrine or paracrine growth factors for their cultivation in vitro (Kaprelyants and Kell, 1996; Kaprelyants et al., 1999; Mukamolova et al., 1998a).

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

**The Cytochemical Approach**

There are occasions in which it is the metabolic activity of the cells that is of concern, whether they are capable of multiplication or not. Clearly a cell whose DNA has been damaged at the origin of replication could not multiply, but the rest of its activities would probably be unaffected. If, for example, these activities included the production of a toxin, then a method that detects metabolic activity would be of more interest than one that requires proliferation to score for cellular presence and activity.

Cytochemical assays have several advantages over proliferation-based assays. They are generally less time-consuming, in some cases delivering instantaneous results. They facilitate (at least potentially) a method of measuring something that might be correlated with other measures of viability (such as culturability) in organisms for which suitable growth conditions have not been established. For organisms that 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 taxonomic traits by using specific antibodies or ribosomal RNA probes (Amann et al., 1995; Wallner et al., 1993). Thus, multidimensional snapshots of mixed populations can be generated, providing information on the species composition and physiological status of cell populations.

However, these rapid assays also often have their own drawbacks that can make them difficult to interpret. 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 (Kaperlyants et al., 1993), where dead cells are operationally defined as cells unable to form a colony on a plate under any condition tested (Barer et al., 1998; Kell et al., 1998). This is due to the fact that assays are normally based on single parameters such as membrane energization (often referred to as membrane potential, despite the absence of any direct evidence for it in bacteria; Kell, 1988, 1992), enzyme activity, or uptake of a substrate. Some of these criteria might be considered necessary to define viability in most cases, but none of them is sufficient to exclude nonviable cells. For example, a cell could display some enzyme activity but may have lost its ability to divide by lethal lesions in the chromosome. Thus, these assays can give rise to false-positive results, making it 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 (e.g., heat, ethanol, chlorine).

Commercial viability assays 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 (e.g., heat or cold). 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 score as nonviable in these kits, whereas repair of the damage during cultivation on a rich medium would allow subsequent growth (i.e., viability). 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 experimental measurements. Using these definitions, however, it should not be surprising if different experiments lead to different results.

Taken together, the cytochemical viability assays might be less time-consuming and more convenient in many cases, but they do not necessarily provide reliable data because the principles on which they are based are not sufficient (nor sometimes even necessary) criteria for viability. Thus, before use, each method for assessing viability 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 DETECTION BY FLOW CYTOMETRY

Despite the problems associated with fluorescent staining protocols for viability measurements, useful information can be obtained providing one carefully selects and tests an appropriate protocol for the problem under investigation. To illustrate this, this section discusses the three general classes of molecules whose uptake and/or fluorescence may be expected to reflect the viability of the cells (see Table 11.3.1), and presents sample procedures for the determination of microbial viability. This is followed by a case study (in the next section) of the use of flow cytometric viability testing in the gram-positive bacterium Micrococcus luteus.

It must be stressed that the protocols given below are provided as a guide only. The large variety of microorganisms that one may wish to study and the different modes of death that may befall them preclude the development of a universal flow cytometric viability test. The most useful advice that can be given to an

| Stain | Mode of action | Excitation source | References |
|-------|---------------|-------------------|------------|
| BacLight kit (Molecular Probes) | Exclusion of PI and staining with SYTO 9 | 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, 1997), Suller et al. (1997) |
| Calcofluor white | Uptake by dead cells | He-Cd (325 nm) | Berglund et al. (1987), Mason et al. (1995a) |
| Carboxynaphthofluorescein diacetate | Enzymic activity | He-Ne (633 nm) or laser diode (635 nm) | Berghersen et al. (1995), 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 (FDA) | 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 (FDG) | Enzymic activity | Argon (488 nm) | Plovins et al. (1994) |
| FUN-1 kit (Molecular Probes) | Metabolic activity | Argon (488 nm) | Millard et al. (1997), Prudencio et al. (1998), Wensisch et al. (1997) |
| Propidium iodide (PI) | Exclusion | Argon (488 nm) or He-Ne (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 Vives-Rego (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 | He-Ne (633 nm) or laser diode (635 nm) | Davey and Kell (1999) |

*He-Cd, helium/cadmium; He-Ne, helium/neon.*
The experimenter is to try a range of stains with sensibly designed control samples (i.e., exposed to the same types of stress that will be present in the experimental samples), and to choose the method that gives the most reliable results.

