

ISOLATION AND FLOW CYTOMETRIC CHARACTERIZATION OF PROTOPLASTS FROM MARINE MACROALGAE¹

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ABSTRACT

Protoplasts were isolated from *Ulva rigida* C. Agardh (Chlorophyta) and two species of Rhodophyta, *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto et Folvik and *Gracilaria tenuistipitata* Chang et Xia var. *liui* with minor modifications (the inclusion of 0.01% agarase in the set of cell-wall-degrading enzymes for the two red algae). Flow cytometric characteristics of freshly isolated protoplasts were determined on a FACScan flow cytometer (FC). The most useful parameters for characterizing protoplasts from marine algae were forward angle light scatter (FSC), orange fluorescence (FL2) and red fluorescence (FL3). Protoplasts from all the species were easily distinguishable when their FSC, FL2, and FL3 signals were combined in the bivariate plots FL3 vs. FSC and FL3 vs. FL2. Two alternative techniques to help identify protoplasts from debris in the FC computer screen were developed (for FC without sorting capability). Both techniques were based on the ability of new FCs to record time. The first one was based on the induction of rapid changes of cell volume in response to osmotic stress. Only intact protoplasts responded to changes in the osmotic pressure. The second one was based on the uptake and hydrolysis of fluorescein diacetate by intracellular esterases. Viable protoplasts showed a hyperbolic accumulation of fluorescein with time. Semimaximal fluorescein accumulation was attained in 30.5 ± 9.5 s. Debris was easily recognized since, contrary to protoplasts, it did not show a time-dependent accumulation of fluorescein.

Key index words: autofluorescence; Chlorophyta; flow cytometry; fluorescein; *Gracilaria tenuistipitata*; *Gracilariopsis lemaneiformis*; macroalgae; protoplasts; Rhodophyta; *Ulva rigida*

Flow cytometry (FCM) is a novel technique in plant biology. Most plant biologists are unaware of its potential, and the scarcity of flow cytometers (FCs) worldwide is due to their, until very recently, high cost. Animal cell biology, on the other hand, has benefited from FCM a great deal due to its biomedical applications. However, analysis of plant cells by FCM can be useful because of the autofluorescent characteristics of the photosynthetic pigments. A

particular pigment composition will produce a specific flow cytometric signature when excited by the FC laser beam. This signature is, in principle, characteristic of each species. In addition, since pigment composition of the plant cells, both quantitatively and qualitatively, is physiologically regulated, changes in their fluorescent characteristics can be expected. However, the potential of FCM in plant cell studies is not restricted to the description and/or identification of the autofluorescent characteristics of different cellular types or species. A number of fluorescent compounds suited for FCM label antibodies, other proteins, hormones, and nucleic acids (Ormerod 1990). Many examples of these fluorescent probes exist in the animal literature. Some examples also exist for plant cells (Galbraith et al. 1983, Laats et al. 1987, Zhang et al. 1992). Dynamic cell properties such as membrane potential, intracellular pH, intracellular $[Ca^{2+}]$, and intracellular esterase activity have also been measured in animal cells by means of specific fluorescent dyes and FCM (for a general overview of this field, see Rabinovitch and June 1990). No *a priori* reason exists for not applying the same techniques to plant protoplasts with minor modifications.

The application of FCM to most plant species needs, as a preliminary step, the isolation of protoplasts, since single-cell suspensions are required. Protoplasts were first isolated from seaweeds using cell-wall-degrading enzymes by Millner et al. (1979), almost 20 years later than in higher plants (Cocking 1960). Although in the last decade great progress has been made in this field, a large gap exists between the studies involving higher plant protoplasts and those with marine macroalgae. At the beginning of the 1990s, protoplasts had only been isolated from two genera of red macroalgae, three genera of green algae, and six genera of brown seaweeds (Butler et al. 1991); however, it was possible to isolate protoplasts from virtually any higher plant species with commercially available enzyme preparations. In this paper we report the isolation of protoplasts from the green alga *Ulva rigida* C. Agardh (Chlorophyta) and the red alga *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto et Folvik and *Gracilaria tenuistipitata* Chang et Xia var. *liui* with basically the same procedure as with higher plants and using commercially

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available enzyme preparations. Protoplasts were characterized by their flow cytometric signature. We show the use of FCM to determine dynamic cell processes such as volume changes in response to osmotic stress and intracellular esterase activity. Esterase activity, considered a marker for "metabolic vigor" (Rotman and Papermaster 1966, Larkin 1976, Bentley-Mowat 1982), was determined by means of fluorescein diacetate (FDA) breakdown. Determination of esterase activity by means of FDA and FCM is a quantitative approach to cell viability determinations, which are usually done in a qualitative way by fluorescence microscopy. Furthermore, esterase activity determination represents a starting point for the use of FCM to measure any specific enzymatic activity for which a fluorescent substrate can be found or designed.

MATERIALS AND METHODS

Ulva rigida C. Agardh was collected from a rocky shore in the south of Spain (Lagos, Málaga) and kept in a cool chamber (15°C) for different periods of time (1 to 5 days in natural seawater at a photon fluence rate of $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). *Gracilaria tenuistipitata* Chang et Xia var. *liui* was cultured in natural seawater supplemented with Provasoli medium (Provasoli 1968). A closed circuit was established, and the medium was continuously circulated through an activated charcoal filter and continuously irradiated with a UV lamp to avoid proliferation of microorganisms. The culture was illuminated with white light ($200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 8:16 h LD) and maintained at 25°C. *Gracilariaopsis lemaneiformis* (Bory) Dawson, Acleto et Folvik was cultured at 27°C in white light ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 14:10 h LD) and in filtered (Whatman GF/C) natural seawater supplemented with $100 \mu\text{M}$ KNO_3 . The algae were transferred to fresh medium every 3 days. Gas exchange was stimulated by continuous pumping of air into the culture medium. The microalga *Dunaliella viridis* Teodoresco, used in the study of volume changes, was grown according to Jiménez and Niell (1990).

