

Assessment of the membrane potential, intracellular pH and respiration of bacteria employing fluorescence techniques

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Introduction

All living cells require energy to grow and multiply, for synthesis of enzymes, nucleic acids, polysaccharides, and other cell components, for cell maintenance and repair of damage, for motility, and for numerous other processes. In microorganisms, there are basically two forms of metabolic energy: energy-rich phosphate bonds such as ATP, and electrochemical energy provided by ion gradients. Fermentative microorganisms, for example, can produce ATP by substrate level phosphorylation. In this process, ATP is formed by transfer of a phosphate group from a chemical compound to ADP. Additionally, many microorganisms have developed another strategy to obtain metabolic energy based on conversion of chemical, light or redox energy to energy stored in ion gradients. The generation of such gradients is generally accomplished by primary transport systems, such as the respiratory chain, anaerobic electron transfer systems, or H⁺-ATPases. In most microorganisms, the ions used to generate gradients are protons. This form of metabolic energy is termed the proton-motive force (pmf). The pmf can be the driving force for the uptake of various compounds by secondary transport systems, but can also be applied to synthesize ATP via the F₀F₁-ATPase. The pmf consists of the electrical potential ($\Delta\Psi$) and the chemical proton potential ($-Z \Delta pH$, where $Z = 2.3 RT/F$) according to equation 1.

$$pmf = \Delta\Psi - \frac{2.3RT}{F} \Delta pH \quad (1)$$

where $\Delta\Psi$ is the membrane potential (in Volt), R is the gas constant (in J K⁻¹), F is the Faraday constant (in C mol⁻¹), T is temperature (in degrees Kelvin), and ΔpH is the pH gradient (pH_{in} – pH_{out}).

This chapter discusses and describes methods for the measurement of each of these parameters.

Membrane potential

The $\Delta\Psi$ in cells can be determined by the distribution of lipophilic ionic molecules between the cells and the suspending medium according to the Nernst equation [21, 24, 55]:

$$\Delta\Psi = -\frac{RT}{nF} \ln \frac{[X]_i}{[X]_o} \quad (2)$$

where n is the number of electrons per mole, $[X]_i$ is the concentration of the indicator inside the cell (in mol m^{-3}), and $[X]_o$ is the concentration outside the cell (in mol m^{-3}). The properties of the ideal membrane potential probe are discussed by Lolkema [35] and summarized here: **(i)** the probe should pass the membrane rapidly, **(ii)** it should not bind to the membrane or other cellular constituents, **(iii)** it should be detectable at very low concentrations, and **(iv)** it should be biologically inert. Generally, small lipophilic charged molecules, such as tetraphenylphosphonium (TPP^+) ions are used for membrane potential measurements. Recently, the use of fluorescent distributional probes has become popular. The choice of probes include rhodamine 123, positively charged carbocyanines such as 3,3-dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$), 3,3-diethyloxacarbocyanine iodide ($\text{DiOC}_2(3)$), and 3,3'-dipropylthiadicarbocyanine iodide ($\text{DiSC}_3(5)$), and the negatively charged bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($\text{DiBAC}_4(3)$) [3, 4, 20, 28, 31, 37, 38, 64]. Cells that have a membrane potential (negative inside) accumulate the cationic rhodamine 123 and also cyanines, whereas oxonols are excluded. In eukaryotic cells, rhodamine 123 accumulates preferentially in the mitochondria, due to the high membrane potential present in this organelle [51], but also the less hydrophobic cyanines are expected to accumulate in mitochondria [46]. Furthermore, a high intramitochondrial carboxycyanine concentration is likely to be toxic to the cells, and the fluorescence is likely to be quenched [55]. Other problems associated with lipophilic membrane potential probes are their potentially strong binding to cell constituents such as membranes, and formation of non-fluorescent aggregates at higher concentrations [21, 24]. To avoid these problems, Krasznai et al. [33] developed a calibration procedure based on the assumption that a direct relation exists between the total cell-related fluorescence and the free intracellular dye concentration, which is in (Nernstian) equilibrium with the extracellular fluorescence. Using this procedure, the membrane potential in rat thymocytes and human lymphocytes could be measured with the oxonol $\text{DiBAC}_4(3)$. The obtained results were in good agreement with those obtained by the patch clamp method.

Flow cytometry is a popular technique for the analysis of individual cells, but as pointed out clearly by Shapiro [56], the flow cytometer measures the amount of fluorescent dye rather than the concentration. The amount of probe, however, is not a good parameter for the measurement of the membrane potential because the fluorescence may fluctuate depending on *e.g.* the size of the cells. This led to the development of a ratiometric method to measure the membrane potential of bacteria using the oxocarbocyanine dye $\text{DiOC}_2(3)$ [43, 44]. This dye supposedly

forms aggregates emitting red fluorescence, dependent on the membrane potential and the size of the cells. The normal green fluorescence of the dye is dependent on the size, but not on the membrane potential. The ratio between the green and the red fluorescence should eliminate the dependence of the fluorescence on the size of the cells [43].

In Gram-negative bacteria, a potential problem for measurement of the membrane potential is that proper distribution of the membrane potential probes is sometimes hindered by the low permeability of the outer membrane. The addition of EDTA or EGTA can help to permeabilize the outer membrane, but such treatments may obviously influence cell viability.