Under ideal conditions, complete separation of viable and dead cells will be achieved (see example in Fig. 11.3.1), and the analysis gates can easily be set to encompass live and/or dead cells. Under less favorable conditions, overlap may occur between the control samples on a single-parameter histogram. It may be possible to separate the two populations by using a dual-parameter dot plot of, for example, fluorescence versus forward light scatter. If this is not found to be the case, it may be necessary to choose a different staining protocol or to accept a “zone of uncertainty” in the results.

As a general rule, the use of 0.22-µm (or smaller) filtered water is recommended for all aqueous solutions. Fluorescent reagents should generally be protected from light to avoid photobleaching, and buffers should be protected from light to inhibit microbial growth. Specific details for preparing and storing dyes are given in the examples below. The most important factor in experimental design is the preparation of suitable control samples. Both positive and negative controls are suggested in the protocols below, but the choice of control samples will depend on the exact purposes of the experiment.

**Dye Exclusion**

The exclusion of dye by an intact membrane is probably the most straightforward viability test to understand and perform. Fluorescent stains normally excluded by living cells are used to assess viability on the grounds that dead cells have leaky membranes that are permeable to the stains. Nucleic acid stains such as propidium iodide (PI) 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). PI is often the dye of choice for viability determinations in animal cells, whether the assay is done using flow cytometry or fluorescence microscopy (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 one cell type is eukaryotic and the other is prokaryotic. In the case of ethidium bromide, for instance, efficient efflux pumps capable of removing the dye from *Escherichia coli* have been demonstrated by Jernaes and Steen (1994), and many other such pumps are known (Lewis, 1994). Thus, the applicability of excluded dyes for microbial viability determinations needs to be carefully considered for each type of organism.

**Figure 11.3.1** Distribution of the fluorescence 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 at a final concentration of 0.5%.
Example 1: Measuring Viability by Dye Exclusion Using TO-PRO-3

TO-PRO-3 is a nucleic acid stain that can be used for viability testing by dye exclusion. This series of dyes has an advantage over many other exclusion dyes in that its fluorescence is enhanced some 1000-fold on binding to nucleic acids (Rye et al., 1992, 1993a,b). Undiluted solutions of TO-PRO-3 (as obtained from the manufacturer) should be stored frozen and protected from light. The diluted solution is stable for several weeks when stored in the same manner. This procedure calls for a flow cytometer with a 633-nm helium/neon (He-Ne) laser or 635-nm laser diode as excitation source, and a detector set to receive emission above 650 nm.

Staining. Place 999 μl of a cell sample or control, at 10^5 to 10^7/ml, into a tube suitable for use with the flow cytometer. Dilute 1 mM TO-PRO-3 (Molecular Probes) to 0.1 mM in a buffer suitable for use with the sample of interest. Add 1 μl per sample (final 0.1 μM) and mix gently (e.g., with a pipet). Stain each sample and control immediately before use.

Calibration. Stain a dead control (e.g., ethanol-fixed or heat-treated cells) and analyze on the flow cytometer, adjusting the PMT voltage as necessary to ensure that the sample fluorescence (at >650 nm) is to the right-hand side of the display. Position an analysis gate to encompass the dead cells. Stain the live control (freshly harvested cells, ≥95% alive) and analyze on the flow cytometer. Position an analysis gate to encompass the live cells. Adjust the gates so that optimum separation of live and dead cells is achieved. If good separation is not achieved, vary the final stain concentration to between 0.05 and 1 μM, alter the PMT voltages, and/or alter the staining time according to the nature of the cell sample.