Protoplast isolation. The thalli were cleaned of epiphytes (this was only necessary for *Ulva*) and incubated in artificial seawater plus 0.6 M mannitol and 4.2 mM HEPES, pH 8 (medium 1) for 1 h. Afterward, portions of 1 g fresh weight were chopped and incubated ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 20°C, 60 rpm) for different periods of time (2 to 8 h) in 100 mL of artificial seawater supplemented with 0.6 M mannitol, 4.2 mM HEPES, 25 mM MES, pH 6.5 (medium 2) plus the enzymes: 2% Onozuka R-10 and 0.1% macerozima R-10 (Yakult Pharmaceutical, Tokyo). The enzymes were added to medium 2 just before the introduction of the plant tissue. The composition of the artificial seawater was 450 mM NaCl, 10 mM KCl, 10 mM CaCl_2 , 30 mM MgSO_4 , and 1.5 mM NaHCO_3 . For *G. lemaneiformis* and *G. tenuistipitata*, the same procedure was followed, but 0.01% agarase (Sigma) was also included in the digestion medium. After enzymatic digestion in medium 2, protoplast suspension plus debris and undigested tissue was filtered consecutively through a 100- μm - and a 45- μm -mesh filter. Mesh filters were rinsed with 10 mL of 0.2 M mannitol and 25 mM MES in sterile filtered seawater, pH 6.5 (medium 3). The protoplast suspension was centrifuged ($100 \times g$) for 5 min and resuspended in the same fresh medium (medium 3). They were further purified by centrifugation for 15 min in a three-phase density gradient at low speed ($50 \times g$). Protoplasts suspended in medium 3 (phase 1) were laid on top of 2 mL of modified medium 3 containing 0.4 M mannitol (phase 2), placed over another 2 mL of modified medium 3 containing 1.2 M mannitol and 50% percoll (phase 3). Finally, protoplasts were recovered with a Pas-

teur pipette and resuspended in medium 3. The method used was basically a combination of the enzymatic digestion procedure of Saga (1984) and the purification protocol of Björk et al. (1990). After isolation, a sample (0.1 mL) of the protoplast preparation was stained with 5 μL of a calcofluor-white stock (0.1% w/v) and observed by fluorescence microscopy for cell wall remains.

Protoplast yield, cytometric measurements, and microscopy. Subsamples of the protoplast suspension were withdrawn at the end of the purification procedure and diluted. Protoplasts were counted using a haemocytometer (Neubauer). An electronic counter (Coulter multisizer II) was used when the determination of protoplast size was also required. Image analysis (BIDS-IV) of fresh, unstained optical microscopy preparations was also used to measure protoplast size. Protoplasts were suspended in medium 3 in all cases.

Optical bright-field microscopy was performed by means of a Nikon Diaphot inverted microscope and a Leitz Dialux 20 microscope. The Leitz microscope was equipped with a light source for fluorescence (Ploemopack). Different filter blocks were used to produce the light quality required for excitation. Filter block A permitted excitation with ultraviolet light (340–380 nm) used for revealing calcofluor-white staining; filter block I2 allowed excitation with blue light (450–490 nm) for autofluorescence. The system was completed with a Leica Wild MP552 photographic camera controlled from a Crison Leucoform unit.

Flow cytometric analysis of isolated protoplasts. Protoplasts were studied with a FACScan FC (Becton Dickinson). The FACScan FC allows five parameters to be measured over time for every particle crossing its laser beam (15 mW argon laser tuned to 488 nm). For each protoplast it can determine forward angle light scatter (FSC), which is strongly correlated with particle size; 90° light scatter, which is strongly dependent on particle refractivity index; green fluorescence collected through a 530-nm filter, with a bandwidth of 30 nm (FL1); orange fluorescence collected through a 585-nm filter, with a bandwidth of 45 nm (FL2); and red fluorescence collected over 607 nm (FL3). The appearance of any sample on the computer screen is highly dependent on the particular photomultiplier gains used. In order to reproduce the results shown in this paper, or as a starting point for anyone interested in the FCM of plant protoplasts, it may be helpful for Becton Dickinson users to know the following information: Acquisition was made using pulse height and log mode for all variables. Gains were settled as follow: FSC = E-01, SSC = 271, FL1 = 450, FL2 = 450, FL3 = 300. FL3 was used as a threshold variable but settled at its minimum level; this ensured that only particles with a certain amount of autofluorescence were measured by the machine's electronics. The program LYSIS from Becton Dickinson was used to collect and analyze these signals. Protoplasts were suspended in medium 3. Natural seawater filtered through a 0.7- μm filter (Whatman GF/F) was used as sheath fluid. Protoplast size was calculated from FSC by means of the following equation: $\log \text{volume } (\mu\text{m}^3) = 0.74 + 0.006(\text{channel number})$, $r = 0.99$ (Jiménez-Gómez 1995). Calibration beads of known sizes (Polymer latex, Coulter) analyzed with the same settings and gains as used in this study were employed to obtain the previous equation.