Intracellular pH

The chemical proton potential can be determined by measuring the pH gradient, *i.e.* the pH difference between the intracellular pH (pH_{in}) and extracellular pH (pH_{out}). Several techniques have been developed to determine the pH_{in} of microbial cells including distribution of weak acids [13, 16], the ^{31}P nuclear magnetic resonance technique [22, 47, 52], and the application of pH-dependent fluorescent probes [11, 39]. Fluorescent probes that have been exploited to measure the pH_{in} in microbial cells include fluorescein, 5 (and 6-)-carboxyfluorescein [cF], 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE), 5 (and 6-)-carboxy-10-dimethylamino-3-hydroxy-spiro[7*H*-benzo[*c*]xanthene-7,1'(3'*H*)-isobenzofuran]-3'-one[cSNARF-1], and 8-hydroxy-1,3,6-pyrene-trisulfonic acid [Pyranine]. Fluorescein and cF are easily incorporated into cells by incubation with their diacetate esters [10]. Furthermore, these probes can be excited at the popular 488 nm excitation line, and are highly fluorescent (*i.e.* they have a high extinction coefficient and quantum yield). However, fluorescein and, to a lesser extent, cF are poorly retained in the cytoplasm. A strategy to circumvent this problem is to employ more polar fluorescent compounds, such as BCECF, which is generally retained much better in the cells. Furthermore, to minimize resulting background problems, chemical elimination of extracellular probe [42] and mathematical correction of the fluorescent signal for efflux [39] have been exploited. On the other hand, in energized yeast cells, there are strong indications that fluorescent probes such as cF and BCECF are actively extruded to the extracellular environment and/or accumulated in intracellular compartments, in particular the vacuoles [9, 14, 40]. To avoid problems with translocation of fluorescent dye, the fluorescent probe cFSE can be used for determination of the pH_{in} of bacteria [11]. Bacteria can easily take up cFSE by incubation with its diacetate ester cFDASE. Once it is incorporated, it is thought that its succinimidyl group forms conjugates with aliphatic amines [11, 27]. The fluorescence can be detected after hydrolysis of the diacetate form by intracellular esterase activity. This approach avoids problems due to leakage or active efflux of the probe and allows accurate calibration of the fluorescence signal.

A recent development is the application of green fluorescent protein (GFP) as a pH probe. It has been found that the fluorescence spectrum of some mutant GFP's respond to changes in pH [34]. Because GFP is small, very stable, does not perturb the metabolism of the cells, and can easily be targeted to specific intracellular compartments, it is quickly becoming a popular tool for pH_{in} measurements. Some examples include the measurement of the pH_{in} of peroxisomes in fungi [69], and *Lactococcus lactis* [45]

Respiration

In microbial cells, energy can be generated via electron transfer systems. Such systems are composed of a series of electron carriers, which are usually membrane bound, such as NADH dehydrogenases, flavoproteins, cytochromes and quinones that can accept electrons from an electron donor and can transfer them to an electron acceptor. The energy released during this process is used to extrude protons to the external environment. The net result is the generation of a proton motive force, which can *e.g.* be used to generate ATP. If O_2 is used as the external electron acceptor, the process is called aerobic respiration. Traditionally, the respiration of bacteria is therefore determined by measurement of the oxygen consumption. Alternatively, tetrazolium dyes may be applied to measure respiration. These dyes are thought to act as an alternative electron acceptor [1], and, consequently, respiring cells can reduce tetrazolium dyes to their respective formazan products. In 1984, Stellmach [61] described the synthesis of the non-fluorescent redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and Rodriquez et al. [50] demonstrated in 1992 that respiring bacterial cells can reduce CTC to the red fluorescent, water-insoluble formazan product 3-cyan-1,5-di-tolyl-formazan (CTF). CTF has an absorption peak at 450 nm, and the fluorescence of the crystals is in the red region (approx. 570–650 nm).

The procedure commonly used for tetrazolium reduction assays involves incubation of the cells (20 minutes to several hours) in the presence of the tetrazolium dye followed by fixation using formaldehyde, paraformaldehyde or formalin, and mounting with paraffin oil or immersion oil on a microscope slide for examination. This is typically combined with counterstaining with DAPI for determination of the total cell count. Formazan deposition may be enhanced by the addition of substrates such as succinate, glucose, and intermediate electron carriers such as phenazine methasulfate [23, 60, 66, 70]. On the other hand, tetrazolium reduction may be reduced by extracellular inorganic phosphate, at concentrations above 10 mM [60]. Depending on the lipophilicity, some formazan products can diffuse out of the cells and form extracellular deposits. This could, in some cases, be suppressed by addition of cobalt ions, which supposedly form a complex with the formazan [66].

The use of CTC also has its limitations. In several cases, addition of CTC proved to be lethal to the microbial cells at concentrations as low as approx. 5 mM [25, 53, 67]. Furthermore, CTC reduction by yeast cells is not very reproducible, most likely

because CTC does not easily enter respiring yeast cells, due to its negative charge [8]. Another important issue is that the exact mechanism of formazan formation is still not completely clear. Smith and McFeters [61] concluded from their study with *Escherichia coli* that CTC was reduced prior to ubiquinone in the electron transfer chain, but could not satisfactorily explain the inhibition of CTC reduction by potassium cyanide, an inhibitor of the terminal oxidase. In this respect, Seidler [58] proposed that under aerobic conditions tetrazolium salts may be reduced by superoxide radicals, *i.e.* if the actual redox potential of the O_2/O_2^- radical couple ($E'_{1/2} \sim -330$ mV) is more negative than that of the tetrazolium/formazan couple. The reduction potential ($E'_{1/2}$) of CTC is approx. -200 mV, indicating that the formazan formation may well be due to superoxide radicals instead of the enzymes from the electron transfer chain.

