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Method for quick evaluation of cell viability in marine macroalgae*

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Various staining-dyes were tested with cultured cells of the economically valuable marine macroalgae, *Enteromorpha intestinalis, Porphyra yezoensis* and *Macrocystis pyrifera* in order to develop a rapid, simple and reliable technique for determining algal cell viability. Pieces of the tissue were immersed in the dye solutions of the cell viability checking kit and observed with a microscope after 10 min. The most suitable dyes tested were FDA for detection of viable cells and phenosafranin for dead cells in *Enteromorpha intestinalis* gametophyte, and neutral red for viable cells and Evans blue for dead cells in *Macrocystis pyrifera* gametophyte. The most suitable dye tested was Evans blue for detection of dead cells, and autofluorescence of phycoerythrin was useful for detection of viable cells in *Porphyra yezoensis* conchocelis.

Key Index Words: Cell viability—Chlorophyta—Enteromorpha intestinalis—Macrocystis pyrifera—*Phaeophyta*—Porphyra yezoensis—*Rhodophyta—staining—tissue culture.*

Accurate evaluation of cell viability is a very important step in studies on algal tissue culture in many ways. Determining cell viability is essential to development of efficient methods for mutagenesis and freeze preservation in cultured algal cells. Usually cell viability is determined by microscopic assessment of cytoplasmic features or plasmolysis capacity in our laboratory. However, sometimes these methods are insufficient to clear-cut evaluation of cell viability. Various staining methods have been used to detect live or dead higher plant cells (WHITHERS 1980), and a few staining methods have been used to detect live or dead macroalgal cells. TTC (OGATA 1956) and neutral red (TERUMOTO 1960, SAKAI and SUGAWARA 1978) were used to detect live algal cells, while erythrosine (MIGITA 1967) was used to dead algal cells.

The purpose of the present paper is to examine various staining dyes in order to develop a rapid, simple and reliable technique for determining cell viability in marine macroalgae.

Materials and Methods

The materials used in this study were an axenic strain of *Enteromorpha intestinalis* (gametophyte), HRFRL culture collection no. EI-001, an axenic strain of *Porphyra yezoensis* (conchocelis), HRFRL culture collection no. PY-401 and axenic male and female strains of *Macrocystis pyrifera* (gametophyte), HRFRL culture collection no. MP-101 (male strain) and no. MP-201 (female strain). They were obtained by the one step antibiotic disk method described previously (SAGA and SAKAI 1982). Ten colonies of the organisms which developed into a mass, 1.0 mm in diameter, were inoculated into plastic vessels (Tissue Culture Flask # 25100, Corning) containing 30 m*l* of ASS 1 medium (Table 1). The cultures were kept for 1 month on a horizontal reciprocal shaker (Thermoshaker Model XY-11, Thermonics Co.; amplitude

Abbreviations: FDA, fluorescein diacetate; TTC, triphenyl tetrazolium chloride.

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Table 1. Composition of ASS 1 per 100 m*l*, pH 8.0.

NaCl	2.5 g	Na_2-glycerophosphate	2 mg
$MgSO_4 \cdot 7H_2O$	1.0 g	$NaHCO_3$	10 mg
KCl	70 mg	Vitamin mix ASS*	0.1 m*l*
$CaCl_2 \cdot 2H_2O$	110 mg	Metal mix ASS**	1 m*l*
$NaNO_3$	10 mg	HEPES	100 mg

* 1 m*l* vitamin mix ASS contains: thiamine HCl 100 μg, nicotinic acid 100 μg, putrescine·2HCl 10μg, Ca·pantothenate 100μg, riboflavin 10 μg, pyridoxine·2HCl 10 μg, pyridoxamine·2HCl 10 μg, *p*-aminobenzoic acid 10 μg, biotin 1 μg, inositol 1 mg, choline-H_2-citrate 100 μg, thymine 100 μg, orotic acid 100 μg, cyanocobalamine 1 μg, folic acid 1 μg, folinic acid 0.1 μg.