Viability and fluorescein diacetate hydrolysis. Besides microscopic observation of membrane integrity and spherical appearance, protoplast viability was tested by means of fluorescein accumulation after FDA uptake into the protoplasts. Time course of FDA breakdown inside the protoplasts was followed by FCM using the FACScan FC previously described. Fluorescein diacetate (Sigma) was used as described by Watson et al. (1990). A first FDA stock solution (12 mM) was prepared in 1 mL spectrograde acetone. This solution was stored in the dark at -10°C. A second stock solution (4.8 μM) was prepared by dissolving 10 μL of the 12-mM stock solution in 25 mL of sterile filtered seawater. From this second stock, 0.6 mL was added to the same volume of sample and immediately passed through the FC. The FL1 from intra-

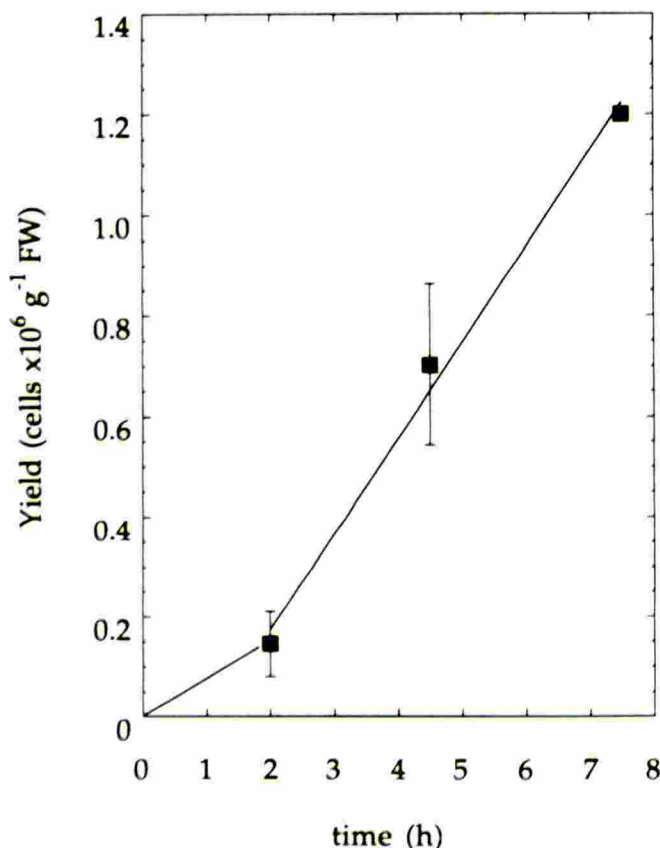


FIG. 1. Protoplast yield from *Ulva rigida* incubated for different periods of time in the digestion medium (medium 2). Standard deviations shown as bars when larger than symbol sizes.

cellular fluorescein was collected and analyzed using the CHRONYS software from Becton Dickinson.

RESULTS AND DISCUSSION

Protoplast yield. Yield of protoplasts isolated from *U. rigida* was considerably dependent upon the conditions of the starting plant material. A number of factors (i.e. low growth rate, senescence, etc.) have been shown to affect protoplast yield, although the specific manner in which they alter yield is poorly understood. Yields for a 7-h incubation period ranged from 1.1×10^6 to 8.6×10^6 protoplasts \cdot g $^{-1}$ fresh weight ($n = 6$). Yield was obviously dependent on the incubation time in the presence of the enzymes. After 2 h in medium 2, yield increased linearly with time (Fig. 1). Usually a balance must be achieved between optimizing yield by increasing incubation time and the physiological performance of the isolated protoplasts. Protoplast preparations from *U. rigida* obtained after 7 h of incubation in the digestion medium were photosynthetically competent (apparent photosynthesis rate = $300 \mu\text{mol O}_2 \cdot \text{g}^{-1} \text{Chl } a \cdot \text{min}^{-1}$), and surprisingly they displayed rates of nitrate reductase activity 5–10 times as high as the untreated blade (results not shown). They were also able to perform cellular division (Fig. 2B). Protoplasts isolated with the method proposed

in this study were naked cells. No cell wall remains were observed when stained with calcofluor-white, which is a specific stain for cellulose. However, after 24 h, protoplasts displayed fluorescence in the presence of calcofluor-white, and after 48 h they were not noticeably different in fluorescence intensity from 9-day-old cells (results not shown). The same isolation procedure, but with the inclusion of 0.01% agarase and an incubation time of 4.5 h, gave very good results (in the range of 10^6 to 10^7 protoplasts \cdot g $^{-1}$ fresh weight) with two red algae, *G. lemaneiformis* and *G. tenuistipitata*. Those protoplasts were also naked cells, as revealed by their exclusion of the calcofluor-white staining (Fig. 3F).

Protoplast size. A single cell type was apparent in the protoplasts isolated from *Ulva* as expected from its thallus with no cell differentiation. Size distributions obtained either with image analysis of optical microscopy preparations or by an electronic particle-counter fitted significantly ($\alpha = 0.01$) to a Gaussian curve (Fig. 4). Both techniques agreed well. The diameter of *Ulva* protoplasts was about $20 \mu\text{m}$ (see Table 1 for details). Strictly speaking, Coulter Counter electronics detects the volume of a particle and provides its diameter after considering the particle to be a perfect sphere. However, microscopical observation clearly revealed that, frequently, *Ulva* protoplasts were not totally spherical (Fig. 2A, B). The average major axis of protoplasts was statistically different from the average minor axis ($\alpha = 0.01$, $n = 159$, paired-sample *t*-test). Flow cytometric analysis of protoplasts can be used to assess cell size. However, to express size in conventional units (e.g. μm) a linear correlation must be obtained between the forward scatter signal and the particle size in μm . Calculations, according to the regression equation obtained by Jiménez-Gómez (1995), gave a mean cell diameter of $18.04 \mu\text{m}$ for *U. rigida* protoplasts, in agreement with image analysis and Coulter Counter measurements (Table 1). Protoplasts from *G. lemaneiformis* were smaller than those from *U. rigida*. They appeared as "perfect" spheres in optical microscopy (Fig. 3A–F) with a mean diameter of $9.6 \mu\text{m}$. Protoplasts from *G. tenuistipitata* (Fig. 3G, H) were even smaller (mean diameter = $3.6 \mu\text{m}$). Protoplasts from both species of red seaweed showed a wider size distribution than did *U. rigida* protoplasts.

