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A Simple and Rapid Dual-fluorescence Viability Assay for Microalgae

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A dual-fluorescence method was developed to assay the viability of microalgae and was tested in five strains of cyanobacteria and a green algae, *Chlamydomonas reinhardtii*. Fluorescence of SYTOX Green, a dye that only penetrates damaged cell membranes, and autofluorescence were observed simultaneously, allowing the discrimination of live and dead cells. We found good correlation in five of six strains between the real percentage of live microalgae in the samples and that measured by the double-fluorescence method. We conclude that our method provides a simple and rapid technique for assessing the viability of microalgae.

Key words: microalgae, SYTOX Green, viability assay, autofluorescence

INTRODUCTION

The titer of microalgae in culture can be measured accurately by viability assays in agar (10) and in liquid media (15). However, both methods require media preparation and incubation for at least 4 days before titers can be evaluated.

Fluorescence-based techniques provide alternative methods for viability assays and allow rapid and direct measurements. Fluorescein diacetate (FDA), which is cleaved by endogenous esterase activity following uptake into cells (12), is the most common fluorescent probe currently used to assay viability in microalgae (2, 4, 9). However, fluorescein, which is formed by intracellular hydrolysis of FDA, rapidly leaks from cells (6). Therefore, an improved fluorescence-based assay method for microalgae would be useful.

The LIVE/DEAD *Bac*Light viability assay kit has been widely employed for bacteria (1, 5, 7). In a stained preparation, live cells fluoresce green whereas dead cells fluoresce red, because this method uses two nucleic acid-binding fluorescent probes, SYTO 9 and propidium iodide. The advantages of this dual-fluorescence assay are that it is easy to perform, rapid, and not affected by endogenous physiological activity. However, the method cannot be used for microalgae because the autofluorescence of pigments such as chlorophyll overlaps that of propidium iodide.

An alternative viability assay using SYTOX Green was tested in 27 strains of microalgae (14). In this method, the dye has a high affinity to nucleic acid, only penetrates damaged cell membranes, and fluoresces bright green when excited with a 450−490 nm source. Therefore, only dead cells are detected by this method. Notably, the fluorescence of SYTOX Green does not overlap chlorophyll autofluorescence. Therefore, SYTOX Green fluorescence and autofluorescence can be used simultaneously as markers for dead and live cells, respectively, in a simple dual-fluorescence viability assay. We evaluated this method using a fluorescence microscope equipped with a double

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band pass filter set in five strains of cyanobacteria and a green algae.

MATERIALS AND METHODS

Strains and culture conditions

Three strains, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7492, and *Anabaena* sp. PCC 7120, were obtained from the Pasteur Culture Collection of Cyanobacteria at the Institut Pasteur (Paris, France) (11). Two strains, *Oscillatoria agardhii* NIES-204 and *Phormidium foveolarum* NIES-32, were provided by the Microbial Culture Collection at the National Institute for Environmental Studies (Tsukuba, Japan) (8). *Chlamydomonas reinhardtii* IAM C-9 was obtained from the IAM Culture Collection at the University of Tokyo (Tokyo, Japan) (3). The three strains from PCC were grown at 30 ℃ in BG-11 medium (13) in 3 cm diameter test tubes under continuous illumination by a fluorescent lamp (30 μE/m^2/s) and aeration containing 1.0 % (v/v) CO_2. *Oscillatoria agardhii* NIES-204, *Phormidium foveolarum* NIES-32, and *Chlamydomonas reinhardtii* IAM C-9 were grown at 25 ℃ in CB-, MDM-, and C-medium, respectively (8), with continuous illumination as above.