** 1 m*l* of metal mix ASS contains: Fe (as Fe-sequestren) 100 μg, B (as H_3BO_3) 100 μg, Mn (as Cl^-) 100 μg, Zn (as Cl^-) 10 μg, Co (as Cl^-) 1 μg, Mo (as Na_2MoO_4) 10 μg, Cu (as Cl^-) 1 μg, Br (as K^+) 1 mg, Sr (as Cl^-) 100 μg, Rb (as Cl^-) 10 μg, Li (as Cl^-) 10 μg, I (as K^+) 1 μg.

40 mm, 40 strokes/min) in an incubator (Cultivation Chamber Model CU-250, Tomy Seiko Co.) which was regulated at 15°C, 14:10 hr L:D cycle and ca. 60 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. The dead organisms were prepared by heat-killing at 70°C for 5 min.

The live and dead organisms were transferred into a CVC-kit (cell viability checking kit) and immersed in the dye solutions for 10 min. The CVC-kit was prepared by the following procedures: the staining-dyes were dissolved, in a concentration of 0.01%, in autoclaved seawater; adjusted the pH to 8.0 and put into each well of a plastic vessel (Cell Wells # 25860, Corning) up to 0.25 m*l*. The compounds tested were Evans blue (Merk Co.), phenosafranin (Katayama Chemical Co.), eosin Y (Wako Pure Chemical Industries), erythrosine (Tokushu Chemical Co.), neutral red (Kanto Chemical Co.) and FDA (Aldrich Chemical Co.). After immersion, the organisms were rinsed several times with autoclaved seawater and transferred onto a glass slide. Then, a coverglass was lowered over the organisms and the slide was observed with a microscope (Vanox T Model-AHBT, Olympus). The FDA-treated organisms were observed with the same microscope using a fluorescence system (Vanox T Model-AH2RFL, Olympus). A mercury-vapor lamp (HBO-200W/2, Osram) was used in combination with a dichroic mirror (DM-500, Olympus), exciter filter (BP-490, Olympus) and absorber filter (AFC & O-515, Olympus).

Results

Evans blue, phenosafranin, eosin Y and

Table 2. Staining of live and dead gametophyte cells with various dyes in *Enteromorpha intestinalis*.

Staining dyes	Live cells		Dead cells	
	PTS	CW	PTS	CW
Evans blue	−	++	+	++
Phenosafranin	−	±	++	±
Eosin Y	−	±	+	±
Erythrosine	−	±	++	±
Neutral red	++	±	−	±
FDA	++	−	−	−

++ stained deeply; + stained moderately; + stained lightly; ± stained infrequently; − not stained; PTS=protoplasts; CW=cell walls.

