

Multiparameter Flow Cytometry of Bacteria

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Summary

The small size of bacteria makes some microbial constituents undetectable or measurable with only limited precision by flow cytometry. Bacteria may also behave differently from eukaryotes in terms of their interaction with dyes, drugs, and other reagents. It is therefore difficult to design multiparameter staining protocols that work, unmodified, across a wide range of bacterial species. This chapter describes reliable flow cytometric methods for assessment of the physiologic states of Gram-negative organisms, on the one hand, and Gram-positive organisms, on the other, based on measurement of membrane potential and membrane permeability. These techniques are useful in the assessment of effects of environmental conditions and antimicrobial agents on microorganisms.

Key Words

Bacteria, cyanine dyes, flow cytometry, membrane permeability, membrane potential.

1. Introduction

Although microscopy made us aware of the existence of the microbial world in the 17th century, it was not until the advent of cytometry in the late 20th century that it became possible to carry out detailed studies of microorganisms at the single-cell level.

In principle, one can use a flow cytometer to measure the same parameters in bacteria or even viruses as are commonly measured in eukaryotic cells. However, the size, mass, nucleic acid, and protein content, and so forth of bacteria are approx 1/1000 the magnitude of the same parameters in mammalian cells, and this affects measurement quality. Low-intensity measurements typically exhibit large variances as a result of photoelectron statistics; some microbial constituents may thus be undetectable or measurable with only limited precision.

Bacteria also tend to behave differently from eukaryotes in terms of their interaction with reagents used in cytometry. Uptake and efflux of dyes, drugs, and other reagents by and from bacteria are affected by the structure of the cell wall, and by the presence of pores and pumps that may or may not be analogous to those found in eukaryotes. Moreover, the outer membrane of Gram-negative bacteria excludes most lipophilic or hydrophobic molecules, including reagents such as cyanine dyes. Although chemicals such as ethylenediaminetetraacetic acid (EDTA) may be used to permeabilize the outer membrane to lipophilic compounds with at least transient retention of some metabolic function, the characteristics of the permeabilized bacteria are distinct from those of organisms in the native state. Gram-positive organisms may take up a somewhat wider range of reagents without additional chemical treatment, but are no more predictable; for example, Walberg et al. (1) found substantial variability in patterns of uptake of different nucleic acid binding dyes by Gram-positive species.

As one might guess from reading the preceding paragraph, it is, difficult, if not impossible, to design multiparameter staining protocols that will work, unmodified, across a wide range of bacterial genera and species. Both authors of this chapter have provided more general discussions of multiparameter flow cytometry of microorganisms elsewhere (2–4); here we concentrate on reliable methods developed in each of our laboratories for assessment of the physiologic states of Gram-negative organisms, on the one hand, and Gram-positive organisms, on the other.

1.1. Defining Bacterial “Viability”: Membrane Permeability vs Metabolic Activity

Both microbiologists and cytometrists would like to be able to characterize microorganisms as viable or nonviable at the single-cell level; this is essential in determining effects of antimicrobial agents or adverse environmental conditions. A number of criteria for “viability” have been suggested; impermeability of the membrane to nucleic acid dyes such as propidium is one, and the presence of metabolic activity, as indicated by the production and retention of fluorescent product from a nonfluorescent enzyme substrate or by maintenance of a membrane potential, is another. However, until recently, relatively few investigators had reported making flow cytometric measurements of more than one of these characteristics in the same cells at the same time.