Autofluorescence signature by flow cytometry. Analysis of *U. rigida*, *G. lemaneiformis*, and *G. tenuistipitata* protoplasts by flow cytometry clearly revealed their differences both in cell size and in pigment composition (Fig. 5). Ironically, one problem with this technique is the large amount of information it can produce. Most FCs on the market measure at least six variables for each cell. Dealing with such a large amount of information is not an easy task, and therefore it is useful to restrict the potential information to that which is essential. From our own experience with both natural phytoplankton communities (Corzo et al., unpubl.) and plant protoplasts, the set of



FIG. 2. Protoplasts from *Ulva rigida* A) immediately after isolation and B) 1 day after isolation showing one of them in a stage of cell division. Bright field. Scale bar = 20 μm .

variables which best discriminate among the autofluorescence characteristics of the different types of plant cells were FSC, FL2, and FL3. They were particularly useful when combined in the bivariate plots FL3 vs. FSC and FL3 vs. FL2 (Fig. 5). Red autofluorescence, mainly due to chlorophylls, was more intense in *U. rigida* than in *G. lemaneiformis* and *G. tenuistipitata* protoplasts on a cell basis. However, since *U. rigida* protoplasts had a cell volume about 6 times as large as *G. lemaneiformis*, the fluorescence signal must be related to protoplast volume to make comparisons of the autofluorescence characteristics of different cell sizes possible and meaningful. Strictly speaking, this calculation should be done for each cell. Available software did not permit such a simple calculation for each single event, and we resorted to the use of the mean values of the FL3 and FL2 frequency distributions for red and orange autofluorescence, respectively. Red autofluorescence was higher in *U. rigida* protoplasts than in *G. lemaneiformis* protoplasts even on a cell-volume basis (Table 2). In contrast, orange autofluorescence, mainly due to phycobiliproteins in red algae, was considerably

more intense in *G. lemaneiformis* protoplasts, as expected. $\text{Log FL2} \cdot \mu\text{m}^{-3}$ was about 60 times as high in *G. lemaneiformis* as in *U. rigida* (Table 2). The same calculation for *G. tenuistipitata* gave very different results. Red and orange fluorescence on a cell-volume basis were one order and two orders of magnitude higher than for *U. rigida* and *G. lemaneiformis*. Mean size and mean fluorescence values for both species of red seaweeds were less representative than for *U. rigida*, since the ranges of both size and fluorescence distributions were wider. There were indications in both cases of the existence of overlapping protoplast subpopulations. Additionally, differences in the physiological state of the starting algal material could also be involved in the large differences observed in protoplast autofluorescence. Growth rate in *G. tenuistipitata* ($9\% \cdot \text{d}^{-1}$) was higher than in *G. lemaneiformis* ($5\% \cdot \text{d}^{-1}$). Flow cytometric characteristics of the protoplasts of both species were readily visible when observed by fluorescence microscopy (Fig. 3D, H).

Volume changes. Since protoplast preparations are never completely clean of debris, identifying which

TABLE 1. Biometry of *Ulva rigida*, *Gracilariopsis lemaneiformis*, and *Gracilaria tenuistipitata* protoplasts immediately after isolation. Three techniques were used: electronic counter (CC), image analysis of optical microscopy (BIDS-IV), and flow cytometry (FCM). No SD values are shown when data are direct calculations from measured or estimated diameters.

	<i>Ulva rigida</i>			<i>Gracilariopsis lemaneiformis</i>	<i>Gracilaria tenuistipitata</i>
	CC	BIDS-IV	FCM	FCM	FCM
Diameter or major axis (μm)	19.84 ± 3.92	19.82 ± 3.24	18.04 ± 6.32	9.6 ± 5.56	3.6 ± 2.86
Minor axis (μm)	—	17.19 ± 2.72	—	—	—
Area (μm^2)	1236.6	—	1022.4	289.5	40.7
Volume (μm^3)	4089.0	3276.5 ± 1537.6	3074.0	467.8	24.4