Analysis. Stain a sample and analyze using the settings determined during calibration. Record the percentages of viable and dead cells.

Dye Uptake

It is well documented that the mitochondria of eukaryotic cells have the ability to accumulate lipophilic cations such as rhodamine 123 concentrically (Chen, 1988; Chen et al., 1982; Grogan and Collins, 1990; Johnson et al., 1980, 1981), in an uncoupler-sensitive fashion. The staining of mitochondria with rhodamine 123 has been used in conjunction with flow cytometry to study their activity (Darzynekiewicz et al., 1981; Iwagaki et al., 1990; Lizard et al., 1990). Viable bacteria also accumulate rhodamine 123, while nonviable ones do not (Diaper et al., 1992). Under certain conditions, the extent to which individual bacteria take up rhodamine 123 quantitatively reflects the extent of their viability—i.e., whether they are immediately culturable, nonculturable, or dormant (Kaprelyants and Kell, 1992). On average, larger cells may be expected to accumulate more molecules of rhodamine 123 than do smaller cells, but since flow cytometry allows collection of both fluorescence (rhodamine 123 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. For an example of results using an uptake dye, see Estimations of Viability of Micrococcus luteus.

In contrast to some of the other viability stains such as acridine orange (Back and Kroll, 1991), the uptake of rhodamine 123 is useful not only because it does not require the use of fixatives to permeabilize the cell, but also because the concentrative uptake is dependent on an intact and energized cytoplasmic membrane. This has the great advantage that living cells can be stained and that further physiological studies may be conducted following staining, if required (Davey et al., 1993).

There are, however, experimental problems with the use of lipophilic cations for microbial viability determinations. For instance, they may be pumped out of viable cells by microbial efflux pumps, causing both viable and nonviable cells to appear to be nonfluorescent. In addition, although 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 pretreated with EDTA (Kaprelyants and Kell, 1992). However, such pretreatment is practically impossible to standardize, 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 preprocessing 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 nonviable cells. The lipophilic anion bis-(1,3-dibutylbarbituric acid) trimethine oxonol, or DiBAC4(3), has been shown to enter eukaryotic membranes only if the membranes are deenergized (Wilson and Chused, 1985). This stain has been used for the rapid assessment of microbial responses to antibiot-
ics (Jepras et al., 1997; Mason et al., 1994, 1995b; Suller et al., 1997), allowing the analysis of heterogeneity within a microbial population in terms of susceptibility to an antibiotic.

**Metabolic Activity**

In certain circumstances the activity of the cells may be of more interest than their membrane integrity. For this purpose, a third class of viability stains is used in mammalian cell biology, often as a positive marker in a dual-staining protocol with ethidium bromide or propidium iodide (Aeschbacher et al., 1986). These stains are themselves nonfluorescent and membrane impermeant, but are metabolically altered inside the cell to become fluorescent and, under ideal conditions, impermeant. One example is fluorescein diacetate (FDA), which is cleaved by intracellular esterases to produce fluorescein. Dead cells do not stain because they lack enzyme activity and/or the fluorescein diffuses freely through their damaged membranes. Flow cytometric analyses of mammalian cells with this class of dyes are well established (e.g., Aeschbacher et al., 1986; Frey, 1997). Diaper and Edwards (1994a,b) used flow cytometry to detect a variety of viable bacteria after staining with FDA and its derivatives, or with ChemChrome B (Chemunex). 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 et al. (1995) showed that FDA and carboxyfluorescein diacetate (CFDA) penetrated yeast rapidly and that esterase activity was probably most limiting; an energy-dependent efflux of carboxyfluorescein from viable cells 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 microorganisms, thus giving the appearance of a lack of metabolic activity in cells that are nonetheless viable.