Propidium (usually available as the iodide [PI]) and ethidium (usually available as the bromide [EB]) are structurally similar nucleic acid dyes; both contain a phenanthridinium ring, and both bind, with fluorescence enhancement, to double-stranded nucleic acids. However, ethidium has only a single positive charge; its *N*-alkyl group is an ethyl group. Ethidium and other dyes with a

single delocalized positive charge are membrane permeant; that is, they cross intact prokaryotic and eukaryotic cytoplasmic membranes, although the dyes may be pumped out by efflux pumps. Propidium bears a double positive charge because its *N*-alkyl group is an isopropyl group with a quaternary ammonium substituent. Like a number of other dyes that also bear quaternary ammonium groups and more than one positive charge (e.g., TO-PRO-1, TO-PRO-3, and Sytox Green, all from Molecular Probes) propidium is generally believed to be membrane impermeant; that is, such dyes are excluded by prokaryotic and eukaryotic cells with intact cytoplasmic membranes. Cells that take up propidium and other multiply charged dyes are usually considered to be nonviable, although transient permeability to these dyes can be induced by certain chemical and physical treatments, for example, electroporation, with subsequent recovery of membrane integrity and viability. Thus, staining (or the lack thereof) with propidium is the basis of a so-called dye exclusion test of viability. Acid dyes, such as trypan blue and eosin, are also membrane impermeant and are used in dye exclusion tests.

A variation on the dye exclusion test employs a nonfluorescent, membrane-permeant substrate for an intracellular enzyme, which crosses intact or damaged cell membranes and which is then enzymatically cleaved to form a fluorescent, impermeant (or slowly permeant) product. The product is retained in cells with intact membranes, and quickly lost from putatively nonviable cells with damaged membranes. One commonly used substrate is diacetylfluorescein, also called fluorescein diacetate (FDA), which yields the slowly permeant fluorescein; nonfluorescent esters of some other fluorescein derivatives are better for dye exclusion tests because their products are less permeant (5). Another substrate is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (6); this is reduced by intracellular dehydrogenases to a fluorescent formazan, and provides an indication of respiratory activity as well as of membrane integrity.

Bacteria normally maintain an electrical potential gradient (membrane potential, $\Delta\Psi$) of >100 mV across the cytoplasmic membrane, with the interior side negative. Charged dyes that are sufficiently lipophilic to pass readily through the lipid bilayer portion of the membrane partition across the membrane in response to the potential gradient. Positively charged lipophilic dyes, such as cyanines, are concentrated inside cells that maintain $\Delta\Psi$, while negatively charged lipophilic dyes, such as oxonols, are excluded. Thus, if two cells of the same volume, one with a transmembrane potential gradient and one without, were equilibrated with a cyanine dye, the cell with the gradient would contain more dye than the one without; if the cells were equilibrated with an oxonol dye, the cell without the gradient would contain more dye. However, cells with different volumes may contain different amounts of dye, irrespective of their $\Delta\Psi$ s, because it is the concentration of dye, rather than the amount of dye, in

the cell that reflects $\Delta\Psi$. The flow cytometer measures the amount, not the concentration.

When the cyanine dye 3,3'-diethyloxacarbocyanine iodide ($\text{DiOC}_2[3]$) is added to cells at much higher concentrations than are normally used for flow cytometric estimation of $\Delta\Psi$, it is possible to detect red (~610 nm) fluorescence in addition to the green (~525 nm) fluorescence normally emitted by this dye (7); the red fluorescence is likely due to the formation of dye aggregates. At high dye concentrations, the green fluorescence is dependent on cell size, but independent of $\Delta\Psi$, whereas the red fluorescence is both size and potential dependent. The ratio of red and green fluorescence, which is largely independent of size, provides a more accurate and precise measurement of bacterial $\Delta\Psi$ than can be obtained from simple fluorescence measurements.

In theory, oxonol dyes should produce little or no staining of cells with normal $\Delta\Psi$ and brighter staining of cells in which the potential gradient no longer exists. However, it is likely that the increased oxonol fluorescence seen in the heat-killed and alcohol-fixed bacteria often used as zero-potential controls reflects changes in size and in lipid and protein chemistry resulting from these treatments, as well as changes in $\Delta\Psi$. Decreases in $\Delta\Psi$ of the Gram-positive *S. aureus* produced by less drastic treatments, for example, nutrient deprivation, were detected by the ratiometric method using $\text{DiOC}_2(3)$ but produced no change in oxonol fluorescence (7). However, oxonol fluorescence does appear to increase with decreasing $\Delta\Psi$ in *Escherichia coli* and other Gram-negative organisms (2).