Preparation of "live" and "dead" cells

A 50 m*l* mid-logarithmic phase (OD_{730} 0.6) culture of microalgae was divided into two equal parts, which were centrifuged at 2,450 × g for 1 min. Both pellets were resuspended, one in 0.5 m*l* culture medium and the other in 0.5 m*l* pure ethanol. After 30 sec, the alcohol-treated cells were centrifuged at 2,450 × g for 1 min and resuspended in 0.5 m*l* culture medium. Heat-treated cells were prepared by boiling for 15 minutes. We assumed that the ethanol-treated cells and heat-treated cells were dead whereas the untreated cells were alive. The cell suspensions were mixed to produce samples containing 0, 25, 50, 75, or 100 % live cells.

Fluorescent labeling

The SYTOX Green fluorescent probe (Molecular Probes Inc., Eugene, OR, USA) was supplied as a 5 mM stock solution in DMSO. 0.5 μ*l* of this stock solution was added to 0.5 μ*l* cell suspension for a final dye concentration of 5 μM, and the mixture was incubated for 5 minutes at room temperature in the dark. No washing was required before or after SYTOX Green staining because no background fluorescence occurred in this experimental system (data not shown).

Dual-fluorescence microscopy

A fluorescence microscope (DMRE, Leica, Germany) equipped with a double band pass filter set at 473−498 and 548−573 nm for excitation, and at 515−535 and 590−620 nm for emission (G/R, Leica, Germany), was used. Fluorescence images were acquired using a CCD camera (DC 500, Leica, Germany). At least 500 cells in random fields were counted for viability calculations in each experiment.

Freeze-thaw cycles

Aliquots (0.5 m*l*) were taken from a mid-exponential phase culture (OD_{730} 0.6) and transferred to cryotubes (Cat. No. 375353, Nunc, Denmark) which were frozen at −20 ℃ without cryoprotectant for 6 h and then thawed for 5 min in a 30 ℃ water bath. This procedure was repeated one or two times for some cryotubes, to produce cyanobacterial samples that had gone through 1, 2, or 3 freeze-thaw cycles.

Plate counts

Colony-forming units/m*l* (CFUs) were assayed just before freezing and after each of the up to three freeze-thaw cycles by spreading 10-fold dilutions of the cell suspensions on BG-11 plates containing 5 mM $NaHCO_3$. Plates were incubated at 25 ℃ for 7 days and colonies were counted. Viability was calculated as: % viability = $(N/N_0) \times 100$, where N_0 is the CFU count before freezing and N is the CFU count after 1, 2, or 3 freeze-thaw cycles. These experiments were performed in triplicate with samples from different cultures.

RESULTS AND DISCUSSION

Detection of live and dead microalgae by dual-fluorescence

Microalgae viability was assayed based on measurements of dual-fluorescence of SYTOX Green and autofluorescence. In SYTOX Green-stained samples, very bright green fluorescence was observed in the samples which contained ethanol-treated cells of all strains except *Anabaena* sp. PCC 7120, while some cells showed red autofluores-

Table 1. Correlation between the percentage of live cells in the sample which contained ethanol-treated cells and the percentage of live cells detected by the double-fluorescence method in six strains of microalgae

Percent live cells in mixture	Percent live cells counted (red/total)[a]					
	Synechocystis sp. PCC6803	*Synechococcus* sp. PCC7942	*Oscillatoria agardhii* NIES-204	*Phormidium foveolarum* NIES-32	*Chlamydomonas reinhardtii* IAM C-9	*Anabaena* sp. PCC7120
0	0 (0)	1.8 (0.3)	0 (0)	0 (0)	0 (0)	45.9 (9.4)
25	26.9 (4.7)	26.9 (4.9)	32.3 (10.3)	32.7 (8.8)	34.6 (4.7)	61.3 (8.3)
50	42.0 (6.1)	48.4 (10.8)	43.0 (12.7)	46.4 (14.3)	51.0 (0.8)	66.3 (7.4)
75	67.3 (6.1)	66.4 (9.1)	59.0 (11.2)	62.4 (8.5)	77.4 (4.2)	85.7 (5.6)
100	96.1 (0.9)	98.4 (0.3)	98.4 (1.1)	98.7 (0.3)	98.6 (0.4)	98.1 (0.6)
Correlation coefficient (R)	0.996	0.996	0.955	0.976	0.994	0.989
Intercept	− 0.046	1.803	1.835	2.599	4.317	45.683
slope	0.930	0.931	0.894	0.908	0.960	0.515

[a] Mean of triplicate analyses. Values in parentheses are standard deviations.