Procedures

In the following section, detailed protocols are described for measurement of the membrane potential of *Listeria monocytogenes* (based on the method described by Bennik et al. [3] with some modifications), the intracellular pH of *Lactococcus lactis* (based on the method described by Breeuwer et al. [9] with some modifications), and the analysis of the respiration of *Listeria monocytogenes* using CTC and flow cytometry.

Membrane potential ($\Delta\Psi$) measurements

Materials:

- 50 mM potassium phosphate buffer (KP_i) pH 7.
- Cell culture: *L. monocytogenes*, grown in BHI in a shaking water bath at 30 °C to optical density at 620 nm of 0.6.
- Fluorescent probe: DiSC₃(5) (Molecular Probes Europe B.V., Leiden, The Netherlands), stock solution 3 mM in DMSO, stored in freezer.
- Water bath, 30 °C.
- Eppendorf tubes 1.5 ml.
- Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
- Spectrofluorimeter (Perkin-Elmer LS 50B, Nieuwerkerk a/d IJssel, The Netherlands).
- Glucose (1 M).
- Valinomycin (stock solution 3 mM in ethanol).

- Nigericin (stock solution 3 mM in ethanol).
- 3 ml fluorescence glass cuvette (4 sides transparent).
- Small magnetic stirrer bar for in cuvette.
- Waste container.
- Ethanol (70 %) for rinsing.

Measurement:

1. Grow *L. monocytogenes* (BHI, 30 °C, shaking) until O.D._{620 nm} :0.6 (approximately: $5 \cdot 10^8$ cells per ml).
2. Harvest 30 ml cells by centrifugation (10 minutes at $2800 \times g$) and wash twice in 50 mM KP_i pH 7 buffer.
3. Resuspend in 30 ml 50 mM KP_i pH 7 buffer and store on ice until use.
4. Turn on the spectrofluorimeter and computer.
Excitation wavelength 643 nm, slit width 10 nm.
Emission wavelength 666 nm, slit width 10 nm.
Measurement time 900 seconds at intervals of 0.5 seconds.
The water bath connected to cuvette holder is set at 30 °C.
5. Clean the cuvette with water (3 times) and 70 % ethanol (3 times), and blow dry with air.
6. Add 3 ml cell suspension to the cuvette (the concentration of cells in the cuvette should be approximately $5 \cdot 10^8$ cells per ml buffer), use small magnetic stirrer to mix cells¹.
7. Add 5 µl DiSC3(5) to a final concentration of 5 µM and wait until the signal is stable (after ± 400 seconds).
8. Add test compound, e.g. 10 mM glucose, and wait again until the signal is stable.
9. To prevent generation of a transmembrane pH gradient, nigericin (10 µl; final concentration 1 µM) may be added.
10. At the end of the assay, valinomycin (10 µl; final concentration 1 µM) is added. The $\Delta\Psi$ is dissipated and the signal will increase. The signal value obtained after dissipation is generally lower than the start value due to binding of the probe to the cells².

An example of a typical experiment is shown in Figure 1. First, the cell suspension is added to the cuvette. Subsequently, the DiSC₃(5) probe is added and the signal rapidly decreases due to (Nernstian) re-distribution of the probe (inside the cells the fluorescence of the probe is quenched which results in decrease of the total signal). Addition of

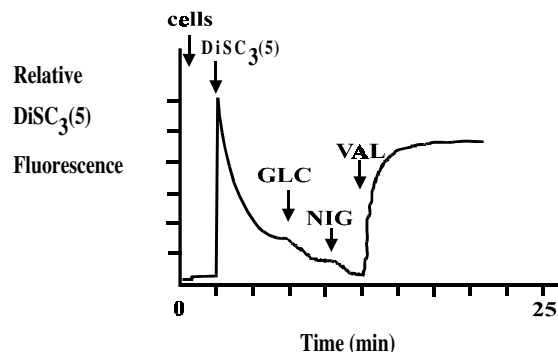


Figure 1. The membrane potential of *Listeria monocytogenes* measured by DiSC₃(5).

glucose induces a further decrease of the signal due to an increase in the membrane potential. Addition of nigericin results in dissipation of the pH gradient, which may be compensated for by increasing the membrane potential. Addition of valinomycin results in dissipation of the pmf and the signal increases again.

Notes

1. Depending on the bacterial species the optimum cell concentration may vary. With *L. monocytogenes*, a relatively high concentration of cells is required to obtain sufficient fluorescence signal.
2. The fluorescence signal after addition of valinomycin corresponds to a $\Delta\Psi$ of zero, but the described method does not include calibration of the membrane potential. It only gives qualitative changes in membrane potential.