1.2. Flow Cytometric Methods for Assessment of the Physiologic States of Gram-Negative and Gram-Positive Organisms

The protocol described here for work with *E. coli* and other Gram-negative organisms (2,8,9) combines the oxonol dye *bis*-(1,3-dibutyl-barbituric acid) trimethine oxonol ($\text{DiBAC}_4[3]$), which is used as an indicator of $\Delta\Psi$, with EB, which is retained by cells with intact membranes in which the efflux pump becomes inactive, as happens when energy metabolism is impaired. PI is used to demonstrate membrane permeability; once PI enters cells, it displaces EB from nucleic acids, presumably because PI has a higher binding affinity owing to its double positive charge. All three dyes are excited at 488 nm; $\text{DiBAC}_4(3)$ fluorescence is measured in a green (~525 nm) fluorescence channel, while EB and PI are, respectively, measured at ~575 nm and >630 nm.

The protocol described here for work with *S. aureus* and other Gram-positive organisms uses the ratio of red (~610 nm) and green (~525 nm) fluorescence of $\text{DiOC}_2(3)$, excited at 488 nm, as an indicator of $\Delta\Psi$ (7), and the far red (>695 nm) fluorescence of TO-PRO-3, excited by a red He-Ne (633 nm) or

diode (635–640 nm) laser, to demonstrate membrane permeability. Dividing the TO-PRO-3 fluorescence signal by the green DiOC₂(3) fluorescence signal produces a normalized indicator of permeability that provides better discrimination between cells with impermeable and permeable membranes than can be obtained from TO-PRO-3 fluorescence alone (10).

2. Materials

Note: All aqueous solutions should be made with deionized distilled water (dH₂O) and filtered through a filter with a pore size no larger than 0.22 μ m. Dye solutions should be stored in the dark.

2.1. For DiBAC₄(3)/EB/PI Staining

1. DiBAC₄(3) (Molecular Probes, Eugene, OR) (FW 516.64): Oxonols may require addition of a base to be soluble.
 - a. Stock solution: 10 mg/mL in dimethyl sulfoxide (DMSO), store at –20°C.
 - b. Working solution: 10 or 100 μ g/mL in dH₂O, 0.5% Tween; store at 4°C.
 - c. Final concentration: 10 μ g/mL.
2. EB (Sigma-Aldrich, St. Louis, MO) (FW 394.3):
 - a. Stock solution: 10 mg/mL in dH₂O, store at –20°C.
 - b. Working solution: 500 μ g/mL in dH₂O, store at 4°C.
 - c. Final concentration: 10 μ g/mL.
3. PI (Sigma-Aldrich) (FW 668.4):
 - a. Stock solution: 2 mg/mL in dH₂O; store at 4°C.
 - b. Working solution: 500 μ g/mL in dH₂O; store at 4°C.
 - c. Final concentration: 5 μ g/mL.
4. Dulbecco's buffered saline (DBS+), pH 7.2, with 0.1% peptone, 0.1% sodium succinate, 0.2% glucose, and 4 mM EDTA added.

2.2. For DiOC₂(3)/TO-PRO-3 Staining

1. DiOC₂(3) (Molecular Probes) (FW 460.31):
 - a. Stock and working solution: 3 mM in DMSO; store at 4°C.
 - b. Final concentration: 30 μ M.
2. TO-PRO[®]-3 iodide (Molecular Probes) (FW 671.42):
 - a. Stock and working solution: 1 mM in DMSO (supplied in this form); store at 4°C.
 - b. Final concentration: 100 nM.
3. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich) (FW 204.6):
 - a. Stock and working solution: 2 mM in DMSO, store at 4°C.
 - b. Final concentration: 15 μ M.
4. Nisin (Sigma-Aldrich) (FW 3354): The preparation sold by Sigma-Aldrich contains 2.5% nisin, with the rest NaCl and dissolved milk solids; the filtered aqueous suspension must be diluted to achieve a final concentration of 25 μ g/mL.