Table 2. Correlation between the percentage of live cells in the sample which contained heat-treated cyanobacterium *Synechocystis* sp. PCC 6803 and the percentage of live cells detected by the double-fluorescence method

Percent live cells in mixture	Percent live cells counted (red/total)[a]
	Synechocystis sp. PCC 6803
0	0 (0)
25	25.3 (6.1)
50	47.7 (5.2)
75	67.5 (2.9)
100	97.3 (1.2)
Correlation coefficient (R)	0.998

[a] Mean of triplicate analyses. Values in parentheses are standard deviations

cence due to endogenous pigments that had remained in the cells after ethanol treatment. This fluorescence usually was weaker than that of live cells without ethanol treatment. Furthermore, we stained samples which contained heat-treated cells (Table 2) and freeze-thawed ones of the strain *Synechocystis* sp. PCC 6803 (Fig. 3, described below). In both cases, the loss of endogenous pigment was smaller than in ethanol-treated samples. In freeze-thawed cultures, the level of autofluorescence of dead cells was similar to that of living cells. However, dead cells could be identified unambiguously by their green fluorescence. On the other hand, all cells showed strong red autofluorescence due to endogenous pigments without ethanol treatment (100% "live" cells). Figure 1 shows dual-fluorescence images of samples of different strains (except *Anabaena* sp. PCC 7120), which were adjusted to contain 50% live cells. Dead cells are readily identified by their bright green (SYTOX Green fluorescence alone) or yellow (SYTOX Green fluorescence plus autofluorescence) color. However, in the filamentous cyanobacterium *Anabaena* sp. PCC 7120, the double-fluorescence method failed. Only about half of the ethanol-treated cells, which had been proven dead by direct culture methods, showed pronounced SYTOX Green fluorescence (Table 1). Figure 2 shows dual-fluorescence images together with the corresponding transmission images of dead *Anabaena* sp. PCC 7120. The levels of background fluorescence were particularly high in these tests. In some dead cells, we could recog-