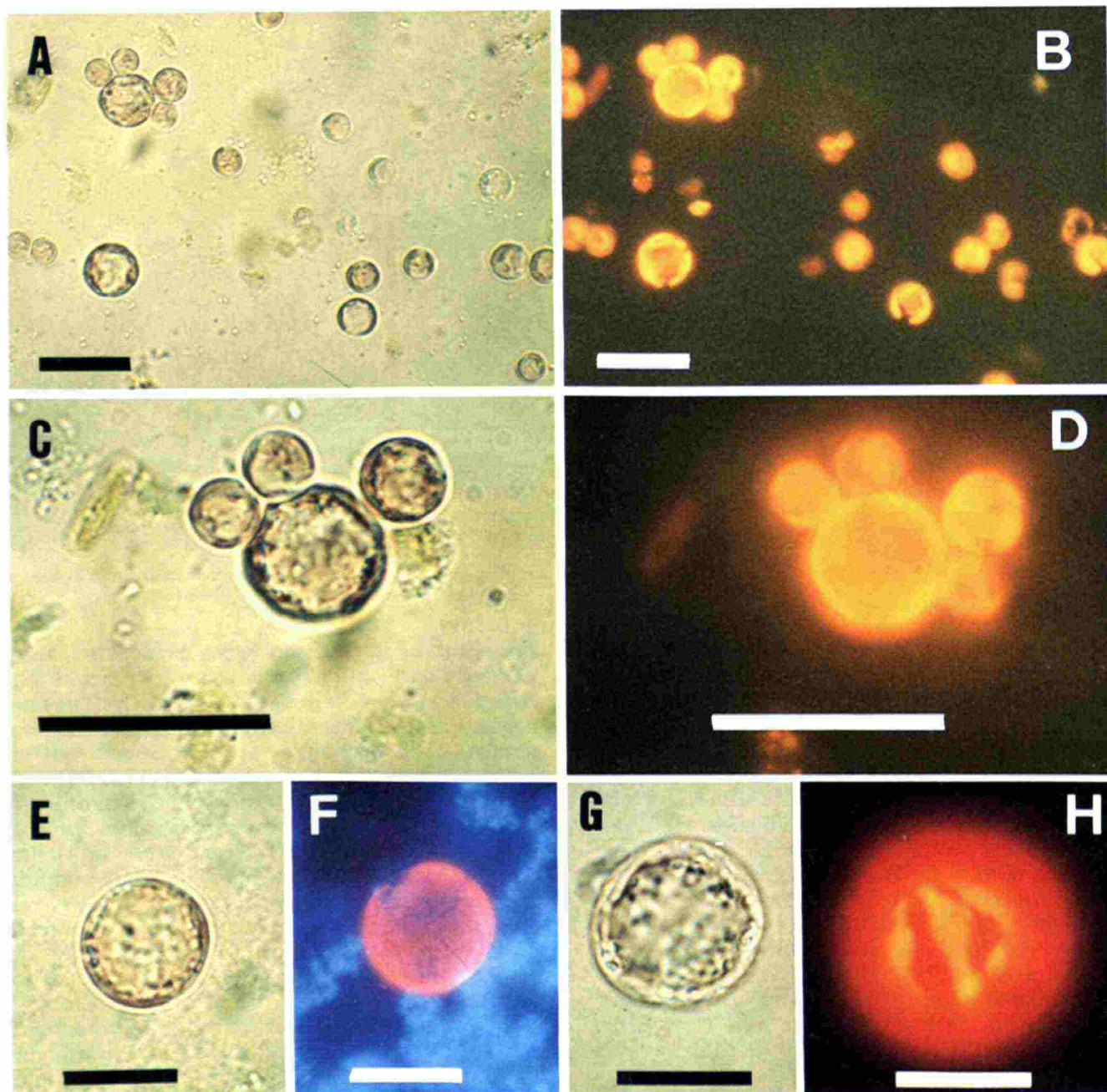


FIG. 3. Protoplasts immediately after isolation from (A–F) *Gracilariopsis lemaneiformis* and (G, H) *Gracilaria tenuistipitata*. A) *G. lemaneiformis* protoplasts in bright field; scale bar = 20 μm ; and B) autofluorescence from the same field. C, D) Partial enlargement from the previous field; scale bar = 20 μm of bright field and its autofluorescence, respectively. E) Single *G. lemaneiformis* protoplast stained with calcofluor-white in bright field, scale bar = 10 μm and F) in dark field. G) Single *G. tenuistipitata* protoplast in bright field, scale bar = 10 μm and H) autofluorescence from the same preparation.

of the dot populations on the computer screen corresponds to the protoplast population is not an easy task when analyzing a sample the first time (Fig. 6). The best check is to sort the population and observe them by microscopy. However, FCs with sorting capability are extremely expensive and not common. This makes it necessary to tackle the identification problem in a different way. Good agreement between microscopic techniques and FCM in protoplast counting is a useful criterion but not conclu-

sive. Calculation of size from the FSC signal may also help to rule out some dot populations. However, since new FCs have the capability of recording the time at which a particular event crosses the laser beam, we can make use of this feature to record virtually continuous changes in any of the other variables that the apparatus is able to measure. In the case of protoplasts, cell-volume changes induced by osmotic stress represent a simple way to confirm identification. We used *Dunaliella viridis* (Chloroph-

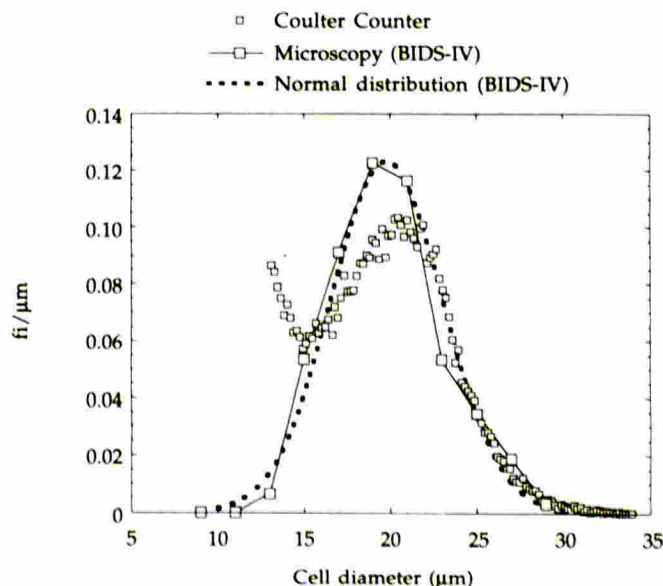


FIG. 4. Size distributions of *Ulva rigida* protoplasts suspended in medium 3. Relative frequency normalized by μm ($f_i \cdot \mu\text{m}^{-1}$) is represented against the major axis (optical microscopy) or against the diameter (Coulter Counter). Theoretical normal distribution calculated for microscopy data is also shown.

yta), an alga with a nonrigid cell wall, to illustrate cell volume regulation in response to instantaneous osmotic changes. Cell volume decreased very quickly in response to the new hypertonic conditions when NaCl in the medium was increased from 0.3 to 2.6 M (Fig. 7A). As expected, cell volume rapidly increased when the external medium was diluted (Fig. 7A). The responses of *U. rigida* protoplasts differed. As expected, protoplasts (region 1 in Fig. 6) burst

when their external medium was quickly diluted (confirmed by microscopic observation). The study of this process by FCM revealed that bursting was a rapid process, too rapid to be seen by FCM. There was a delay of about 5 s between any manipulation in the external medium and the moment in which the first cells exposed to that change reached the laser beam (this temporal delay might be reduced by shortening the tubing length). What the FC detected was an irreversible decrease in volume due to the release of the single *U. rigida* chloroplast (Fig. 7B). However, surprisingly, *U. rigida* protoplasts were insensitive to hypertonic changes, at least in the range studied here (Fig. 7C). The size changes in response to osmotic stress allowed us to distinguish protoplasts from debris since the latter (events located in regions 2 and 3 in Fig. 6) did not show any alteration in its FSC signal in the same experiments (results not shown).