5. Valinomycin (Sigma-Aldrich) (FW 1111):
 - a. Stock and working solution: 2 mM in DMSO; store at 4°C.
 - b. Final concentration: 5 μ M.
6. Mueller–Hinton broth (Gibco™ Invitrogen Corporation, Carlsbad, CA) with 50 mg/L of Ca²⁺ (MHbC).

3. Methods

3.1. Functional Assessment of *E. coli* and Gram-Negatives Using DiBAC₄(3)/EB/PI Staining (see Note 1)

3.1.1. Sample Preparation; Disaggregation of Bacteria by Ultrasound and Staining Procedure

1. For samples in liquid media, dilute 10 μ L of sample in 200 μ L of DBS+. Resuspend samples from solid media in DBS+ and then dilute further.
2. Optimize the fraction of single organisms in samples by gentle sonication in a Sanyo MSE Soniprep 150 apparatus (Sanyo, Chatsworth, CA) operating at 23 kHz. Place a 3-mm exponential probe, with its tip 5 mm below the liquid surface of a 2-mL sample, in a disposable polystyrene 7-mL flat-bottom container. Sonicate the sample for 2 min at 2 μ m amplitude.
3. Add dyes: 10 μ g/mL of DiBAC₄(3), 10 μ g/mL of EB, and 5 μ g/mL of PI.
4. Keep the samples at 25°C for 30 min before running on the flow cytometer.

3.1.2. Flow Cytometry

1. Use 488 nm as the excitation wavelength.
2. Use forward and/or side scatter signals for triggering. A software gate excluding low-level scatter signals may be set to remove events due to noise and particulate contaminants in samples.
3. Set up detector filters so that PI fluorescence is measured above 630 nm, EB fluorescence at 575 nm, and DiBAC₄(3) fluorescence at 525 nm.
4. Adjust hardware or software compensation to minimize fluorescence of each dye in channels used primarily for measurement of other dyes.
5. For viability determination, single cells may be sorted directly onto nutrient agar plates.

3.1.3. Results

Figure 1 shows the results of a sorting experiment in which *Salmonella typhimurium* stored for 25 d on nutrient agar at 4°C was resuspended in DBS, sonicated to break up aggregates, and stained with DiBAC₄(3), EB, and PI.

The dye combination delineates cells in different functional stages. Active pumping cells do not stain significantly with any of the dyes. Deenergized cells take up ethidium, but not DiBAC₄(3) or propidium. Depolarized cells take up ethidium and DiBAC₄(3), but not propidium, and permeabilized cells and “ghosts,” that is, cells with damaged membranes, take up both DiBAC₄(3) and