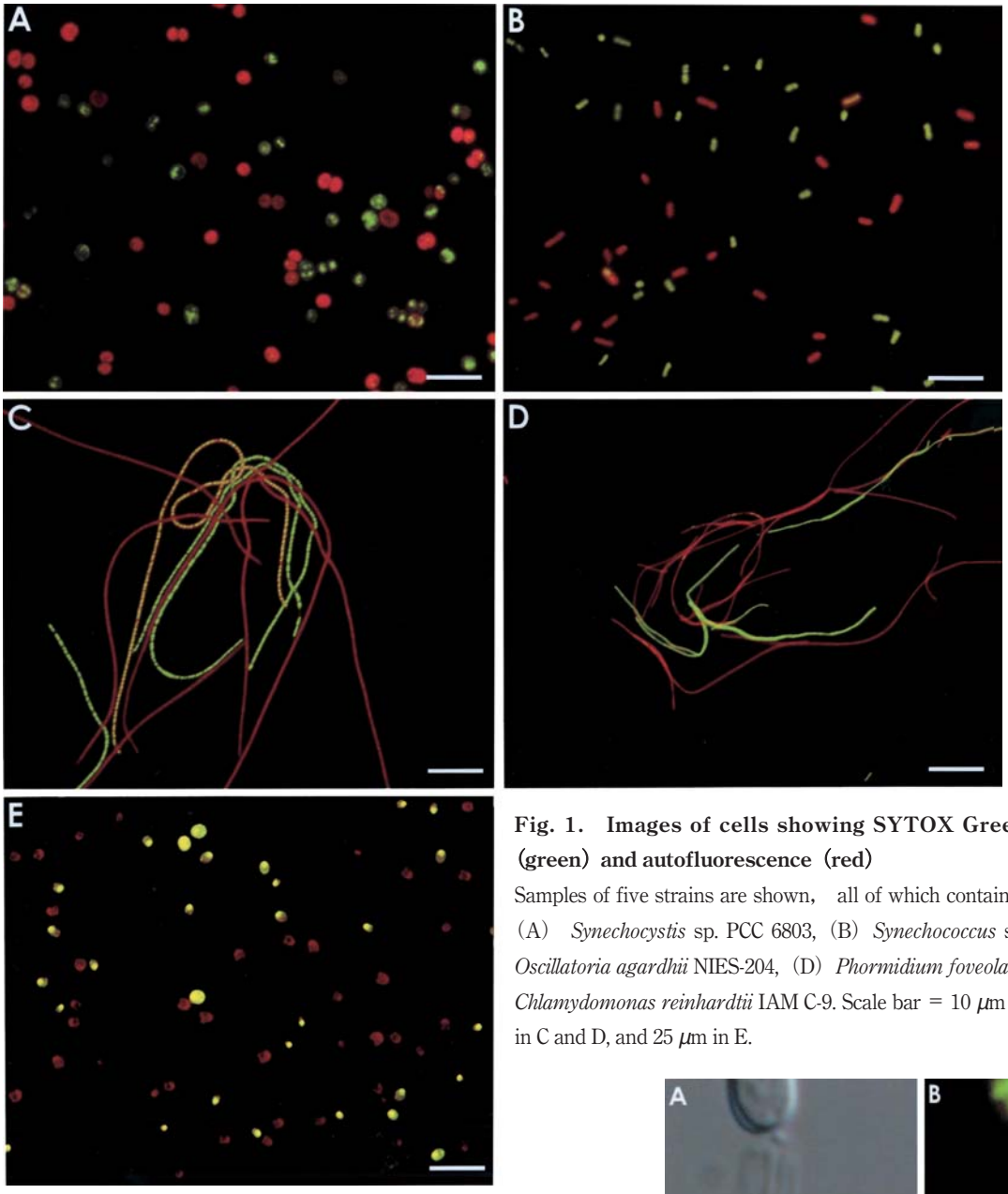

Fig. 1. Images of cells showing SYTOX Green fluorescence (green) and autofluorescence (red)

Samples of five strains are shown, all of which contain 50 % viable cells. (A) *Synechocystis* sp. PCC 6803, (B) *Synechococcus* sp. PCC 7942, (C) *Oscillatoria agardhii* NIES-204, (D) *Phormidium foveolarum* NIES-32, (E) *Chlamydomonas reinhardtii* IAM C-9. Scale bar = 10 μm in A and B, 50 μm in C and D, and 25 μm in E.

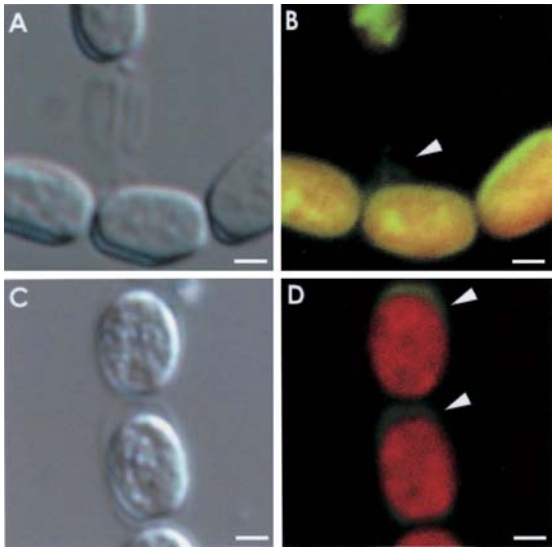

Fig. 2. Images of dead *Anabaena* sp. PCC 7120 cells showing SYTOX Green fluorescence (green) and autofluorescence (red)

