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MINIREVIEWS

Life, Death, and In-Between: Meanings and Methods in Microbiology▽

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Determination of microbial viability by the plate count method is routine in microbiology laboratories worldwide. However, limitations of the technique, particularly with respect to environmental microorganisms, are widely recognized. Many alternatives based upon viability staining have been proposed, and these are often combined with techniques such as image analysis and flow cytometry. The plethora of choices, however, adds to confusion when selecting a method. Commercial staining kits aim to simplify the performance of microbial viability determination but often still need adaptation to the specific organism of interest and/or the instruments available to the researcher. This review explores the meaning of microbial viability and offers guidance in the selection and interpretation of viability testing methods.

The determination of viability in microbial samples is one of the most routine and straightforward analyses carried out in microbiology laboratories worldwide. The "gold standard" method involves the growth of colonies on a nutrient agar surface during a period of incubation (16, 34) and is among the first methods taught to microbiology students and trainees. Viability determinations using this method may be qualitative ("Are colonies formed?") or quantitative (e.g., "What is the concentration of viable cells in the sample?"). The plate count method is based upon the premise that a single bacterium can grow and divide to give an entire colony, and this amplification provides a high level of sensitivity (28) with the capability to detect viable bacteria at densities of ~10 per ml without the necessity for preanalysis concentration. However, despite its widespread use, it cannot be considered a universal approach, as 95% of all cultivated and published species belong to just 5 of the 53 recognized bacterial phyla (23). Furthermore, it has long been recognized that microbial cells may exist in "cryptobiotic" (21), "dormant" (18–20, 26), "moribund" (33), or "latent" (41) states, in which they will not form colonies on nutrient media but may have other measurable activity (and therefore can still have an important role to play in disease or economic loss). In the case of environmentally acquired samples, it has been estimated that 1% (or fewer) of the microscopically observable organisms are scored as viable by the plate count method (1). Nevertheless, in industry, detection and quantification of viable cells of well-characterized species are important for quality control purposes (6), while in environmental samples, despite limitations, enumeration of viable bacteria provides information on soil and water quality, environmental contamination, and bioremediation (25).

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DEFINING VIABILITY

Despite its frequent use, the term viability is difficult to define and Schrödinger's classic book (35) is testament to the difficulties of answering the question "What is life?" Taking a tangential approach, we can consider the question "What is death?" It is not simply an absence of life (13), but we might reasonably answer that it is the cessation of life, i.e., the absence of viability where it had previously existed. From this, we can see that the definitions of life and death are inseparable, and, indeed, the *Oxford English Dictionary* defines life as "The condition or attribute of living or being alive; animate existence. Opposed to death or inanimate existence." In human medicine, technological advances made the cardiopulmonary definition of death untenable, and thus it was replaced by a definition of whole-brain death (total and irreversible cessation of brain function), which is more difficult to identify than the absence of heartbeat or respiration (42). For microbes, too, the distinction between life and death is problematic, on both a practical and a philosophical level. While we are safe, at least for now, in the assertion that "The only certainty in life is death," the definitions of the two states remain somewhat nebulous; the route from life to death, and the potential for reversing part of the route, remains uncertain.

For practicality in microbiology, repeated division of a cell on an agar surface to produce a visible colony is usually taken as incontrovertible evidence of viability. However, while it is clear that the founder cell giving rise to a colony must have been alive at the outset, it may not be the case that, at the time of performing the plate count, this specific individual is still alive. Interpreting the situation where there is an absence of colony formation is not at all clear-cut (Table 1). Nevertheless, bearing in mind that it is usually impossible to test the viability of an individual cell more than once, absence of viability on a population basis may be defined as failure to form colonies under any condition tested (18).

In 1976, John Postgate stated: "At present one must accept that the death of a microbe can only be discovered retrospectively: a population is exposed to a recovery medium, incu-

TABLE 1. Interpretation of the results of plate counting

Observed result	Usual interpretation	Alternative interpretations
A colony is formed	A viable cell gave rise to the colony	At least one viable cell gave rise to the colony—but it may have been two or more cells coinciding at the same place on the plate or a clump of cells that contained at least one viable individual
No colony is formed	There were no viable cells in the sample	(i) The growth medium and/or incubation conditions were incorrect (ii) The cells were damaged/stressed and therefore unable to grow on solid medium (iii) The population density was low and therefore cell-cell communication could not take place, resulting in no observable growth (iv) Insufficient time was allowed for visible colony development in slowly growing cells

bated, 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" (32).