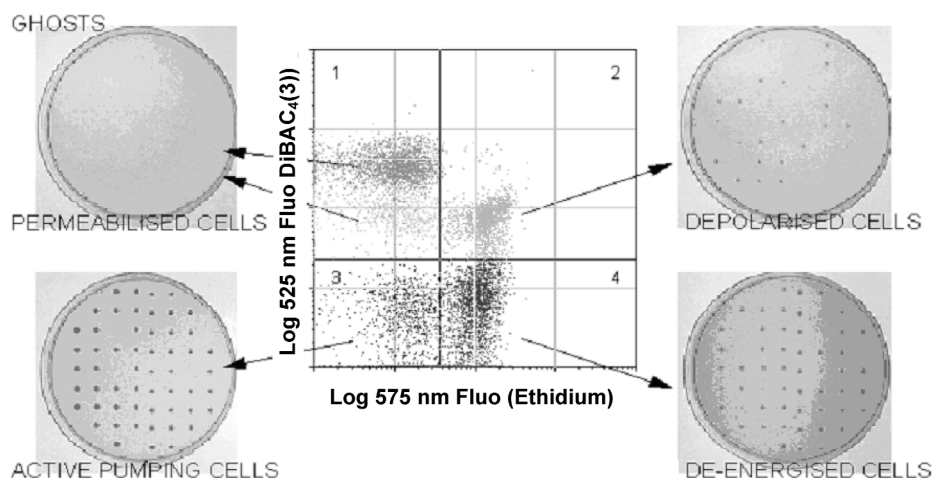


Fig. 1. DiBAC₄(3)/EB/PI staining delineates different functional stages of *Salmonella typhimurium* (stored for 25 d on nutrient agar at 4°C). Cells corresponding to the different functional stages were sorted onto nutrient agar plates to monitor recovery.

propidium. In most cases, all but the permeabilized cells are capable of recovery. In the experiment shown in the figure, approx one third of the electrically depolarized cells grew on agar plates; depolarization therefore indicates a decline in cell functionality, but certainly not cell death. Recovery of actively pumping and deenergized cells typically approaches 100%; deenergized cells lose pump activity but maintain $\Delta\Psi$ at least briefly. Fewer than 1% of events sorted from the regions containing permeabilized cells and ghosts will form colonies on agar.

3.2. Measurement of $\Delta\Psi$ and Permeability Using DiOC₂(3) and TO-PRO-3 (see Note 2)

3.2.1. Sample Preparation

1. Dilute samples in MHBc to a target concentration of 10^6 – 10^7 cells/mL.
2. Add dyes: 30 μ M DiOC₂(3) and 100 nM TO-PRO-3.
3. Keep the samples at room temperature (~25°C) for 5 min before running on the flow cytometer.

3.2.2. Flow Cytometry and Data Analysis

1. Use an instrument with 488 nm (argon-ion or solid-state laser) and red (633 nm from a He–Ne laser or approx 635 nm from a diode laser) excitation beams.
2. Use forward or side scatter as the trigger signal. A software gate may be set to exclude low-level scatter signals produced by noise and debris.