Esterase activity. A different approach to distinguishing viable cells from debris and/or nonviable cells, also valid when a rigid cell wall exists, is the use of vital fluorescent dyes. Fluorescein diacetate (FDA) was initially used for testing viability in mammalian cells (Rotman and Papermaster 1966). Its use was extended to plant protoplasts (Larkin 1976) and phytoplankton (Bentley-Mowat 1982) later. Fluorescein diacetate is a hydrophobic, nonpolar, colorless, nonfluorescent compound with a molecular weight of 416. It penetrates the plasmalemma easily and once inside the cell, nonspecific esterases break down the FDA molecule to produce fluorescein, which is a fluorescent compound. Fluorescein, interestingly, is a polar compound and does not leak

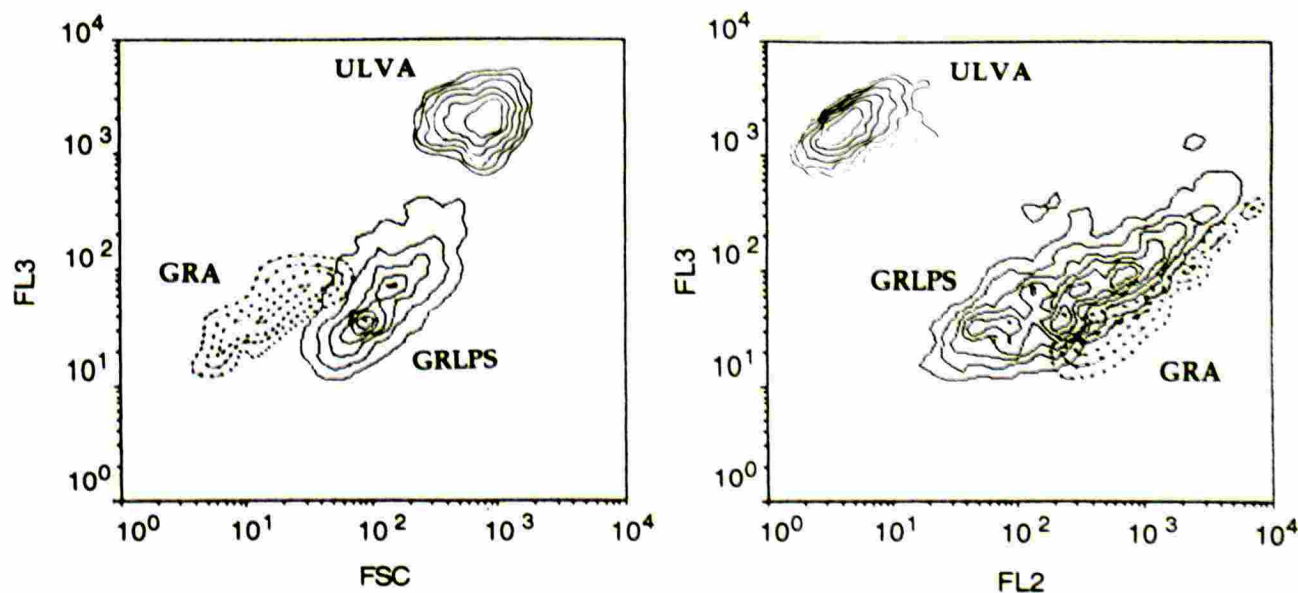


FIG. 5. Flow cytometry characterization of *U. rigida* (ULVA), *G. lemaneiformis* (GRLPS), and *G. tenuistipitata* (GRA) protoplasts. Protoplast populations are shown as density plots in (left) a red fluorescence (FL3) vs. forward angle light scatter (FSC) representation, and (right) a red fluorescence vs. orange fluorescence (FL2) representation. This figure was obtained by combining in the same plots the results of the analyses of typical samples from each of the species. Debris was not represented for clarity. Variables, in arbitrary units, are given in 4-decade logarithmic scales.

TABLE 2. Characteristics of the autofluorescence signals from *Ulva rigida*, *Gracilariopsis lemaneiformis*, and *Gracilaria tenuistipitata* protoplasts obtained by flow cytometry. Fluorescence is given in arbitrary units, in a 4-decade log scale, by the FACScan flow cytometer and divided by cell volume. Cell volume was obtained from the forward scatter FC signal. Data are means \pm SD; number of samples analyzed are in parentheses.

Species (no. of samples)	Red fluorescence (FL3) arbitrary units μm^{-3}	Orange fluorescence (FL2) arbitrary units μm^{-3}
<i>U. rigida</i> (7)	0.600 \pm 0.123	0.019 \pm 0.002
<i>G. lemaneiformis</i> (3)	0.182 \pm 0.048	1.135 \pm 0.196
<i>G. tenuistipitata</i> (3)	2.220 \pm 0.455	49.68 \pm 6.22

out of healthy cells easily. Esterases are common enzymes in both plant and animal cells; their natural substrates are organic esters, and their catalytic activities are essential to the phospholipid turnover in membranes. Rapid phospholipid turnover is correlated with high metabolic activity, and unspecific esterase activity has been shown to be a good index of "metabolic vigor" in both animal and plant cells (Rotman and Papermaster 1966, Larkin 1976, Bentley-Mowat 1982). Dorsey et al. (1989) found a good correlation between fluorescein accumulation rate and ^{14}C fixation rate in several species of marine microalgae. This clearly supports the use of the FDA breakdown by cells not only as a measurement of the specific esterase activity but more generally as a marker of healthy states. As a fluorescent probe, fluorescein is suited for FCM. The use of FDA in flow cytometric studies provides not just a qualitative indication of staining, as is the case with fluorescence microscopy, but a quantitative assessment of the internal esterase activity on a cell-by-cell basis.