Thirty-five years may have elapsed but, with very limited exceptions (e.g., imaging of the destructive analysis of microbes [40]), these words remain true. Notwithstanding this, however, many attempts have been made to develop rapid methods, usually based on the exclusion, uptake, or metabolism of colored, fluorescent, or fluorogenic stains, designed to provide information that correlates with reproductive viability (12). Microscopy, to ascertain the extent of staining of the cells, has the advantage that results can be obtained in minutes rather than the days required for plate counts; however, where there are many samples to process, the microscopy is labor-intensive and can lead to operator fatigue (Table 2). Methods of overcoming these limitations include image analysis and flow cytometry.

IMAGE ANALYSIS

Image analysis is the automated extraction of information from images and can be used to identify, for example, the number of cells in an image, their size, morphology, color, and intensity, etc. This approach overcomes the tedium of manually counting cells of a particular type or intensity, providing rapid acquisition of data relating to statistically significant numbers of cells.

Although image analysis was once the domain of high-powered, expensive commercial software, there are now multiple examples of free programs for such analysis (e.g., imageJ [http://rsb.info.nih.gov/ij/], *daime* [http://www.microbial-ecology.net/daime/], and CellC [http://sites.google.com/site/cellcsoftware/]). To take advantage of this method, Singh et al. (37) modified an existing direct viable counting method to make it compatible with image analysis. With a range of bacterial species it was shown that the viable cell counts determined using image analysis were higher than those obtained by either the direct manual count of viable cells or spread plate methods but that image analysis was an efficient and quantitative method for viability determination in bacteria.

FLOW CYTOMETRY

Flow cytometry has its origins in the analysis of microorganisms (15, 38, 39) but has developed over the last 30 years as a technique primarily optimized for, and associated with, the

TABLE 2. Comparison of methods for determining viability of microorganisms

Method	Speed	No. of cells analyzed	Ease of use	Typical costs (excluding labor)
Plate counting	Preparation of dilutions and plating take minutes. Hundreds of plates can be prepared per day. Incubation of plates for 1 to 7 days typical before results are obtained.	Viable counts are typically based on plates with 30 to 300 cells.	Minimal training required in aseptic technique and safe handling of microbes.	Plastic consumables and media components. Incubation at growth temp.
Microscopy	Dilution (if necessary) and staining take minutes. Some stains may require incubation of, e.g., 10 to 30 min. Manual microscopic analysis may take several minutes per sample. A hundred samples could conceivably be processed in a day. Results are obtained immediately.	Typically 100 to 500 cells per sample are scored as viable or dead. Image analysis can be used to automate the process of identifying and scoring viable/dead cells.	Minimal training in safe handling of microbes and stains (some of which are carcinogenic).	Microscope slides and coverslips. Stains. Cost of purchasing and maintaining microscope or fluorescence microscope.
Flow cytometry	Dilution (if necessary) and staining take minutes. Some stains may require incubation of, e.g., 10 to 30 min. Manual sample presentation may take several minutes per sample. Automated samplers can be loaded with, e.g., a 96-well plate of samples. Hundreds of samples can be processed in a day. Results are obtained immediately, although postacquisition analysis of data is common.	Typically 10,000 to 100,000 cells per sample are analyzed. As stain uptake is quantified, intermediate results between live and dead are possible.	In addition to the above, training is needed in operation and quality control of flow cytometer. Experience required for protocol development and data analysis.	Sample tubes and stains. Costs of purchasing and maintaining flow cytometer.