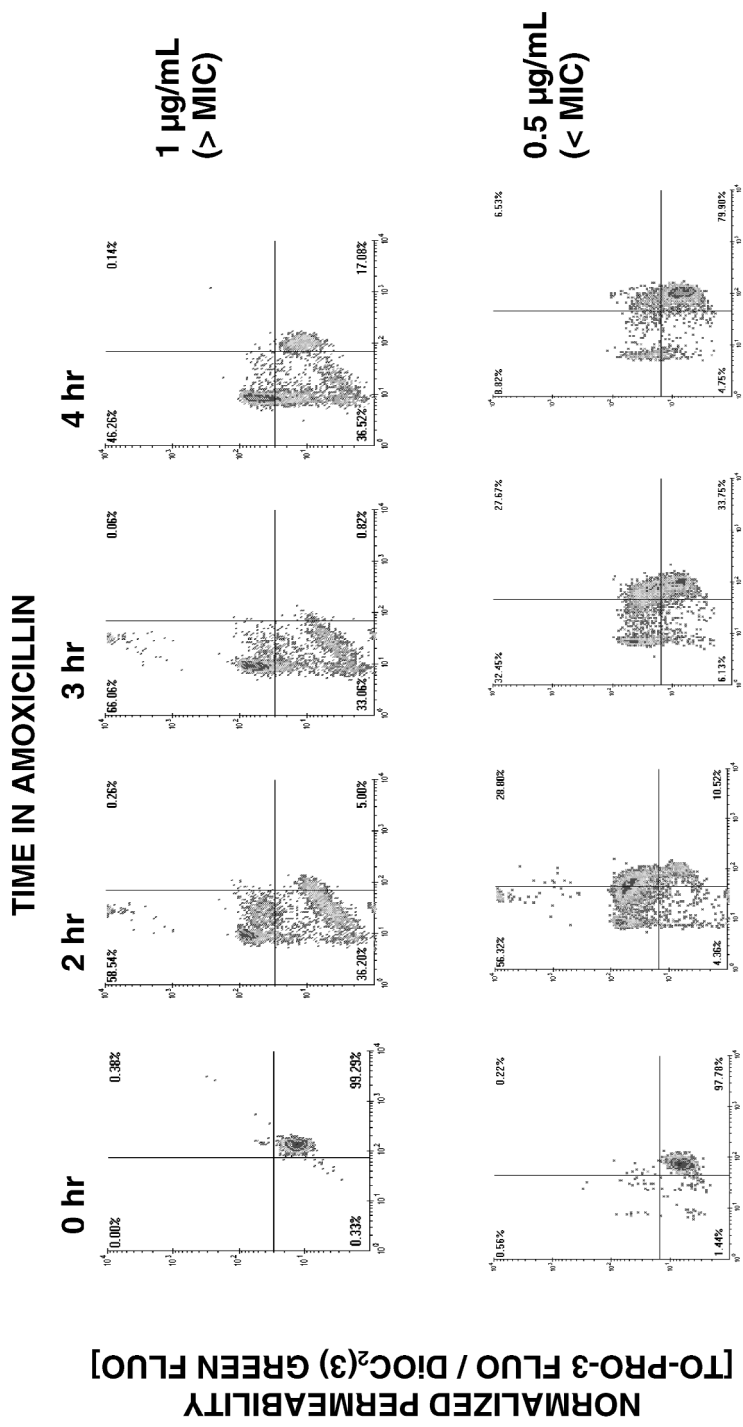
3. DiOC₂(3) is excited at 488 nm; its green fluorescence is detected through a 525- to 530-nm bandpass filter with approx 20 nm bandwidth and its red fluorescence is detected through a 610-nm bandpass filter with approx 20 nm bandwidth.
4. TO-PRO-3TM is excited by the red laser, and its far red fluorescence is detected through one or two 695-nm longpass color glass filters.

In instruments in which data are collected using logarithmic amplifiers, a quantity proportional to the ratio of [DiOC₂(3) red fluorescence]/[DiOC₂(3) green fluorescence] is calculated by adding a constant to the red fluorescence channel value and subtracting the green fluorescence channel value (7). The addition of a constant value is necessary to keep values of the calculated parameter on the same scale as is used for the raw fluorescence measurements. For a 256-channel logarithmic scale, with 64 channels per decade, a constant value of 96 is convenient; the calculated parameter, which serves as a measure of $\Delta\Psi$, then represents the log of ($10^{3/2} \times [\text{red fluorescence/green fluorescence}]$). A normalized permeability value, based on the ratio of [TO-PRO-3 fluorescence]/[DiOC₂(3) green fluorescence], is derived in the same manner. If high-resolution linear data are available, ratios may be calculated directly by division and multiplied by appropriate scaling constants.

Different mechanisms for perturbing $\Delta\Psi$ and permeability are incorporated into control samples. After 5–15 min of incubation, CCCP (15 μM) reduces $\Delta\Psi$ to zero, but does not affect permeability; nisin (25 $\mu\text{g/mL}$) reduces $\Delta\Psi$ to zero, and also renders organisms permeable to TO-PRO-3.

Measurements of $\Delta\Psi$ using DiOC₂(3) may be calibrated by controlled application of valinomycin in the presence of different external potassium ion concentrations (7). The red/green fluorescence ratio is measured for cells in a range of buffers containing 5 μM valinomycin and various concentrations of potassium; the concentration of sodium ion is adjusted to keep the combined molarity of potassium and sodium at 300 mM.