(A) and (C), transmission images corresponding to the dual-fluorescence images (B) and (D). Scale bar = 1 μm

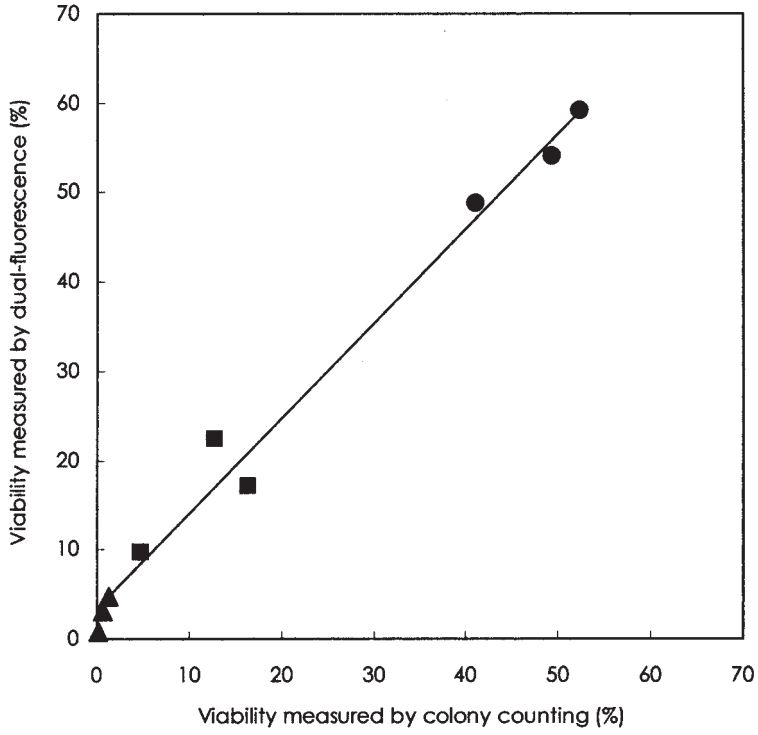

Fig. 3. Correlation between the percentages of viable cells as measured by CFU counts and by dual-fluorescence after 1 (circles), 2 (squares), or 3 (triangles) freeze-thawing cycles in the cyanobacterium *Synechocystis* sp. PCC 6803

CFU counts were determined from triplicate plating and each dual-fluorescence measurement from at least 500 cells. Values are expressed as percentages of the number of viable cells in samples that were not frozen. The linear regression line between the two methods has a slope of 1.033, intercept of 5.702, and correlation coefficient of 0.978.

nize intracellular SYTOX Green fluorescence (Fig. 2B), and sometimes we observed green fluorescence in the vicinity of cells (arrowhead, Fig. 2B). In other cases, no intracellular SYTOX Green fluorescence was detectable (Fig. 2D), but green fluorescence occurred between the cell wall and the cell membrane. This was probably due to DNA leaking out of the destroyed protoplasts.

To evaluate the validity of our method, the viability measured by dual-fluorescence in the six strains was compared to expected values in mixtures of live and dead cells with fractions of live cells of 0, 25, 50, 75, and 100 %. There were good correlations between expected and measured values in all strains except *Anabaena* sp. PCC 7120 (Table 1, 2). These results demonstrated the suitability of our method for the assessment of the fraction of viable cells in cultures of microalgae.

Application of the dual-fluorescence method to freeze-thawed cyanobacteria

To test the applicability of the dual-fluorescence method for studies of environmental stress in cyanobacteria, the viability of *Synechocystis* sp. PCC 6803 after freezing-thawing as determined by the dual-fluorescence method was compared to the results of CFU counts. Cyanobacterial viability decreased after each freeze-thaw cycle (Fig. 3). Both methods yielded comparable results, as expressed by their close correlation. Therefore, the dual-fluorescence method described here is an effective technique for quantifying cyanobacteria viability in environmental studies.