**Example 2: Measuring Viability by Assessing Metabolic Activity with CFDA**

Carboxyfluorescein diacetate (CFDA) is cleaved by intracellular enzymes to produce fluorescent carboxyfluorescein. In using this approach, the experimenter must be aware that dormant cells (Kaprelyants et al., 1993) or cells with low metabolic activity will probably score as nonviable. The CFDA solution in DMSO should be stored frozen and protected from light, and is stable for several months. This procedure calls for a flow cytometer with a 488-nm argon laser or other suitable excitation source, and a detector set to collect emission at ~525 nm.

**Staining.** For cell samples, use 10^6 cells/ml suspended gram-positive bacteria in 50 mM KH_2PO_4, pH 7.4, or gram-negative bacteria in TE buffer (APPENDIX 2A). For dead controls, use ethanol-fixed or heat-treated cells; for live controls, use freshly harvested cells, ≥95% alive. Place 990 µl of cell samples and controls into tubes suitable for use with the flow cytometer. Add 10 µl of 1 mM CFDA in dimethyl sulfoxide (Molecular Probes; final 10 µM) to each and mix gently (e.g., with a pipet). Incubate for 30 min at the normal growth temperature of the microorganism.

**Calibration.** Analyze the dead control on the flow cytometer, adjusting the PMT voltage as necessary to ensure that the sample fluorescence (at ~525 nm) is to the right-hand side of the display. Position an analysis gate to encompass the dead cells. Analyze the live control and position an analysis gate to encompass the live cells. Note that with CFDA live cells are brightly fluorescent and dead cells are nonfluorescent or weakly fluorescent. Adjust the gates so that optimum separation of live and dead cells is achieved. If good separation is not achieved, adjust the stain concentration, alter the PMT voltages, and/or alter staining time according to the nature of the cell sample.

**Analysis.** Analyze cell samples using the settings determined during calibration. Record the percentages of viable and dead cells.

**Commercial Kits**

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 (Molecular Probes) gives a two-color viability assessment of both gram-negative and gram-positive bacteria, where live cells are labeled green with SYTO 9 and dead cells are labeled red with PI. 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 microbiology (Braux et al., 1997; Buchmeier and Libby, 1995b; Suller et al., 1997), allowing the analysis of heterogeneity within a microbial population in terms of susceptibility to an antibiotic.
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Estimation of Microbial Viability Using Flow Cytometry

Example 3: Measuring Viability with the BacLight Kit

A convenient approach to viability determination may be obtained using the Molecular Probes BacLight kits. A set of eleven 2-ml calibration samples ranging from 100% live to 100% dead cells should be prepared by mixing live and dead cells in different ratios. Full details of the sample preparation and reagent addition differ depending on the specific kit purchased (see http://www.probes.com/pis/mp07007a.pdf). The protocol provided below is based on this information, in case Internet access is not available. BacLight kit reagents should be protected from light. The storage instructions provided with the kit should be followed exactly; in some cases, freezing or desiccation may be required. This procedure requires a flow cytometer with a 488-nm argon laser or other suitable excitation source, and detectors set to collect emission below 590 and above 610 nm.

**Staining.** Follow the instructions provided with the kit purchased. Incubate at room temperature in the dark for 15 min.

**Calibration.** Analyze the calibration samples as described in the kit instructions. Produce a calibration curve of percentage live bacteria measured versus the actual percentage of live bacteria present in the calibration sample.

**Analysis.** Analyze the experimental samples in the same way as the calibration samples and determine the “true” percentage of viable cells from the calibration curve.

Designing a Cytotoxic Protocol

When selecting a stain for a particular application there are several factors that need consideration. Some—such as the extinction coefficient, quantum yield, and photostability—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; a list of viability stains that are compatible with common flow cytometric light sources is shown in Table 11.3.1.

One factor that is of particular relevance in the measurement of viability is the toxicity of the stain. Protocols used to assess viability should clearly not perturb viability. 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. However, levels of cellular autofluorescence are generally higher for unfixed cells than for, say, 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, driving the development of red-excited fluorophores (Fabian et al., 1992; Patonay and Antoine, 1991; Shealy et al., 1995a,b). Such dyes can be exploited in flow cytometry using a 633-nm He-Ne laser or a 635-nm laser diode.