Figure 2 plots the membrane potential of amoxicillin-treated *S. aureus* against normalized permeability. The strain of *S. aureus* used was amoxicillin sensitive; aliquots were exposed to concentrations of amoxicillin above (1 $\mu\text{g/mL}$) and below (0.5 $\mu\text{g/mL}$) the minimal inhibitory concentration (MIC). In cultures treated with either dose, at time zero, most cells show low values of permeability and relatively high values of $\Delta\Psi$, appearing in the lower right quadrant of the display. After 2 h at an amoxicillin concentration above MIC (top strip), many cells have lost $\Delta\Psi$ completely, and most have lost $\Delta\Psi$ to some extent (lower and upper left quadrants); over 58% of the total have become permeable (upper left quadrant). By 4 h, some regrowth has occurred; about 17% of the events measured show normal $\Delta\Psi$ and no permeability. The situation is quite different at a sub-MIC amoxicillin concentration (bottom strip). At 2, 3, and even 4 h, a substantial fraction of events (as high as 28%) are in the



MEMBRANE POTENTIAL [DiOC₂(3) RED FLUO / DiOC₂(3) GREEN FLUO]

Fig. 2. DiOC₂(3)/TO-PRO-3 staining reveals the response of *S. aureus* exposed to different concentrations of amoxicillin for different lengths of time. Membrane potential (the ratio of DiOC₂(3) red to DiOC₂(3) green fluorescence, log scale, x-axis) is plotted against normalized permeability (the ratio of TO-PRO-3 far red to DiOC₂(3) green fluorescence, log scale, y-axis).

upper right quadrant, indicating a $\Delta\Psi$ greater than zero with permeability to TO-PRO-3. By 4 h, most cells (>79%) have regained normal $\Delta\Psi$ and lost permeability. Bacterial counts over this time period rule out the accumulation of a high- $\Delta\Psi$, impermeable population by expansion of the small population of such cells present after 2 h. Although some intermediate- $\Delta\Psi$, permeable events may represent aggregates of high- $\Delta\Psi$, impermeable viable cells and permeable, low- $\Delta\Psi$ dead cells, many of these events appear to be accounted for by TO-PRO-3 uptake into viable cells. This, parenthetically, suggests a novel approach to antimicrobial therapy (3,4,11).

4. Notes

1. DiBAC₄(3)/EB/PI staining: This methodology has been shown to work in some Gram-positive species, for example, *Micrococcus lysodeikticus*, and in yeasts (9). Some adjustment of DiBAC₄(3) concentration may be needed depending on the concentration of organisms and lipophilic components in samples; concentrations as low as 0.1 $\mu\text{g/mL}$ have been used with samples containing small numbers of cells.
2. DiOC₂(3)/TO-PRO-3 staining: This method has been used in instruments with 488 nm and red illuminating beams separated in space; it is not known whether it will work in instruments with collinear 488 nm and red beams. Both DiOC₂(3) and TO-PRO-3 adhere to the tubing used in most flow cytometers, and the high concentration of cyanine dye used in this protocol may necessitate replacement of some tubing if repeated cleaning with dilute chlorine bleach, ethanol, detergents, and so forth still leaves dye in the system. Some bacterial types (e.g., *S. aureus*) may be grown in culture following exposure to 30 μM DiOC₂(3). The method has been used with some Gram-negative species, for example, *E. coli*, with 5 mM EDTA added to staining solutions, and delineates cells with various apparent values of $\Delta\Psi$ as well as discriminating those that do and do not take up TO-PRO-3. However, Gram-negative bacteria appear to be damaged by the calibration buffers used with Gram-positive organisms, and it has not been possible to derive calibration curves for the former.

Acknowledgments

The authors thank Chris Hewitt, Dave Novo, Nancy Perlmutter, and Jared Silverman, who have played vital roles in the development and application of the methods described here.

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<http://www.springer.com/978-1-58829-234-6>

Flow Cytometry Protocols

Hawley, T.S.; Hawley, R.G. (Eds.)

2004, XIV, 434 p., Hardcover

ISBN: 978-1-58829-234-6

A product of Humana Press