Internal pH (pH_{in}) measurements

Materials:

- 50 mM potassium phosphate buffer (KP_i) pH 7.
- Cell culture:
Lactococcus lactis ML3, grown at 30 °C in M17 + 5 g/l glucose to optical density at 620 nm of 0.6.
- cFDASE stock solution in acetone (3.8 mg/ml). This solution should be kept at –20 °C in acetone resistant vials, and is stable for at least half a year. To avoid evaporation of the acetone, the solution should be returned to the freezer immediately after use.
- Acetone (reagent grade).
- Water bath, 30 °C
- Eppendorf tubes 1.5 ml.

- Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
- Spectrofluorimeter with ratio option, *i.e.* the emission is measured at two different excitation wavelengths at very short time intervals and the ratio of the intensities is calculated and displayed.
- Glucose (1 M).
- Valinomycin (stock solution 3 mM in ethanol).
- Nigericin (stock solution 3 mM in ethanol).
- 3 ml fluorescence glass cuvette (4 sides transparent).
- Citric acid monohydrate (50 mM).
- Potassium chloride (4 M).
- Disodium hydrogen phosphate dihydrate (50 mM).
- Glycine (50 mM).
- NaOH (3 M).
- HCl (4 M).

Fluorescent labeling:

1. Harvest 1 ml cell suspension by centrifugation in an Eppendorf tube ($11300 \times g$, 2 minutes), wash once in 50 mM potassium phosphate buffer pH 7.0, and resuspend the cells again in 1 ml KP_i pH 7 buffer.
2. Add 10 μ l cFDASE previously diluted 10 \times in acetone (20 μ l cFDASE + 180 μ l acetone) to 1 ml sample and incubate 15 minutes at 30 °C³.
3. Wash 2 \times in KP_i pH 7, resuspend the cells again in 1 ml KP_i pH 7 buffer, and add 10 μ l glucose (1 M), and incubate 15 minutes at 30 °C⁴.
4. Wash 1 \times in KP_i pH 7 to remove extruded cFSE, resuspend the cells in 1 ml KP_i pH 7 buffer, and add again 10 μ l glucose (1 M), and incubate 15 minutes at 30 °C.
5. Wash 1 \times in KP_i pH 7, resuspend the cells again in 1 ml KP_i pH 7 buffer, and place Eppendorf tube on ice⁵.

Measuring:

6. Measure the pH_{in} using the spectrofluorimeter. A glass cuvette filled with 3 ml buffer of desired pH is placed in the stirred and thermostated cuvette holder of the spectrofluorimeter. After 1 minute cells (diluted to a concentration of approx. 10^7 cells per ml) are added, and after time x, y, z, etc. glucose or other reagents are added⁶. The fluorescence intensities are measured at excitation wavelengths of 500 and 440 nm by rapidly altering the

monochromator (ratio option) between both wavelengths (<2 seconds). The emission wavelength is 530 nm, and the excitation and emission slit widths are 5 and 10 nm, respectively.

The incubation temperature is 30 °C. It is a good habit to equilibrate the pH_{in} and pH_{out} at the end of each assay by addition of valinomycin (1 μM final concentration) and nigericin (1 μM final concentration) in the cuvette. Additionally, the extracellular fluorescence signal (background) is determined by filtration (do this very gently!) of the cell suspension through a 0.22 μm pore-size membrane filter into a clean cuvette and measurement of the filtrate (take out the cuvette during the measurement)⁷.

Calibration:

7. Prepare a series of citrate phosphate glycine buffers ranging from pH 3 to 9 at 0.5 pH unit intervals.
Preparation: take 75 ml $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (50 mM) and add 2.5 ml KCl (4 M) and 75 ml glycine (50 mM).
 - a. buffers between 5 and 7: add citric acid monohydrate (50 mM) until desired pH and add water to total volume of 200 ml.
 - b. buffers higher than pH 7: add citric acid monohydrate (50 mM) until pH 7, and add NaOH until desired pH. Add water to total volume of 200 ml.
 - c. buffers lower than pH 5: add citric acid monohydrate (50 mM) until pH 5, and add HCl until desired pH. Add water to total volume of 200 ml.
8. Add 3 ml buffer pH (3 to 9) to the cuvette and after 1 minute add cells. After 5 minutes, the pH_{in} and pH_{out} are equilibrated by addition of valinomycin (1 μM final concentration) and nigericin (1 μM final concentration) in the cuvette. After 10 minutes, the extracellular fluorescence signal (background) is determined by gentle filtration of the cell suspension through a 0.22 μm pore-size membrane filter by use of a 5 ml syringe into a clean cuvette and measurement of the filtrate (take out the cuvette during the measurement).

A typical example of a pH_{in} measurement of *Lactococcus lactis* is shown in Figure 2. First, buffer (50 mM KP_i buffer pH 6.5) is added to the cuvette. The signals at 490 nm and 440 nm excitation of the buffer are very low (because there is no fluorescent probe present), and the 490-to-440 ratio signal should be close to 1. When the fluorescent cells