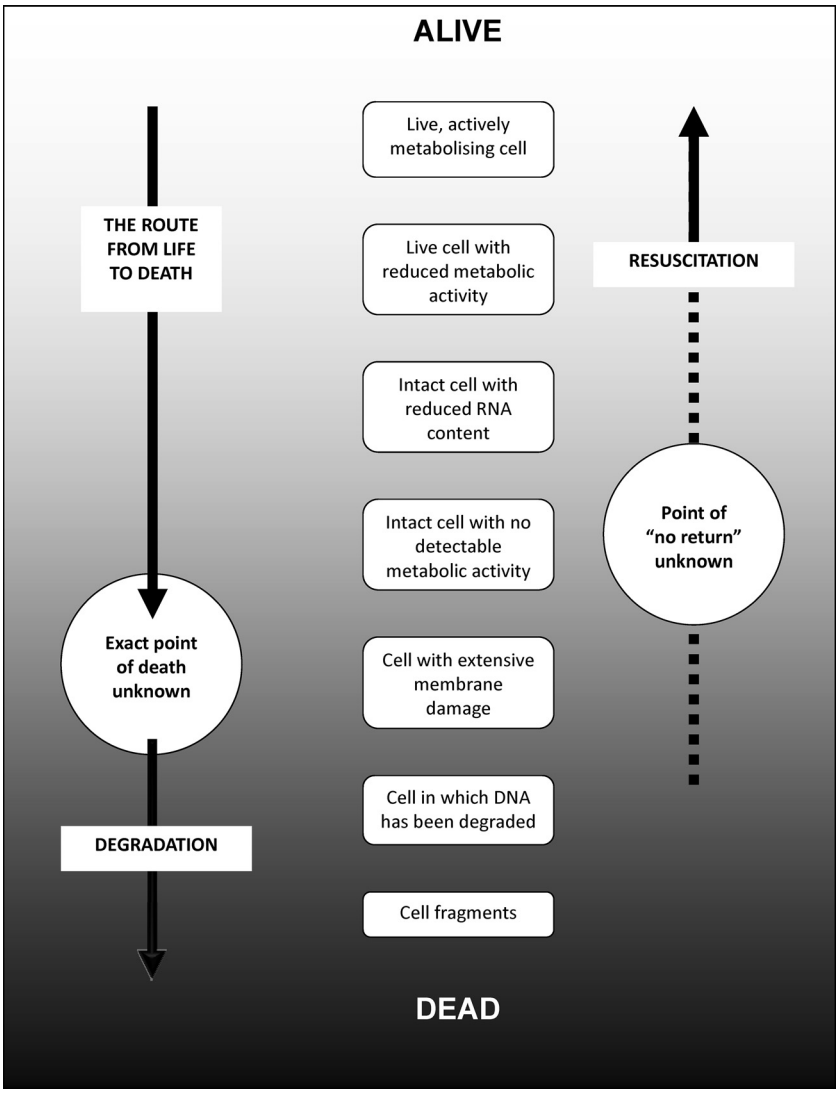

FIG. 1. Groups of cells within a microbial population may exhibit heterogeneous uptake of fluorescent stains and thus be classified into more subpopulations than "live" and "dead." The route from "live" to "dead" contains many steps (not all of which will occur or be observed to occur in all cases), and while the extremes are relatively clear-cut, the reversibility of these steps and indeed the moment of death are far from easy to define. Metabolic activity can be demonstrated by cleavage of fluorescein diacetate or uptake of rhodamine 123, RNA content can be measured using pyronin Y, and membrane damage can be measured by entry of stains that are normally excluded, such as propidium iodide.

analysis of clinical samples (2). In particular, with the development of appropriate fluorescently labeled monoclonal antibodies, it has become a common method for the diagnosis and tracking of HIV infection (8), including coinfection with tuberculosis (14). Notwithstanding this bias toward human medicine, manifested by an overwhelming dominance of "nonmicrobial" applications (27), there are many reasons why flow cytometry is advantageous for the study of microbes and, in particular, for the determination of their viability. Flow cytometry analyzes individual cells (11, 22, 27), thereby permitting the determination of sample heterogeneity. As viability is ultimately a characteristic of an individual cell, an approach such as this is essential for meaningful results to be obtained. However, unlike other "single-cell" methods such as microscopy, the level of automation and method of sample handling and presentation means that thousands of cells can be analyzed per second.

The principle is straightforward: at the measurement point in the flow cytometer the stream of cells intersects a beam of light from one or more light sources (lasers and arc lamps). Light is scattered and fluorescence is emitted from the cells as a consequence, and the emitted light can be separated according to its wavelength. By the judicious selection of compatible (spectrally distinct) cocktails of fluorescent probes, multiparametric measurements can be used to quantify uptake of fluorescent dyes that discriminate subpopulations of cells according to characteristics of interest. In the case of viability measurements this might include measurement of metabolic activity, membrane energization, RNA and/or DNA content, membrane permeability, etc. This rapid analysis at the single-cell level allows distributions of multiple cell properties to be determined, allowing identifications of subpopulations of cells that may be characterized on a spectrum from "maximum viability" through to death and, potentially, degradation (Fig. 1).