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REFERENCES

1. Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R. LIVE/DEAD *Bac*Light: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Meth. **37**: 77－86 (1999).
2. Day, J.G., McLellan, M.R.(eds.), Cryopreservation and freeze-drying protocols. Meth. Mol. Biol. **38**: 81－89 (1995).
3. Editional Board of the IAM Catalogue of Strains Center for Bioinformatics Institute of Molecular and Cellular Biosciences, the University of Tokyo,(eds.), IAM Catalogue of Strains, Third Edition (2004)
4. Fleck, R.A., Benson, E.E., Bremner, D.H., and Day, J.G. Studies of free radical-mediated cryoinjury in the unicellular green alga *Euglena gracilis* using a non-destructive hydroxyl radical assay: a novel approach for developing protistan cryopreservation strategies. Free Rad. Res. **32**: 157－170 (2001).
5. Gardiner, G.E., O' Sullivan, E., Auty, M.A.E., Fitzgerald, G.F., Collins, J.K., Ross, R.P., and Stanton, C. Comparative survival rates of human-derived probiotic Lactobacillus paracasei and L. salivarius strains during heat treatment and spray drying. Appl. Environ. Microbiol. **66**: 2605－2612 (2000).
6. Haugland, R.P., Gregory, J., and Spence Michelle T.Z. (eds.), Handbook of Fluorescent Probes and Research Products Ninth Edition, p605, Molecular Probes, Inc, OR.
7. Hope, C.K., Clements, D., and Wilson, M. Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. J. Appl. Microbiol. **93**: 448－455 (2002).
8. Kasai, F, Kawachi, M, and Watanabe, M.M., (eds.), NIES-Collection List of Strains Seventh Edition Microalgae and Protozoa (2004).
9. Mori, F., Erata, M., and Watanabe, M.M. Cryopreservation of cyanobacteria and green algae in the NIES-collection. Microbiol. Cult. Coll. **17**: 45－55 (2002).
10. Morris, G.J. The cryopreservation of *Chllorella* 1. Interactions of rate of cooling, protective additive and warming rate. Arch. Microbiol. **107**: 57－62 (1976).
11. Pasteur Culture Collection of Cyanobacteria Strain in Axenic Culture, Catalogue and Taxonomic Handbook (1992), Collection Nationale de Culture, Catalogue and Insitut Pasteur.
12. Rotman, B. and Papermaster, B.W. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad., Sci. USA. **55**: 134－141 (1966).
13. Stanier, R.Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. Purification and properties of unicellular blue-green algae (order *Choroococcales*). Bacteriol. Rev. **35**: 171－205 (1971).
14. Veldhuis M.J., Cucci T.L., and Sieracki M.E. Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. J. Phyocol. **33**: 527－541 (1997)
15. Watanabe, M.M., and Sawaguchi, T. Cryopreservation of a water-bloom forming cyanobacterium, *Microcystis aeruginosa* f. *aeruginosa*. Phycol. Res. **43**: 111－116 (1995).

簡便で迅速に行える微細藻類の２重蛍光による生存率測定法

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５株の藍藻と１株の緑藻クラミドモナスを用い２重蛍光による微細藻類の生存率測定法を評価した．細胞膜に傷害がある場合のみ膜を透過して蛍光を発するSYTOX Greenと自家蛍光を同時に観察することで，生きた細胞と死んだ細胞を見分けることが可能であることがわかった．さらに実際の生存率と２重蛍光法で測定した生存率との相関係数を調べたところ６株のうち５株について非常によい相関が得られた．このことから我々の方法は微細藻類の生存率を簡便に短時間に測定するために有効な方法であると考えられる．