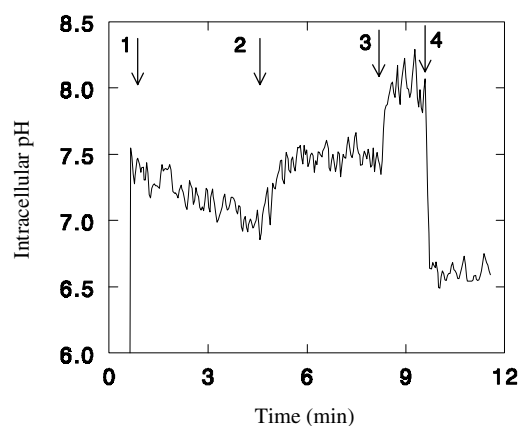


Figure 2. The 490-to-440 nm ratio of cFSE labeled *L. lactis* cells measured at excitations of 490 nm and 440 nm at an emission wavelength of 525 nm. Measurements in the cuvette were performed at 30 °C in 3 ml of 50 mM potassium phosphate buffer, pH 6.5. The following additions were made at the times indicated by the arrows: 1, cell suspension (100 μ l); 2, lactose (10 mM); 3, valinomycin (1 μ M); and 4, nigericin (1 μ M). At the end of the assay (not shown) the cuvette was removed for a short time from the spectrofluorometer and the filtrate was measured after filtration of the cell suspension through a disposable disc filter (0.22 μ m pore size).

are added to the cuvette, the 490-to-440 ratio signal increased to about 3. Glucose addition resulted in a clear increase of the ratio. Addition of valinomycin further increased the ratio, as the cells try to compensate for the dissipation of the membrane potential by exporting protons. Upon addition of nigericin the pH gradient is dissipated.

Notes

3. In the cells the cFDASE is converted into cFSE and 2 acetate molecules. The cFSE gives a bright green fluorescence when excited at 460 to 490 nm. It is common practice to check the labeling by use of a fluorescence microscope with a standard FITC filter set. A part of the cFSE will bind to aliphatic amines, but most of the cFSE remains unbound.
4. In this step the cFSE, which is not bound, is actively extruded to the extracellular environment. This is necessary to minimize leakage or extrusion of probe during the measurement.
5. It is a good habit to check the cFSE labeling of the cells using a fluorescence microscope equipped with a standard FITC filter set. cFSE-labeled cells appear as green to yellow labeled cells.
6. The number of cells to be added to the cuvette depends on the signal-to-noise ratio of the 490/530 nm intensity signals and 440/530 nm intensity signals. In our spectrofluorimeter the 440/530 nm signal of KP_i buffer pH 7 without any addition is about 0.9 (on a scale from 0 to 1000). The 440/530 signal of the cells should be higher than

15 to obtain a stable ratio signal. This signal can be increased obviously by adding more cells, but it should be realized that at a certain moment the optical density will become too high and other effects will occur such as concentration quenching, which may disturb the measurement.

7. At the end of the experiment, the background fluorescence is determined by filtration through the 0.22 μm disposable disc filters. The fluorescence in the filtrate is generally 10 to 20 % of the total signal before filtration, and may result from (unbound) cFSE, which may leak from the cells during the measurement. It was not possible to determine the impact of the filtration itself on the leakage of cFSE from the cells, but using centrifugation instead of filtration does not generally yield better results.

Assessment of respiration

Materials:

- PBS : 0.2 g KCl, 0.2 g KH_2PO_4 , 1.5 g Na_2HPO_4 et 8 g NaCl per liter, adjusted to pH 7.2 with concentrated HCl.
- 50 mM potassium phosphate buffer (KP_i) pH 7, filter sterilized through 0.2 μm membrane filter.
- Cell culture: *L. monocytogenes*, grown in Brain Heart Infusion broth (BHI) at 30 °C to optical density at 620 nm of 0.6.
- Eppendorf tubes 1.5 ml.
- Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
- Water bath 30 °C.
- Fresh CTC solution: weigh 0.015 g and solubilize in 10 ml filter sterilized milliQ water (5 mM), add 0.47 ml glucose (20 % solution in water) to give a final concentration of 50 mM. This solution is not very stable, and should be used the same day. CTC was obtained from Polysciences, Warrington, USA.
- Flow Cytometer (FACSCalibur, Becton Dickinson, Erembodegem-Aalst, Belgium).
- Sheath fluid (FACSFlow).
- Cleaning solutions (FACSSave and FACSRinse).

Procedure:

Labeling of cells with CTC

1. Harvest 1 ml cell suspension by centrifugation in an Eppendorf tube ($11300 \times g$, 4 minutes), wash in PBS, and resuspend again in 1,5 ml PBS. The suspension can be stored on ice.

2. Take 0.15 ml cell suspension and centrifuge ($11300 \times g$, 4 minutes), and resuspend the cells in 1.5 ml CTC solution + glucose (50 mM)⁸.
3. Incubate the cell suspension for 45 minutes at 30 °C in the dark.
4. Check the labeling of the cells using fluorescence microscopy with a standard FITC filter (excitation 460–490). The CTC should be visible as red crystals inside the cells.
5. Store cells on ice before analysis.

Flow cytometric analysis

6. Turn on the flow cytometer using the standard start up procedure.
7. In the acquisition menu, set the number of events to be acquired to 5000 or max. 2 minutes.
8. Set the amplifiers [forward scatter (FSC), side scatter (SSC), FL1, FL2, and FL3] to logarithmic mode. The settings for the gain should be E01, 380, 600, 550, and 690 for FSC, SSC, FL1, FL2, and FL3, respectively.
9. In the threshold window, set SSC as the primary threshold with a cut off value (channel number) of 108. Only signals with an intensity greater than or equal to this threshold channel number will be processed by the flow cytometer.
10. Before measurement dilute the cells to approx. 10^5 cells per ml in milliQ water.
11. CTC positive cells are detected by the FL3 (red) fluorescence parameter.