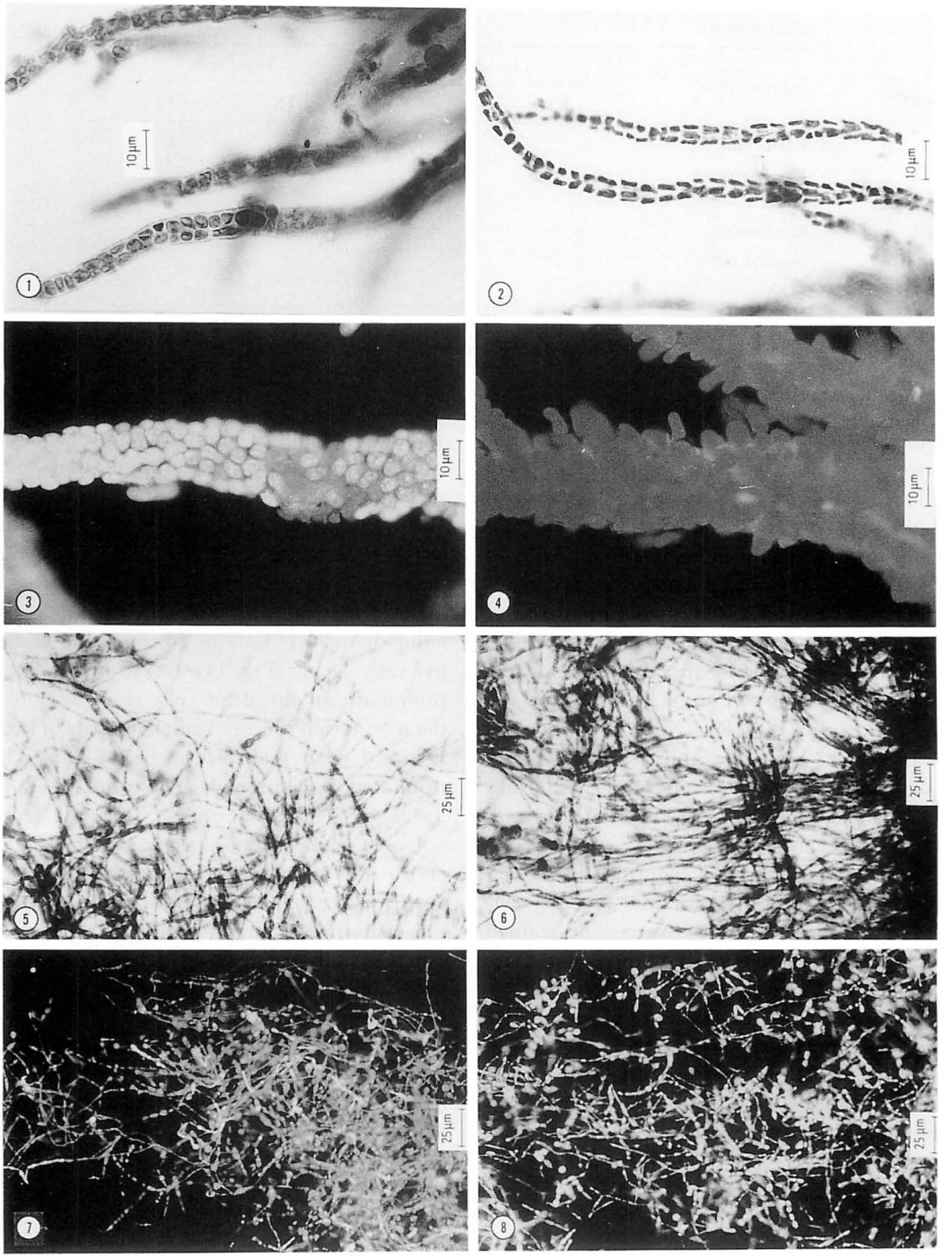


Fig. 1. Live *Enteromorpha intestinalis* gametophyte cells treated with phenosafranin. Fig. 2. Dead *Enteromorpha intestinalis* gametophyte cells treated with phenosafranin. Fig. 3. Live *Enteromorpha intestinalis* gametophyte cells treated with FDA. Fig. 4. Dead *Enteromorpha intestinalis* gametophyte cells treated with FDA. Fig. 5. Live *Porphyra yezoensis* conchocelis cells treated with Evans blue. Fig. 6. Dead *Porphyra yezoensis* conchocelis cells treated with Evans blue. Fig. 7. Autofluorescence of live *Porphyra yezoensis* conchocelis cells. Fig. 8. Autofluorescence of dead *Porphyra yezoensis* conchocelis cells.

Table 3. Staining of live and dead conchocelis cells with various dyes in *Porphyra yezoensis*.

Staining dyes	Live cells		Dead cells	
	PTS	CW	PTS	CW
Evans blue	−	±	++	±
Phenosafranin	−	−	+	−
Eosin Y	−	−	++	−
Erythrosine	−	−	++	−
Neutral red	++	−	−	−
FDA	−	−	−	−

++ stained deeply; ++ stained moderately; + stained lightly; ± stained infrequently; − not stained; PTS=protoplasts; CW=cell walls.