Laser diodes can be used to construct smaller, cheaper, and more robust flow cytometers such as the Microcyte (see Internet Resources 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, 1996, 1999). Flow cytometric analyses of samples of *M. luteus* stained with TO-PRO-3 are shown in Figure 11.3.2.

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 stain and subsequent analysis. The optimum...
concentration will inevitably be a compromise between a high one (for maximum signal) and a low one (for 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 stains 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.

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, as it allows the properties of individual cells in a population to be distinguished. Because fluorescence and light scattering are measured on a quasicontinuous scale, it is possible to fully quantify the heterogeneity of a sample rather than making 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 (e.g., Kaprelyants and Kell, 1992, for full details). In order to obtain samples of low initial

Figure 11.3.2 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 analyzed on a Microcyte flow cytometer. Under these conditions unstained samples did not give any detectable fluorescence. Three samples include cells freshly harvested from the culture, cells freeze-thaw treated prior to staining, and cells permeabilized by fixation in 70% ethanol. The frozen sample was kept at −20°C for 30 min, rapidly defrosted by plunging into a 50°C water bath, and then refrozen and rethawed in the same manner. For fixed cells, 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 normalized to 1 million cells.
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 can be obtained in which <0.01% of the cells 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 (prestarvation) value (Kaprelyants and Kell, 1993a). In fact, as illustrated below, starved populations consist of different subpopulations that can be visualized flow cytometrically.

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 energization–sensitive probe rhodamine 123. Figure 11.3.3A shows the typical fluorescence distribution of nonstarved M. luteus cells stained with rhodamine 123 and analyzed by flow cytometry. Regions 1, 2, and 3 were demarcated following the analysis of a freshly harvested sample of viable cells stained with rhodamine 123. In the absence of uncoupler, the cells exhibited a level of fluorescence between channels 80 and 136 (Fig. 11.3.3A); the use of carbonyl cyanide m-chlorophenylhydrazone (CCCP) showed that this fluorescence was fully uncoupler sensitive (Fig. 11.3.3B).

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 rhodamine 123, judged flow cytometrically (Kaprelyants and Kell, 1992). Similar results were obtained by the flow cytometric study of M. luteus cells stained with 5-cyano-2,3-ditolyltetrazolium chloride (CTC), the reduced form of which is a fluorescent formazan that allows one to monitor the respiratory activity of individual cells (Kaprelyants and Kell, 1993b; Rodriguez et al., 1992).

Estimation of Dormant and Dead Cells

It has been shown that 10% to 50% of M. luteus cells in 3-month-old populations can be resuscitated to normal, colony-forming bacteria under conditions that exclude any significant regrowth of initially viable cells (Kaprelyants and Kell, 1992, 1993a, 1996; Kaprelyants et al., 1993, 1994, 1996, 1999; Mukamolova et al., 1998a,b; Votyakova et al., 1994, 1998). This indicates the persistence of a significant percentage of cells in the dormant state, a hypothesis that was confirmed using the most probable number (MPN) method by the resuscitation of cells from samples that, 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 compared with those estimated with the agar plate method (Table 11.3.2). 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 slow-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 that 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), is a small secreted protein with a molecular weight of ~17 to 18 kDa (Kaprelyants et al., 1999; Mukamolova et al., 1998a).

### Table 11.3.2 Resuscitation of Dormant M. luteus Cells in Liquid Medium

| Culture | Time of starvation | Total count (cells/ml) | Viable count by cfu (cells/ml) | Viable count by MPN (cells/ml) |
|---------|---------------------|------------------------|-------------------------------|-------------------------------|
| 1       | 2 months            | 5.3 × 10⁹              | 5 × 10⁶                       | 3.5 × 10⁹                     |
| 2       | 4.5 months          | 10¹⁰                   | 1.3 × 10⁶                     | 9.2 × 10⁹                     |
| 3       | 6 months            | 1.2 × 10¹⁰             | 3.6 × 10⁴                     | 9.2 × 10⁹                     |
| 4       | 9 months            | 6.2 × 10⁹              | 5.2 × 10⁵                     | 5.4 × 10⁹                     |

*Abbreviations: cfu, colony-forming units; MPN, most probable number.