Note

8. *Cell aggregation as result of the CTC labeling may be a problem with some bacteria [65]. This can be avoided by using low cell concentrations ($<10^7$ cells/ml). In some cases, sonication at low intensity for short time periods (which should not harm the cells) may help.*

Application of the Methods

The membrane potential, the pH gradient, and the electron transfer chain activity of microorganisms can be useful as indicators for cell viability in addition to traditional methods such as plate counting. Membrane potential probes such as DiBAC₄(3), for instance, have been applied to evaluate the antimicrobial effects of bacteriocins or

antibiotics [4, 28, 64]. The cFSE method has been widely applied for the measurement of the pH_{in} in Gram-positive bacteria [4, 15, 26, 36, 41, 48, 54, 59, 68] and yeasts [7, 12]. In Gram-negative bacteria such as *E. coli*, the use of the cFSE method is complicated by the difficulty of the prefluorochrome cFDASE (molecular weight 557) to pass the outer membrane in the Gram negative cell wall. Nevertheless, Riondet et al [49] showed that a short incubation with EDTA is perhaps an approach to overcome this problem.

The Tetrazolium dyes may be used to measure respiration. CTC is the most popular redox dye and has e.g. been used to determine the number of respiring *Micrococcus luteus*, *Listeria monocytogenes* and *Pseudomonas fluorescens* in pure cultures [6, 29, 32], respiring bacteria in water [2, 30, 50, 57, 60], soil [71], microbial communities in bioreactors [17], biofilms [19, 63, 72], and as indicator of viability in anaerobic bacteria [5].

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References

1. Altman FP (1976) Tetrazolium salts and formazans, Progress Histochem Cytochem 9: 1–56.
2. Bartscht K, Cypionka H, Overmann J (1999) Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. FEMS Microbiol Ecol 28: 249–259.
3. Ben Amor K, Breeuwer P, Verbaarschot P, Rombouts FM, Akkermans ADL, De Vos WM, Abee T (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. Appl Environ Microbiol 68: 5209–5216.
4. Bennik MHJ, Verheul A, Abee T, Naaktgeboren-Stoffels G, Gorris LGM, Smid EJ (1997) Interactions of nisin and pediocin PA-1 with closely related lactic acid bacteria that manifest over 100-fold differences in bacteriocin sensitivity. Appl Environ Microbiol 63: 3628–3636.
5. Bhupathiraju VK, Hernandez M, Landfear D, Alvarez-Cohen L (1999) Application of tetrazolium dye as an indicator of viability in anaerobic bacteria. J Microbiol Meth 37: 231–243.
6. Bovill RA, Shallcross JA, Mackey BM (1994) Comparison of the fluorescent redox dye 5-cyano-2,3-ditolyltetrazolium chloride with *p*-iodonitrotetrazolium violet to detect metabolic activity in heat-stressed *Listeria monocytogenes* cells. J Appl Bacteriol 77: 353–358.
7. Bracey D, Holyoak CD, Nebe-von Caron G, Coote PJ (1998) Determination of the intracellular pH (pH_i) of growing cells of *Saccharomyces cerevisiae*: the effect of reduced-expression of the membrane H^+ -ATPase. J Microbiol Meth 31: 113–125.
8. Breeuwer P. Unpublished results.

9. Breeuwer P, Drocourt JL, Rombouts FM, Abee T (1994) Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. *Appl Environ Microbiol* 60: 1467–1472.
10. Breeuwer P, Drocourt JL, Bunschoten N, Zwietering MH, Rombouts FM, Abee T (1995) Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. *Appl Environ Microbiol* 61: 1614–1619.
11. Breeuwer P, Drocourt JL, Rombouts FM, Abee T (1996) A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated probe 5-(and-6)-carboxyfluorescein succinimidyl ester. *Appl Environ Microbiol* 62: 178–183.
12. Breeuwer P, Abee T (2000) Assessment of the intracellular pH of yeast cells employing fluorescence ratio imaging analysis. *J Microbiol Meth* 39: 253–264.
13. Bruno MEC, Kaiser A, Montville TJ (1992) Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. *Appl Environ Microbiol* 58: 2255–2259.
14. Bunthof CJ, Van Den Braak S, Breeuwer P, Rombouts FM, Abee T (1999) Rapid fluorescent assessment of the viability of stressed *Lactococcus lactis*. *Appl Environ Microbiol* 65: 3681–3689.
15. Chitarra LG, Breeuwer P, Van Den Bulk RW, Abee T (2000) Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. *J Appl Microbiol* 88: 809–816.
16. Cook GM, Russell JM (1994) The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis*. *Curr Microbiol* 28: 165–168.
17. Cook KL, Garland JL (1997) The relationship between electron transport activity as measured by CTC reduction and CO₂ production in mixed microbial communities. *Microb Ecol* 34: 237–247.
18. Créach V, Baudoux AC, Bertru G, Rouzic BL (2003) Direct estimate of active bacteria: CTC use and limitations. *J Microbiol Methods* 52: 19–28.
19. De Beer D, Srinivasan R, Stewart PS (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 60: 4339–4344.
20. Deere D, Porter J, Edwards C, Pickup R (1995) Evaluation of the suitability of bis-(1,3-dibutylbarbituric acid)trimethine oxonol, (DiBAC₄(3)[−]), for the flow cytometric assessment of bacterial viability. *FEMS Microbiol Lett* 130: 165–170.
21. Ehrenberg B, Montana V, Wei M.-D, Wuskell JP, Loew LM (1988) Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys J* 53: 785–794.
22. Greenfield NJ, Hussain M, Lenard J (1987). Effects of growth state and amines on cytoplasmic and vacuolar pH, phosphate, and polyphosphate levels in *Saccharomyces cerevisiae*: a ³¹P-nuclear magnetic resonance study. *Biochim Biophys Acta* 926: 205–214.
23. Gribbon LT, Barer MR (1995) Oxidative metabolism in non-culturable *Helicobacter pylori* and *Vibrio vulnificus* cells studied by substrate-enhanced tetrazolium reduction and digital image processing. *Appl Environ Microbiol* 61: 3379–3384.
24. Gross D, Loew LM (1989) Fluorescent indicators of membrane potential: microspectrofluorimetry and imaging. In Lansing Taylor D, Wang Y (eds) *Methods in Cell Biology*, Vol. 30. pp. 193–218. Academic Press, Inc., London, UK.
25. Hatzinger PB, Palmer P, Smith RL, Penarrieta CT, Yoshinari T (2003) Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *J Microbiol Meth* 52: 47–58.
26. Hornbaek T, Dynesen J, Jakobsen M (2002) Use of fluorescence ratio imaging microscopy and flow cytometry for estimation of cell vitality for *Bacillus licheniformis*. *FEMS Microbiol Lett* 215: 261–265.
27. Haugland RP (1992) Succinimidyl esters and carboxylic acids. In Larison KD (ed) *Handbook of fluorescent probes and research chemicals*. pp. 24–33. Molecular Probes Inc., Eugene, Oreg, USA.