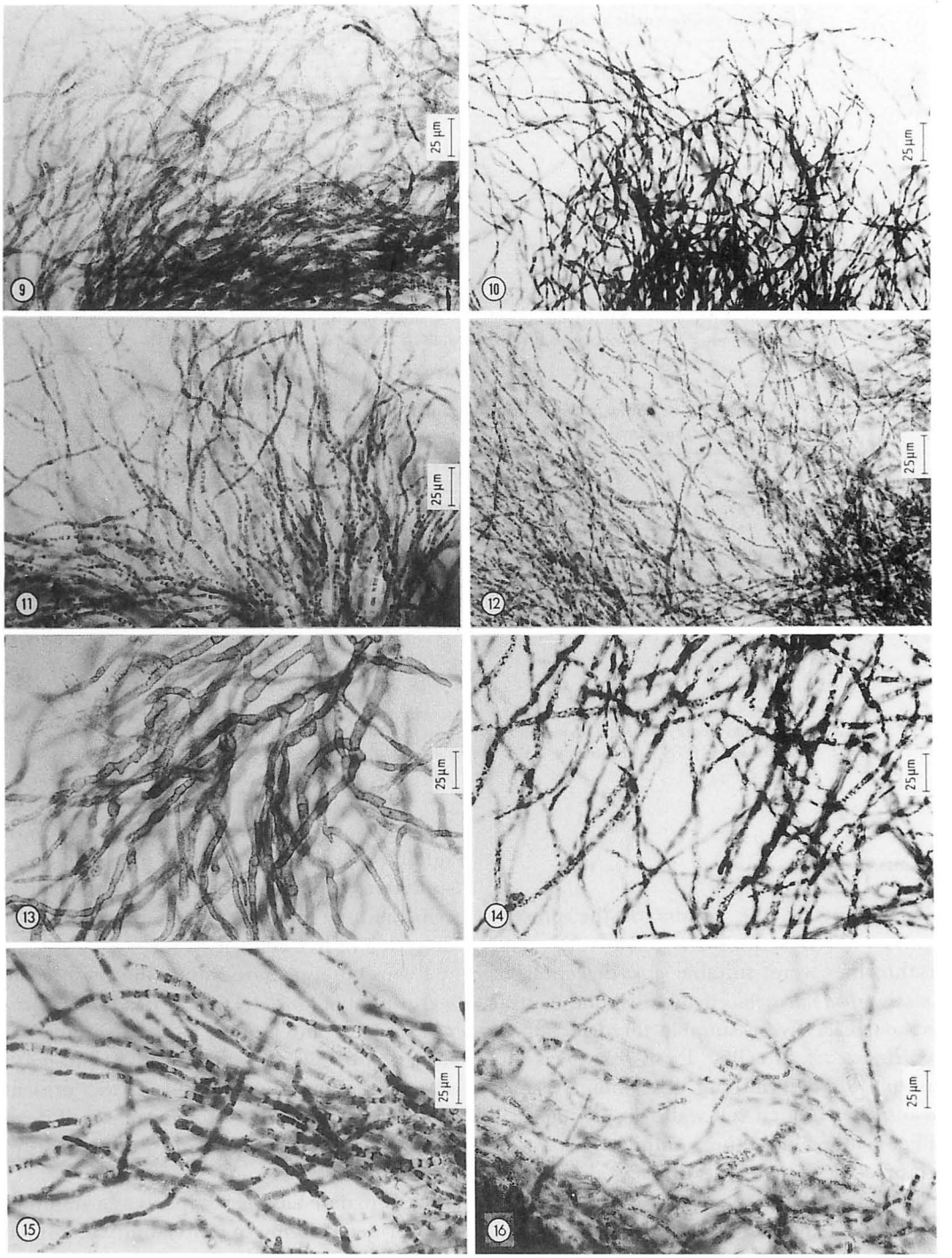
erythrosine stained protoplasts of dead *Enteromorpha intestinalis* cells, and phenosafranin stained them especially deeply (Fig. 2). But these dyes did not stain protoplasts of live *E. intestinalis* cells (Fig. 1). Evans blue stained cell walls of both live and dead cells deeply, and phenosafranin, eosin Y and erythrosine stained them infrequently. On the other hand, neutral red and FDA stained protoplasts of the live cells deeply (Fig. 3), but they did not stain protoplasts of the dead cells (Fig. 4). Neutral red stained cell walls of both live and dead cells infrequently, but FDA did not stain them at all. The results obtained by the present study are summarized in Table 2.