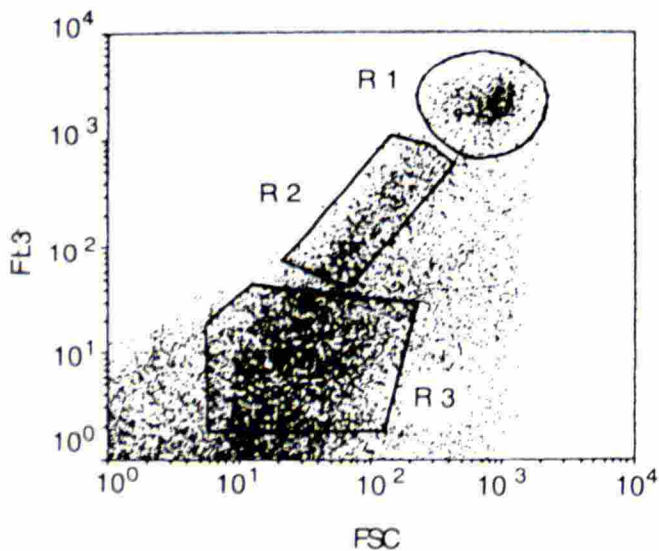


FIG. 6. Flow cytometric appearance in a dot-plot (FL3 vs. FSC) of a preparation of protoplasts from *Ulva rigida*. Protoplasts were located within region 1 (R1). Remaining events concentrated in two other main subpopulations (R2 and R3) and both of them were found to be debris. Variables, in arbitrary units, are given in 4-decade logarithmic scales.

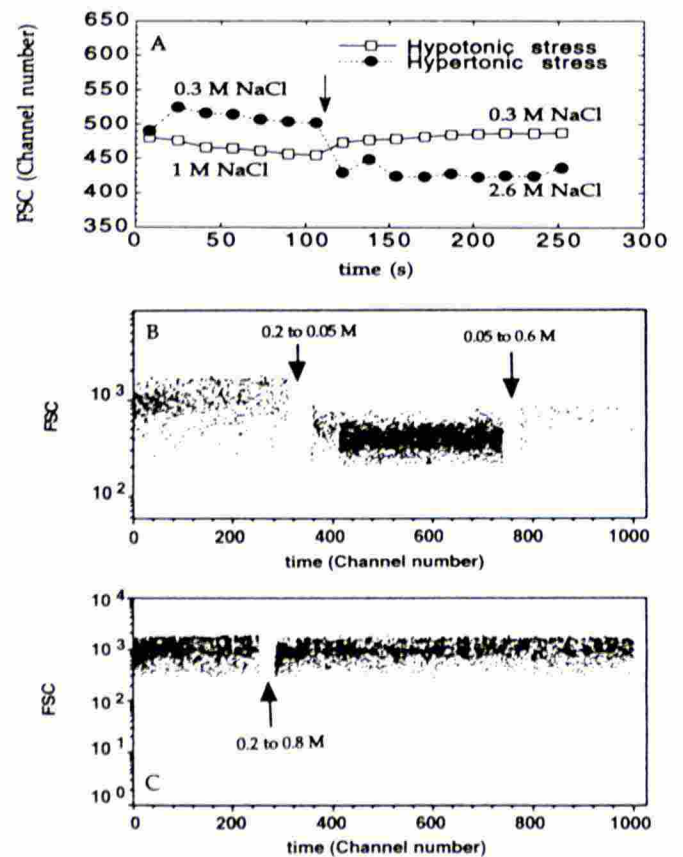


FIG. 7. Time course of forward angle light scatter (FSC) of A) *Dunaliella viridis* and B, C) *Ulva rigida* in response to osmotic changes. Instantaneous changes, indicated by the arrows, in NaCl (*D. viridis*) and D-mannitol (*U. rigida*) concentrations were used to alter osmotic pressure. Experiments shown in A were recorded using the CHRONYS software. This program samples at different time intervals to save memory and to provide an output consisting of the mean channel for the recorded variable for each sampling period (about 5000 cells were measured in every sampling period) and the central time in real units of every sampling interval. This easily permits the use of conventional graphic programs to plot results. Experiments displayed in B and C were recorded by means of the LISYS software, which works in a continuous mode. A certain length of time is required to alter the osmotic concentration. In that period of time the machine stops recording, resulting in empty zones on the plots. Time in this program is expressed as channel number in a scale of 1024 channels which corresponds to 512 s; therefore, transformation to real times (s) is channel number/2.

In an example of the development of fluorescence within *U. rigida* protoplasts as a consequence of FDA breakdown, data were expressed as the mean relative increase in fluorescence per cell at each time unit (Fig. 8). Mean relative fluorescence $\cdot \text{cell}^{-1}$ saturated with time according to a hyperbolic function. The semimaximal mean relative fluorescence $\cdot \text{cell}^{-1}$ was attained in 30.5 ± 9.5 s. This is a considerably low $t_{0.5}$ value compared with a range of 4–6 min obtained for several phytoplanktonic species using the same technique (Dorsey et al. 1989, Yentsch and Campbell 1991). This difference could be due both to the fact that protoplasts are naked cells and therefore fluorescein diacetate may enter the cell more

easily, and to interspecific differences. On the other hand, fluorescence from debris (regions 2 and 3, Fig. 6) in the same samples, after a small initial increase, remained constant throughout the experiments (Fig. 8).