28. Jepras RI, Paul FE, Pearson SC, Wilkinson MJ (1997) Rapid assessment of antibiotic effects on *Escherichia coli* by bis-(1,3-dibutylbarbituric acid) trimethine oxonol and flow cytometry. *Antimicrob Agents Chemotherapy* 41: 2001–2005.
29. Jørgensen F, Nybroe O, Knfchel S (1994) Effect of starvation and osmotic stress on viability and heat resistance of *Pseudomonas fluorescens* AH9. *J Appl Bacteriol* 77: 340–347.
30. Joux F, Lebaron P, Troussellier M (1997) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. *FEMS Microbiol Ecol* 22: 65–76.
31. Kaprelyants AS, and Kell DB (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometr. *J Appl Bacteriol* 72: 410–422.
32. Kaprelyants AS, Kell DB (1993) The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualization of respiratory activity in individual cells of *Micrococcus luteus*. *J Micrbiol Meth* 17: 115–122.
33. Krasznai Z, Márián T, Balkay L, Emri M, Trón L (1995) Flow cytometric determination of absolute membrane potential of cells. *J Photochem Photobiol B: Biol* 28: 93–99.
34. Kneen M, Farinas J, Li Y, Verkman AS (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys J* 74: 1591–1599.
35. Lolkema JS, Hellingwerf KJ, Konings WN (1982) The effect of probe binding on the quantitative determination of the proton-motive force in bacteria. *Biochim Biophys Acta* 681: 85–94.
36. Luppens SBI (2002) PhD thesis. Suspensions or Biofilms and other factors that effect disinfectant testing on pathogens, Wageningen University, The Netherlands.
37. Mason DJ, López-Amoróz R, Allman R, Stark JM, Lloyd D (1995) The ability of membrane potential dyes and calcofluor white to distinguish between viable and non-viable bacteria. *J Appl Bacteriol* 78: 309–315.
38. McFeters GA, Yu FP, Pyle BH, Stewart PS (1995) Physiological methods to study biofilm disinfection. *J Industr Microbiol* 15: 333–338.
39. Molenaar D, Abee T, Konings WN (1991) Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. *Biochim Biophys Acta* 1115: 75–83.
40. Molenaar D, Bolhuis H, Abee T, Poolman B, Konings WN (1992) The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in *Lactococcus lactis*. *J Bacteriol* 174: 3118–3124.
41. Molina-Gutierrez A, Stippl V, Delgado A, Ganzle MG, Vogel RF (2002) In situ determination of the intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum* during pressure treatment. *Appl Environ Microbiol* 68: 4399–4406.
42. Noël J, Tejedor A, Vinay P, Laprade R (1989) Fluorescence measurement of intracellular pH on proximal tubule suspensions. *Renal Physiol Biochem* 12: 371–387.
43. Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM (1999) Accurate flow cytometric membrane potential measurement in bacteria using diethyloxycarbocyanine and a ratiometric technique. *Cytometry* 35: 55–63.
44. Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrob Agents Chemother* 44: 827–834.
45. Olsen KN, Budde BB, Siegmundfeldt H, Rechinger KB, Jakobsen M, Ingmer H (2002) Noninvasive measurement of bacterial intracellular pH on a single-cell level with green fluorescent protein and fluorescence ratio imaging microscopy. *Appl Environ Microbiol* 68: 4145–4147.
46. Peña A, Urbine S, Pardo JP, Borbolla M (1984) The use of cyanine dye in measuring membrane potential in yeast. *Arch Biochem Biophys* 231: 217–225.
47. Rabaste F, Sancelme M, Delort A, Blais J, Bolard J (1995). Intracellular pH of *Candida albicans* blastospores as measured by laser microspectrofluorimetry and ³¹P-NMR. *Biochim Biophys Acta* 1268: 41–49.
48. Rechinger KB, Siegmundfeldt H (2002) Rapid assessment of cell viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* by measurement of intracellular pH in individual cells using fluorescence ratio imaging microscopy. *Int J Food Microbiol* 75: 53–60.