†Performed in the presence of RPF (for details see Mukamolova et al., 1998a).
Figure 11.3.3 Distribution of the fluorescence of *M. luteus* following staining with 0.3 µM rhodamine 123. Flow cytometry was performed using a Skatron Argus 100 instrument that was set up as described in the manufacturer’s manual. The PMT voltage for the fluorescence channel was 700 V, and the full scale of the abscissa represents 3.5 decades in fluorescence intensity. (A) 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) Cells were prepared as in (A), but 15 µM CCCP was added prior to analysis. (C) Cells were grown in lactate minimal medium and then starved for 5 months before dilution and staining.
As shown in Figure 11.3.3, the proportion of dormant cells can also be determined using flow cytometry. Figure 11.3.3C shows a typical distribution of the fluorescence of *M. luteus* cells starved for 5 months, stained with rhodamine 123, and analyzed by flow cytometry. A bimodal fluorescence distribution is evident. Region 1 (channels 0 to 80) represents cells that bind rhodamine 123 nonspecifically. For comparison, under nonstarvation conditions (Fig. 11.3.3B), 98% of fresh, late–logarithmic phase *M. luteus* cells stained with the same concentration of rhodamine 123 followed by treatment with a suitable concentration of the uncoupler CCCP exhibited fluorescence in this region. Although starved cells also occurred in region 2 (channels 80 to 136), their sensitivity to CCCP was very low, with only 2% to 5% of the cells in region 2 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 regions 1 and 2 represent bacteria in different physiological states (Kaprelyants et al., 1996). To determine whether this was indeed the case, a cell-sorting approach was used (see Use of Cell Sorting in Viability Studies, below).

**Estimation of Injured Cells**

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

**Membrane-impermeant probes**

In the case of bacteria, a damaged or leaky membrane (see Dye Exclusion, above) may not be a sufficient criterion for defining a cell as nonviable, but it can nevertheless be used as an indication of stress-induced injury. The permeability barrier of cells starved for 5 months was monitored by staining with PI (MacDonell and Hood, 1982; 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 (Fig. 11.3.1). Observation of different starved cultures of *M. luteus* revealed that resuscitation was not successful in some cultures where the percentage of PI-positive cells was close to 100%, even in the presence of RPF. This indicates a correlation between the state of the permeability barrier and the ability of starved cells to recover, and thus may allow the use of PI staining for discrimination between dormant and dead cells in some populations.

**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 a 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).

This approach, however, can only reflect injuries connected with damage to the outer portion of the cell envelope of gram-negative bacteria (Ignatov et al., 1981; Ray and Speck, 1973), whereas it is damage to the cytoplasmic membrane that is more important in determining the viability of bacteria after freezing (Ignatov et al., 1981). 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 behavior of frozen/thawed *M. luteus* cells after the first 5 min of incubation in the presence of CTC revealed that ∼25% of the population gave significant fluorescence in the presence of NADH, but only 1% did so in its absence. After 17 min of incubation with CTC, the absolute percentage of cells fluorescing above channel number 20 had increased in both cases (i.e., with or without NADH), but the difference had not. Further incubation of the cells resulted in a decreased difference in the distribution pattern for the two types of sample. The kinetics of CTC reduction in the two samples are summarized in Figure 11.3.4.

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., 1981, 1982). These cells very rapidly reduce CTC to formazan, to a concentration comparable to that in intact cells (as judged by the channel number of the...
fluorescence), whereas some endogenous substrates left in the cells after freezing and thawing permit a slower reduction of CTC in the samples without NADH. Thus, at least 25% to 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) was 90% to 95% (Ignatov et al., 1982; Ray and Speck, 1972). Increased permeability of *M. luteus* cells resulting from exposure to the freeze/thaw stress can also be seen in Figure 11.3.2.

