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Note

Propidium ion enters viable cells with high membrane potential during live-dead staining

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ABSTRACT

Live-dead staining with propidium iodide can give erroneous results for bacteria showing high membrane potentials. We observed uptake of propidium ions across intact cell membranes for *Dinoroseobacter shibae* and *Bacillus subtilis*. Apparently, a high membrane potential facilitates breakthrough of the double-charged propidium ion and can mark viable cells as dead.

Live-dead staining after Boulos et al. (1999) is a widely used method to assess the viability of microbial cells, with several hundred citations within the last 17 years. The staining procedure involves the addition of two fluorescent dyes, one of which does not normally permeate intact cell membranes (propidium iodide), while the counterstain (e.g., Sybr Green I or SYTO 9) does.

Both, viable cells and cells with a leaky membrane – classified as dead – will take up the counterstain and show green fluorescence. When applied together with propidium iodide, the leaky cells will also be infiltrated by propidium ions and show red fluorescence by canceling out the counterstain.

We performed live-dead staining with anoxically incubated cells of *Dinoroseobacter shibae*. In this state, a large portion of the cells showed uptake of propidium ions, indicating cell death. It was known that these cells had a boosted membrane potential (Kirchhoff and Cypionka, 2017) and were able to form up to 12 mM of intracellular ATP within 2 min upon re-aeration (Holert et al., 2011). The recovered cells stained as before anoxic incubation and appeared vital again. Similar to that, with *Bacillus subtilis* we observed cells with high and low membrane potential within growing filaments in the early growth phase, staining live and dead side by side.

From these observations we hypothesized that a boosted membrane potential ($\Delta\Psi$, inside negative) results in a reversible permeability for propidium ions in viable cells, which can produce misleading results.

Dinoroseobacter shibae DFL 12^T (Biebl et al., 2005) was grown in artificial seawater medium (SWM) with 10 mM succinate as substrate (see supplement for all media). Cells were cultivated in a diurnal light/dark rhythm in a shaker at 25 °C and 125 rpm (Soora and Cypionka, 2013). *Bacillus subtilis* (DSM-10) was grown in LB medium (lysogeny broth) at 33 °C and 125 rpm.

To compare fresh, de- and re-energized cells, the three energetic states were prepared separately. Fresh cells were taken directly from the exponential phase (grown for 18 h, OD_{436} 0.8). Two aliquots (5 ml each) of the same culture were transferred into a glass reaction tube, protected from light and N_2-flushed for 2 h to provide anoxic conditions. Cells from one of these parallels were subsequently flushed with air under light exposure for 5 min (420 mmol photons m^{-2} s^{-1}), to allow recovery of the cells before staining. Additionally, we conducted control experiments for both, live-dead- and membrane-potential staining, with heat-treated cells (65 °C, 8 min). Pictures were taken with a Canon EOS 600D camera and the EOS utility software (ver. 2.10.2.0). Final image processing was performed with PICOLAY (Cypionka et al., 2016).

Live-dead staining was performed after Boulos et al. (1999), modified as follows: We used propidium iodide and Sybr Green I as counterstain. To 1 ml cell culture 10 µl Sybr Green I solution (1:200 diluted with Tris-HCl buffer) were added, followed by thorough mixing. Afterwards, 15 µl of propidium iodide (1 mg/ml Tris-HCl buffer) were added to the cells and mixed again. Subsequently, the cells were incubated for 10 min in the dark and analyzed by fluorescence microscopy (Leica DMRB, Leitz) at excitation wavelength of 450–490 nm, dichroic filter 510 nm, long-pass filter 515 nm (Leica-Nr.: 513808).

The membrane potential was assessed as described by Kirchhoff and Cypionka (2017). In short; we used the $\Delta\Psi$-sensitive dye, 3,3′-Diethyloxacarbocyanine Iodide ($DiOC_2(3)$, TCI, Germany) and visualized the membrane potential via fluorescence microscopy.

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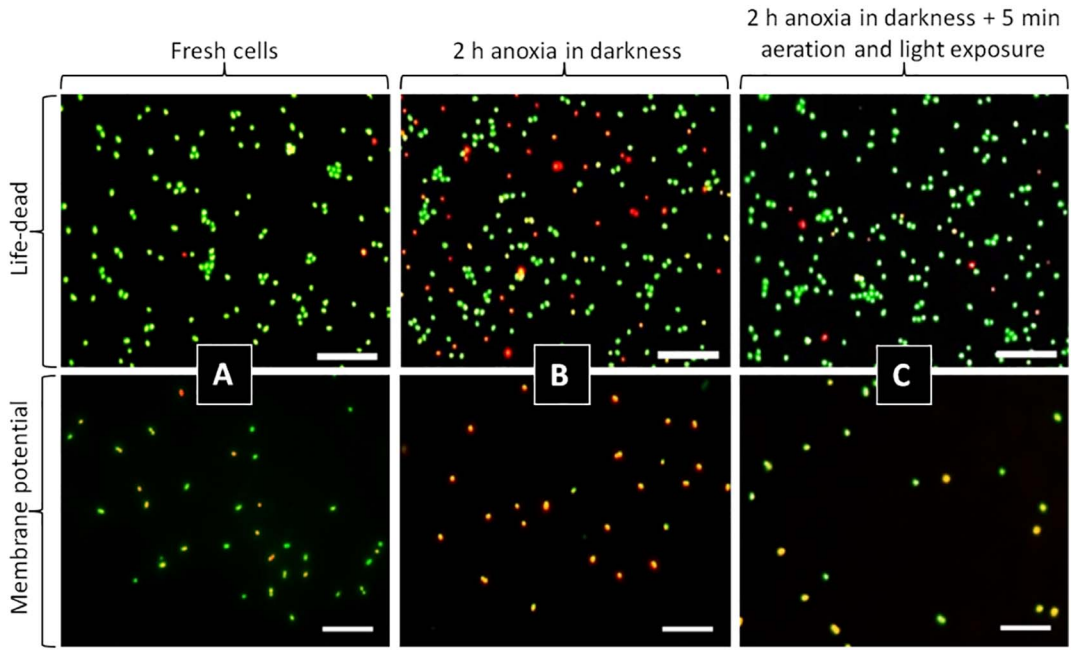