Evans blue, phenosafranin, eosin Y and erythrosine stained protoplasts of dead *Porphyra yezoensis* cells, and Evans blue stained them especially deeply (Fig. 6). But these dyes did not stain protoplasts of live *P. yezoensis* cells (Fig. 5). On the other hand, neutral red stained protoplasts of the live cells. The color of autofluorescence of live *P. yezoensis* cells is orange (Fig. 7), and the color of autofluorescence of dead *P. yezoensis* cells is yellow (Fig. 8). All the dyes tested did not stain cell walls of both live and dead *P. yezoensis* cells except Evans blue. The results aforementioned are summarized in Table 3.

Evans blue, phenosafranin, eosin Y and erythrosine stained protoplasts of dead male *Macrocystis pyrifera* cells, and Evans blue stained them especially deeply (Fig. 10). But these dyes did not stain protoplasts of live male *M. pyrifera* cells (Fig. 9). On the other hand, neutral red stained protoplasts of the live cells deeply (Fig. 11), but it did not stain protoplasts of the dead cells (Fig. 12). All the dyes tested did not stain cell walls of both live and dead male *M. pyrifera* cells. The results aforementioned are summarized in Table 4.

Evans blue, phenosafranin, eosin Y and erythrosine stained protoplasts of dead female *M. pyrifera* cells, and Evans blue stained them especially deeply (Fig. 14). But these dyes did not stain protoplasts of live female *M. pyrifera* cells (Fig. 13). On the other hand, neutral red stained protoplasts of the live cells deeply (Fig. 15), but it did not stain protoplasts of the dead cells (Fig. 16). All the dyes tested did not stain cell walls of both live and dead female *M. pyrifera* cells. The results aforementioned are summarized in Table 5.

Fig. 9. Live *Macrocystis pyrifera* gametophyte cells treated with Evans blue (male strain). Fig. 10. Dead *Macrocystis pyrifera* gametophyte cells treated with Evans blue (male strain). Fig. 11. Live *Macrocystis pyrifera* gametophyte cells treated with neutral red (male strain). Fig. 12. Dead *Macrocystis pyrifera* gametophyte cells treated with neutral red (male strain). Fig. 13. Live *Macrocystis pyrifera* gametophyte cells treated with Evans blue (female strain). Fig. 14. Dead *Macrocystis pyrifera* gametophyte cells treated with Evans blue (female strain). Fig. 15. Live *Macrocystis pyrifera* gametophyte cells treated with neutral red (female strain). Fig. 16. Dead *Macrocystis pyrifera* gametophyte cells treated with neutral red (female strain).


9
25 µm
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16
25 µm

Table 4. Staining of live and dead gametophyte cells with various dyes in *Macrocystis pyrifera* (male strain).

Staining dyes	Live cells		Dead cells	
	PTS	CW	PTS	CW
Evans blue	−	−	++	−
Phenosafranin	−	−	+	−
Eosin Y	−	−	±	−
Erythrosine	−	−	+	−
Neutral red	++	−	−	−
FDA	−	−	−	−

++ stained deeply; + stained moderately; + stained lightly; ± stained infrequently; − not stained; PTS=protoplasts; CW=cell walls.

Table 5. Staining of live and dead gametophyte cells with various dyes in *Macrocystis pyrifera* (female strain).

Staining dyes	Live cells		Dead cells	
	PTS	CW	PTS	CW
Evans blue	−	−	++	−
Phenosafranin	−	−	+	−
Eosin Y	−	−	±	−
Erythrosine	−	−	+	−
Neutral red	++	−	−	−
FDA	−	−	−	−

++ stained deeply; + stained moderately; + stained lightly; ± stained infrequently; − not stained; PTS=protoplasts; CW=cell walls.