**USE OF CELL SORTING IN VIABILITY STUDIES**

While flow cytometric analysis allows the investigator to perform a rapid and quantitative version of 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 that 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 nonviable fractions of the histogram. This allows determination of the culturability of exactly those cells whose cytological properties have already been determined directly.

To this end, cultures whose fluorescence was of the type displayed in Figure 11.3.3C were sorted into two populations: (1) cells whose rhodamine 123 staining was sensitive or partially sensitive to CCCP (regions 2 + 3), and (2) cells whose rhodamine 123 staining was not sensitive to CCCP (region 1). 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 2 + 3, but not for cells in region 1. This constitutes direct evidence that dormant cells
are concentrated in regions 2 + 3 (Kaprelyants et al., 1996).

Some cell sorters, such as the Coulter Epics Elite, have a motorized 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 microtiter plates, it is also possible to modify the stage and collection protocol to allow microbial cells to be collected directly onto agar plates (Fig. 11.3.5). Thus, an event from a tightly defined region on a histogram can be correlated directly to the growth (or absence of growth) of a colony on an agar plate. This approach has been pioneered by Nebe-von Caron and colleagues, and has great potential for many microbiological applications (e.g., Nebe-von Caron and Anderson, 1996; Nebe-von Caron and Badley, 1996; http://www.cyto.purdue.edu/flowcyt/research/microflow/gerhard/genmic10.htm).

Although the sorting approach offers many advantages, it is important to also be aware of its potential pitfalls. 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

![Flow cytometric cell sorting using the Autoclone module of the Coulter Epics Elite can be used to place individual cells onto ∼60 to 65 discrete locations on a standard 90-mm agar plate. (B) An agar plate with *M. luteus* colonies following sorting.](image-url)
as propidium iodide, and the clump will thus be fluorescent and 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 an additional stress that may convert an injured cell into a nonviable 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, in large part because of the problems of defining viability in microbial cells. Despite the difficulties mentioned above, the view to which the authors subscribe is that only culturability can provide a gold standard for positive viability (Kell et al., 1998). Although the flow cytometric approach has much to offer for the determination of microbial viability, it must be emphasized that no single stain nor even cocktail is likely to be a universal indicator of viability, especially if its interpretation must reflect our ability to induce the cells to divide (Table 11.3.3).

A cell that is killed by exposure to environmental extremes such as heat or pH 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 history.

Although most instruments designed specifically for the analysis of microorganisms (e.g., the Bio-Rad Bryte or the Aber Instruments Microcyte) do not permit cells to be sorted, microbes may be sorted using the standard commercial instruments (UNIT 11.4). 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 the adoption of this approach is strongly recommended.

To conclude, although there are currently no perfect stains, careful protocol development does currently allow 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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Table 11.3.3 Advantages and Disadvantages of Viability Determination Methods

| Method               | Advantages                                      | Disadvantages                        |
|----------------------|-------------------------------------------------|---------------------------------------|
| Multiplication assay | Provides sufficient proof that cell was alive   | Slow                                  |
|                      | Generally straightforward to interpret          | Requires prior knowledge of growth requirements |
|                      | Can be used without knowledge of growth requirements | Underestimates viable cell numbers |
| Cytological assay    | Rapid                                           | Can be difficult to interpret         |
|                      | Can be used without knowledge of growth requirements | Viability is not measured directly |
|                      | Total count can be determined simultaneously    | False positives and false negatives may occur |
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Another short but useful review of applications of flow cytometry in environmental microbiology, concentrating on aquatic systems.

INTERNET RESOURCES

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

Aberystwyth, U.K., flow cytometry site with 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 (the Microcyte) 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 Molecular Probes viability stains, including viability kits.

Contributed by Hazel M. Davey, Dieter H. Weichert, and Douglas B. Kell
University of Wales
Aberystwyth, Wales, United Kingdom

Arseny S. Kaprelyants
Russian Academy of Sciences
Moscow, Russia