Although the process is simple in principle, there are two

stumbling blocks (beyond cost of/access to a flow cytometer) that may be limiting the wider use of flow cytometry for microbial viability measurements. The first is the huge diversity of possibilities in terms of stain selection, concentration, staining time, etc., described in the literature—the wide choice has arisen in part because no single stain or staining method has been found to be suitable for all organisms (12). The *modus operandi* of different fluorescent stains has been extensively described in other reviews (12, 24, 29, 36) and will not be covered in detail here. While having many options can be a benefit, it is also a barrier in that it creates confusion; however, multiple stains can be used together to allow several viability-related parameters to be assayed for each cell that is analyzed. Multiple stains will provide a more complete picture of physiological changes than can be achieved with a single stain or indeed by the presence or absence of growth on an agar surface. It would be expected that a cell at the top of Fig. 1 (alive) would be fluorescent when stained with rhodamine 123 or fluorescein diacetate but would not stain with propidium iodide or $DiBAC_4(3)$. In a cell with extensive membrane damage, the opposite staining pattern would be expected.

Recognizing that culturability was not the best proxy for viability with environmental samples, Barbesti and colleagues (4) immunolabeled bacteria prior to staining for DNA content (SYBR green I) and membrane permeability (propidium iodide [PI]). This allowed simultaneous detection of bacteria and their viability status. More recently (30), several distinct physiological states have been demonstrated in *Pseudomonas fluorescens* using combinations of SYBR green, PI, ethidium bromide, and $DiBAC_4(3)$. These included intact cells with normal energy metabolism, deenergized cells, depolarized cells, and permeabilized cells.

In order to simplify the process of stain selection, a number of companies have developed commercial kits which contain reagents in appropriate combinations to stain a variety of microorganisms. Following addition of the reagents to the sample, it is incubated and then analyzed, usually to provide total and viable counts (and hence also the percentage of viability) from a single analysis. Live/dead kits such as *Bac*Light, Fung*aLight*, and the yeast viability kit (all Invitrogen) provide premixed or individual stains. For example, *Bac*Light contains SYTO9 (which stains all cells green) and also PI, on the basis that the latter enters only cells with membranes damaged sufficiently to cause cell death. Thus, a total cell count can be obtained from the green fluorescence signal and a dead cell count from the red fluorescence signal, allowing the percentage of viability to be readily determined. Specific patterns of staining have been related to intermediate damage, such as permeabilization of the outer membrane of Gram-negative organisms (5). While *Bac*Light is designed primarily for bacteria, it has also been reported to work with *Saccharomyces cerevisiae* (43); however, recently reversible damage sufficient to allow PI entry has been demonstrated with this organism (10). Some flow cytometer manufacturers also produce kits for the purpose of monitoring microbial viability; for example, the Becton Dickinson cell viability kit contains thiazole orange to stain all cells and PI to stain dead cells. This approach has recently been used to enumerate and determine the viability of intracellular *Campylobacter jejuni* following lysis of the host cell (31).

The second limiting factor which deserves consideration is that, irrespective of whether kits or individual fluorescent stains are used, some method development or protocol adjustment is often required. This is perhaps not surprising due to the structural differences between diverse microorganisms and our lack of knowledge, particularly of those which we cannot grow in the laboratory. Stains cannot work as we would wish unless they can reach their target, and the complexities of cell walls, outer membranes, and active ion pumps can prevent this, leading to erroneous interpretation of negative staining results. This can be off-putting to newcomers to flow cytometry in that published methods or suggested protocols included within kits need to be adjusted to take account of the particular flow cytometer hardware and software and the species, growth conditions, or source of the microbes. Figure 2 shows the steps typically carried out for localization of protocols. The preparation of controls is usually straightforward, but where environmental or stressed samples are ultimately to be analyzed, careful consideration should be given to the expected mode(s) of death. Control live samples are usually harvested from exponential or early-stationary-phase cultures where close to 100% viability is expected. Dead control samples may be obtained by heating (3), addition of ethanol (9), etc.