Testing of cell metabolic activity with FDA in combination with FCM could be used as a rapid screening protocol to assess the effect of different isolation procedures or any other treatment on metabolic activity of protoplasts. This technique is not time consuming, and as FC equipment is becoming more accessible day by day, a great benefit could be obtained by generalizing its use. The fact that measurements are made on a cell-by-cell basis instead of bulk determination makes the selection of protoplast subpopulations that are resistant to any assay factor or treatment feasible. This selected subpopulation might be sorted and cultured later. In principle, one might think of the possibility of sorting a protoplast subpopulation in an amount large enough to allow physiological experimentation, as has been done with microalgae (Li 1994). However, our own experience with higher plant protoplasts (*Zea mays* root protoplasts) is that cell-sorting is a very stressful protocol. Most protoplasts burst and in no way was it possible to recover a number great enough to perform any physiological experiment in which a bulk parameter had to be measured. Recent improvements in sorting technology, as well as liquid sorting, could improve the recovery rate.

CONCLUSIONS

Flow cytometry has a great potential in the study of plant protoplasts. Flow cytometers permit the measurement of at least five variables at the same time plus the time course for every single protoplast. Therefore, a rather complete description of protoplast features can be obtained. The estimation of protoplast size of *U. rigida* from the FSC signal agreed convincingly with that of other techniques. Although flow cytometric determinations of size are less accurate than those obtained either with image analysis of microscopic preparations or with electronic counters, they suffice for most applications. The additional advantage of FCM is that it provides the autofluorescent characteristics of cell being analyzed. In addition, since the analyses by FCM of similar samples are highly repetitive, the location in the bivariate plots of each protoplast population is a consistent first clue for identification of cells from debris in protoplast preparations from any one of the species used here, and probably from related species as well (Figs. 5, 6). The autofluorescent signature of protoplasts provided by FCM clearly discriminates among the three species used in this study, even though *G. lemaneiformis* and *G. tenuistipitata* are taxonomically rather close (Fredericq and Hommersand 1989, Goff et al. 1994). Nevertheless, the ability to distinguish among different cell types and/

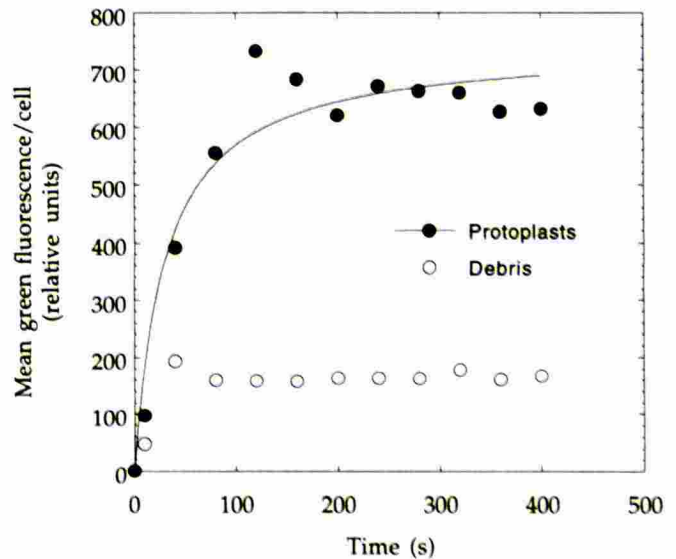


FIG. 8. Time course of fluorescein accumulation after FDA breakdown in isolated protoplasts (region 1 in Fig. 6) and debris (region 2 or 3 in Fig. 6) from *Ulva rigida*. Protoplasts were suspended in medium 3, and the experiment was started when FDA was added at a final concentration of $2.4 \mu\text{M}$.

or physiological states within the same species is more interesting than to discriminate between different species. Flow cytometric analysis of *U. rigida* protoplasts showed a single cell type, as expected from its thallus with no apparent cell differentiation. On the other hand, different cell types seem to exist and have been studied using microscopic observation in the two species of red seaweeds investigated in this study; however, surprisingly, the flow cytometric analysis did not show clear cell differentiation in either species. *Gracilariopsis lemaneiformis* and *G. tenuistipitata* displayed wider size distribution and broader orange and red fluorescence distributions than with *U. rigida* (Fig. 5). This may be an indication of a certain degree of cell differentiation, although a continuous range in cell size and fluorescence seems to exist. An alternative explanation for the apparent inability of FCM to discriminate different cell types in *G. lemaneiformis* and *G. tenuistipitata* could be that the protoplast isolation procedure might have led to the isolation of just one cell type. In fact, we did not observe protoplasts from medullary cells. Coury et al. (1993) reported the discrimination of two protoplast types by FCM in the red alga *Gelidium robustum*, although they did not mention which technique was used to confirm identification on the computer screen. This is, however, an important problem which needs to be solved, because debris may easily be mistaken for a viable cell population. A completely unambiguous confirmation can only be obtained by cell-sorting. Nevertheless, since this capability is restricted to the higher quality and, therefore, higher cost FCs, a need exists to develop alternative strategies to identify which of the dot populations (on the computer screen) correspond to the

protoplast population (in the sample). We propose two criteria here: 1) Counts by FCM and alternative techniques should agree and 2) simple physiological experiments to which debris should not respond should be conducted.

Using the second approach we have shown that a hypotonic shock is the simplest way to discriminate protoplasts from debris, since the protoplasts burst and this can be recorded by the FSC signal. However, the osmotic shock cannot be applied to a cell with a rigid cell wall. In this case, a simple alternative is to record the intracellular esterase activity by FDA hydrolysis, which generates a green fluorescence signal. Studying the time course of intracellular FDA breakdown through flow cytometry, apart from constituting an identification criterion for viable cells, provides a quantitative measure of "metabolic vigor," which may be used to survey the effects of isolation protocols and/or any other factor on protoplast viability.

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