49. Riondet C, Cachon R, Wache Y, Alcaraz G, Divies C (1997) Measurement of the intracellular pH in *Escherichia coli* with the internally conjugated fluorescent probe 5- (and 6-) carboxyfluorescein succinimidyl ester. *Biotechnol Techniques* 11: 735–738.
50. Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl Environ Microbiol* 58: 1801–1808.
51. Ronot X, Benel L, Adolphe M, Mounolou JC (1986) Mitochondrial analysis in living cells: the use of rhodamine 123 and flow cytometry. *Biol Cell* 57: 1–8.
52. Salhany JM, Yamane T, Shulman RG, Ogawa S (1975). High resolution ^{31}P nuclear magnetic resonance studies of intact yeast cells. *Proc Nat Acad Sci USA* 72: 4966–4970.
53. Servais P, Agogue H, Courties C, Joux F, Lebaron P (2001) Are the actively respiring cells (CTC+) those responsible for bacterial production in aquatic environments? *FEMS Microbiol Ecol* 35: 171–179.
54. Shabala L, Budde B, Ross T, Siegmundfeldt H, McMeekin T (2002) Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts. *Int J Food Microbiol* 75: 89–97.
55. Shapiro HM (1990) Cell membrane potential analysis. In Darzynkiewicz Z, Crissman HA (eds) *Methods in Cell Biology*, vol. 33. pp. 25–35. Academic Press, Inc., London, UK.
56. Shapiro HM (2000) Microbial analysis at the single-cell level: tasks and techniques. *J Microbiol Meth* 42: 3–16.
57. Schaule G, Flemming HC, Ridgway HF (1993) Use of 5-Cyano-2,3-Ditoly Tetrazolium Chloride for quantifying planktonic and sessile respiring bacteria in drinking water: *Appl Environ Microbiol* 59: 3850–3857.
58. Seidler, E (1991) The tetrazolium-formazan system: design and histochemistry. *Prog Histochem Cytochem* 24: 1–86.
59. Siegmundfeldt H, Rechinger KB, Jakobsen M (1999) Use of fluorescence ratio imaging for intracellular pH determination of individual bacterial cells in mixed cultures. *Microbiol UK* 145: 1703–1709.
60. Smith JJ, McFeters GA (1996) Effects of substrates and phosphate on INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) and CTC (5-cyano-2,3-ditoly tetrazolium chloride) reduction in *Escherichia coli*. *J Appl Bacteriol* 80: 209–215.
61. Smith, JJ, McFeters GA (1997). Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditoly tetrazolium chloride) reduction in *Escherichia coli* K-12. *J Microbiol Meth* 29: 161–175.
62. Stellmach J (1984) Fluorescent redox dyes. 1. Production of fluorescent formazan by unstimulated and phorbol ester- or digitonin-stimulated Ehrlich ascites tumor cells. *Histochem* 80: 137–143.
63. Stewart PS, Griebel T, Srinivasan R, Chen CI, Yu FP, de Beer D, McFeters GA (1994) Comparison of respiratory activity and culturability during monochloramine disinfection of binary population biofilms. *Appl Environ Microbiol* 60: 1690–1692.
64. Suller MTE, Lloyd D. (1999) Fluorescence monitoring of antibiotic-induced bacterial damage using flow cytometry. *Cytometry* 35: 235–241.
65. Susan M, Horobin RW, Seidler E, Barer MR (1993) Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. *J Appl Bacteriol* 74: 433–443.
66. Thom SM, Horobin RW, Seidler E, Barer MR (1993) Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of bacterial viability and activity. *J Appl Bacteriol* 74: 433–443.
67. Ullrich S, Karrash B, Hoppe HG, Jeskulke K, Mehrens M (1996) Toxic effect on bacterial metabolism of the redox dye 5-cyano-2,3 ditolyl tetrazolium chloride. *Appl Environ Microbiol* 62: 4587–4593.
68. Ultee A, Kets EPW, Smid EJ (1999) Mechanisms of Action of Carvacrol on the Food-Borne Pathogen *Bacillus cereus*. *Appl Environ Microbiol* 65: 4606–4610.

69. Van Der Lende TR, Breeuwer P, Abee T, Konings WN, Driessen AJM (2002) Assessment of the microbody luminal pH in the filamentous fungus *Penicillium chrysogenum*. *Biochimica et Biophysica Acta-Mol Cell Res* 1589: 104–111.
70. Vistica DT, Shekan P, Scudiero D, Monks A, Pittman A, Boyd MR (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production *Canc Res* 51: 2515–2520.
71. Winding A, Binnerup SJ, Sørensen J (1994) Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Appl Environ Microbiol* 60: 2869–2875.
72. Yu FP, McFeters GA (1994) Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. *J Microbiol Meth* 20: 1–10.

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