Fig. 1. Live-dead- and membrane-potential staining of *D. shibae* at different energetic states. (A) Fresh cells from the exponential phase. (B) Cells after 2 h of anoxic incubation. (C) Cells aerated for 5 min after 2 h of anoxia. The number of cells that take up propidium ions increases after N_2-gassing, but declines again after subsequent aeration. The same can be observed for $DiOC_2(3)$-uptake, indicating boosted $\Delta\Psi$ during anoxia. Scales: 10 µm.

With live-dead staining, nearly all cells of *Dinoroseobacter shibae* taken from the exponential growth phase appeared green, indicating no uptake of propidium ions (Fig. 1). After 2 h of anoxic incubation, up to 35% of the culture took up propidium ions, resulting in red fluorescence, indicating death of the cells. Some cells shifted from green to yellow/orange fluorescence, indicating minor uptake of propidium ions. Cells that were aerated for 5 min in the light, subsequently to the N_2-gassing, stained green like fresh cells, and therefore appeared vital. The results of the staining were the same, independent of whether the propidium iodide was added before, or after the addition of Sybr Green I to the sample.

For comparison we performed membrane potential staining with cells treated in the same way as described above. Fresh cells took up moderate amount of $DiOC_2(3)$. Most cells appeared green, and few stained red and orange (Fig. 1), indicating the normal state of the membrane potential. As shown in a previous study (Kirchhoff and Cypionka, 2017), after 2 h of anoxic incubation *D. shibae* shows a drastically boosted membrane potential, rendering the cells' interior more negatively charged then outside. Upon subsequent aeration the cells recover and stain like fresh cells again, maintaining a normal membrane potential.

As expected, cells that were treated with 5 µM CCCP after 2 h of anoxic incubation, did not show an increased membrane potential. At the same time, these cells did not show increased uptake of propidium ions.

To exclude that our observations are specific for *D. shibae*, we investigated the viability and the membrane potential of *B. subtilis* in the early growth phase, when the cells still formed multicellular filaments. We found that a fraction of the cells took up propidium ions and appeared to be dead, although originating from a very fresh and actively growing culture (Fig. 2A).

When performing membrane potential staining of the same culture (Fig. 2B–C), it became obvious that neighboring cells in the growing filaments show differences in dye-uptake and therefore have different membrane potentials.

In the present study we have shown that the results of live-dead staining can be biased by the membrane potential of the stained cells. An explanation for the observed effect could be given by the electrochemical properties of the propidium ion. The membrane-permeable Sybr Green I molecule (Fig. 3A) carries a single positive charge, which is surrounded by hydrophobic aromatic rings. In contrast, the propidium molecule carries two positive charges, one of which appears open to the surroundings and should prevent its membrane permeation (Fig. 3B). On the other hand, an increased membrane potential will amplify the ion-motive force for cations, particularly if they carry two charges as the propidium ion. Thus, a boosted $\Delta\Psi$ might facilitate a breakthrough of propidium molecules. This suggestion is supported by the fact that cells with a depleted membrane potential, due to CCCP treatment, lose their propidium ion permeability.

Our findings with *B. subtilis* indicate that the observed phenomenon could be of general relevance. The fact that a fraction of the cells take up propidium ions and fluoresce red while being located within a growing filament and having viable neighbors (Fig. 2A), suggests that these cells are not dead. It is already known, that cell cultures can have heterogeneity in their physiological states (Quirós et al., 2007).

The observation that cells can become reversibly permeable for propidium ions is not completely new. It was found, that permeability for propidium ions in *Saccharomyces cerevisiae* can be induced by membrane damage like heat or chemicals, followed by repair of the membrane (Davey and Hexley, 2011). We can exclude propidium-ions-uptake due to a damaged membrane, since the membrane potential was significantly boosted during that state and the cells maintained their ability to regenerate ATP.

Summing up, our findings help to explain variations in live-dead staining results. The fact that similar observations were made between *Gram*-negative *D. shibae* and *Gram*-positive *B. subtilis*, suggests that this newly found limitation of live-dead staining should be generally considered.

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Conflict of interest statement

The authors declare that the research was conducted in the absence