Discussion

According to the results of the present study, phenosafranin, eosin Y and erythrosine were suitable for detection of *Enteromorpha intestinalis* dead cells, and neutral red and FDA were suitable for detection of live *E. intestinalis* cells. Evans blue was unsuitable for determining cell viability, since Evans blue stained cell walls deeply and it is difficult to evaluate the staining condition of protoplasts. The most suitable staining-dyes tested in the present study were FDA for detection of the live cells and phenosafranin for detection of the dead cells in *E. intestinalis*.

Evans blue, phenosafranin, eosin Y and erythrosine were suitable for detection of dead *Porphyra yezoensis* cells, and neutral red and autofluorescence of the cells were suitable for detection of live *P. yezoensis* cells. The most suitable staining-dye tested in the present study was Evans blue for detection of dead cells, and the autofluorescence was useful for detection of live cells in *P. yezoensis*.

Evans blue, phenosafranin and erythrosine were suitable for detection of dead *Macrocystis pyrifera* cells, and neutral red was suitable for detection of live *M. pyrifera* cells. The most suitable staining-dyes tested in the present study were Evans blue for detection of dead cells and neutral red for detection live cells in *M. pyrifera*.

FDA to date has been applied as a staining-dye for determining of cell viability in animal cells (ROTMAN and PAPERMASTER 1966) and plant cells (WIDHOLM 1972, NAG and STREET 1973, WITHERS and KING 1979, SIMINOVITCH 1979). FDA probably stains live algal cells in the same manner that it does animal cells

(ROTMAN and PAPERMASTER 1966) and plant cells (WIDHOLM 1972). FDA, the nonpolar molecule, enters into the live cells where esterase cleaves off the acetate residues leaving fluorescein which then accumulates. FDA itself can not fluoresce but these fluorescein molecules can fluoresce. Neutral red has been applied as staining-dye for determining cell viability in animal cells (ETOH 1978) and plant cells (SIMINOVITCH 1979), and it probably stains live algal cells in the following manner: the live cells take up neutral red and concentrate it in their vacuoles (SIMINOVITCH 1979).

Evans blue, phenosafranin, eosin Y and erythrosine have been applied as staining-dyes for determining cell viability in many kinds of plant cells (GAFF and OKONG'O-OGOLA 1971, WIDHOLM 1972, HAUPTMANN and WIDHOLM 1982). These staining-dyes stain only dead protoplasts and are probably excluded from live protoplasts by the intact plasma membrane (WIDHOLM 1972).

The color change of the autofluorescence in *Porphyra* cells probably represents fluorescence from the red algal pigment phycoerythrin (POLNE and GIBOR 1982).

In the present study, a rapid, simple and reliable technique for determining cell viability was established employing the CVC-kit in the marine macroalgae. Although we have applied this method to a few species of economically valuable marine algae, it should be useful with other marine macroalgae.

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嵯峨直恒・坂西芳彦・荻島　隆：大型海産藻類の細胞生存率の簡易評価法

細胞の生存率を正確かつ簡便に評価することは，海藻の組織培養の種々の局面において必要な技術である。ボウアオノリ，マクロシスティス・ピリフェラ，スサビノリの緑褐紅にわたる有用海藻の無菌培養細胞を材料として，種々の染色剤による生細胞と死細胞の染め分けを検討した。ボウアオノリ（配偶体）では生細胞の検出にはフルオレセインジアセテートが，死細胞の検出にはフェノサフラニンが有効であった。マクロシスティス・ピリフェラ（配偶体）では生細胞の検出にはニュートラルレッドが，死細胞の検出にはエバンスブルーが有効であった。スサビノリ（コンコセリス）では生細胞の検出にはフィコエリスリンの自家蛍光が，死細胞の検出にはエバンスブルーが有効であった。また，染め分けに有効な数種の染色剤の入ったマルチプレート CVC-kit（細胞生存率評価キット）を作製し，大型海産藻類の細胞生存率の簡易評価法を開発した。(085 北海道釧路市桂恋116　水産庁北海道区水産研究所)