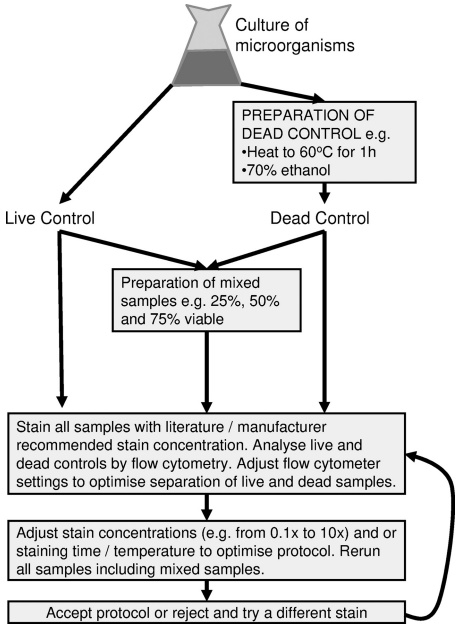

FIG. 2. Flowchart indicating the steps in adjusting a published protocol for a new flow cytometer, microorganism, or experimental condition.

It is the second phase of protocol development that can be the more time-consuming (and frustrating). Here, a published method must be adapted to work with the available flow cytometer and the specific organisms of interest. If the "off-the-shelf" protocol does not give good discrimination between the live and dead control samples (and sensible results with mixed samples of known viability), then it must be adjusted through trial and error. The usual approach is that stain concentration or staining conditions are varied to improve discrimination between the controls (Fig. 2). Alternatively (or additionally), the cells may need pretreatment (e.g., EDTA can be used to

permeabilize the outer membrane of the Gram-negative cell wall, improving stain uptake; addition of a carbon source may be required for active stain uptake in starved cells, etc.). This iterative process of protocol development and optimization can be quite off-putting to the flow novice.

Whether we are discussing microorganisms or macroorganisms, it is usually easier to distinguish between live and killed individuals than between organisms that are alive and those which have recently died of natural causes. In the case of staining, while control live and dead samples may be clearly separable, environmental stresses often give rise to heterogeneous populations, with some cells showing an intermediate uptake of viability stains (5, 10). When a population of cells is exposed to stress, depending on the magnitude of the stress, there may be a heterogeneous response in which some cells are killed, others are damaged, and yet others may show no observable phenotypic change (20). It has recently been shown (10), even for well-characterized, eukaryotic laboratory organisms when cells are under stress, that PI may enter cells during or immediately after application of the stress but that a short period of recovery will allow membrane damage to be repaired such that PI cannot enter the cells.

FUTURE PROSPECTS

While flow cytometry holds many advantages and exciting opportunities for the microbiologist, it has not yet become as widely used as this potential deserves. Instruments have historically been costly and complex to operate, and in some cases commercial instruments have lacked the sensitivity required for the analysis of microbial cells. These shortcomings are being addressed, often by the smaller but more specialized manufacturers. An alternative approach, imaging in flow, has been demonstrated successfully for larger microbes such as the yeast *Saccharomyces cerevisiae* (7) but does not yet have the resolution for meaningful measurements of viability in bacteria. This may be considered a "best of both worlds" approach, combining the rapid and automated throughput of flow cytometry with the ability to visualize cells that give rise to the data rather than relying on representation of them on a dot plot.

CORRELATION BETWEEN METHODS

As described above, plate counts, although usually considered to be the "gold standard" measure of viability, actually indicate only how many of the cells can replicate under the conditions provided for growth. Even for laboratory-grown cells the movement from growth in liquid broth to viability determination on an agar surface may present problems. For environmental samples, the difference between presampling conditions and the conditions under which viability is determined are likely to be even more disparate. As a consequence, the plate count method often gives an underestimation of the true viability of a cell sample. Jones (17) suggests that for stressed cells, plate counts may indicate viability in less than 50% of the true viable population. Viability staining meanwhile provides information on how many of the cells can exclude, accumulate, or metabolize a stain. Unsurprisingly, therefore, while many stains have been evaluated and many have been deemed appropriate or even superior alternatives to plate counting, any expectation that identical results will be obtained is unlikely to be achieved—with the exception (possibly) of results for 100% dead samples. This fact must be borne in mind when designing, evaluating, and interpreting stain-based methods.

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