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Development of a rapid and effective method for preparing delicate dinoflagellates for scanning electron microscopy

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Abstract We developed a rapid and effective procedure for scanning electron microscopy of three delicate dinoflagellates, *Karlodinium micrum*, *Akashiwo sanguinea*, and *Heterocapsa niei*. Good results were obtained when the specimens were fixed with a modified Párducz's fixative (2% osmium tetroxide:saturated mercuric chloride = 5:1 *v*/*v*) for 10 min, washed in 0.05 M sodium cacodylate trihydrate buffer for 2 min, dehydrated in *tert*-butanol for 10 min and dried with hexamethyldisilazane in air for 3 min in a fume hood because reagents are very toxic. This method could be completed in 25 min. Compared with other preparative techniques, the present protocol has significant advantages for SEM observation by limiting distortion of delicate specimens and reducing the preparation time.

Keywords Delicate dinoflagellates · Hexamethyldisilazane · Párducz fixative · Sodium cacodylate buffer · *tert*-Butanol

Introduction

Free-living dinoflagellates are a very old and successful haplontic group of eukaryotic microorganisms adapted to a variety of pelagic and benthic habitats from arctic to tropical seas and estuaries as well as fresh to hypersaline waters (van den Hoek et al. 1995). Dinoflagellates were first described during the late 1800s, based on light microscope observations. However, morphological identification characteristics (i.e., cingular position, sulcal placement, apical groove, and peducle) are difficult to resolve by light microscopy. For accurate identification the use of scanning electron microscopy (SEM) is needed (Steidinger et al. 1996a, b). Moreover, unarmored dinoflagellates lack thick cellulosic plates in the thecal vesicles, and cannot adequately be preserved for SEM studies using a general preparation procedure involving pre- and post-fixation, and critical point drying (CPD; Smith and Finke 1972).

The general preparation procedure for SEM can frequently result in distortion of the cell wall during fixation and drying procedures. To overcome these problems, alternative preparative techniques have been developed such as freezing methods (Huang et al. 1994; Robards and Sleytr 1985; Sargent 1988; Suzuki et al. 1995). A chemical preparation method using new fixing and drying reagents has reduced surface tension during evaporating, and has been effectively used to dry soft materials including delicate microalgae, insect tissues, rat hepatic endothelial cells, and the cilia of rat trachea (e.g., Botes et al. 2002; Braet et al. 1997; Bray et al. 1993; Nation 1983). These methods have the advantage of simplicity, limited equipment requirement and are low cost compared with freezing methods. However, further improvements are needed to enhance the maintenance of original cell morphology and save preparation time. Therefore, the present study aimed to develop a more rapid, simple, and effective technique for preparing samples for SEM.

Materials and methods

Delicate dinoflagellates including the unarmored *Karlodinium micrum* and *Akashiwo sanguinea* and the armored *Heterocapsa niei* were isolated from coastal waters of

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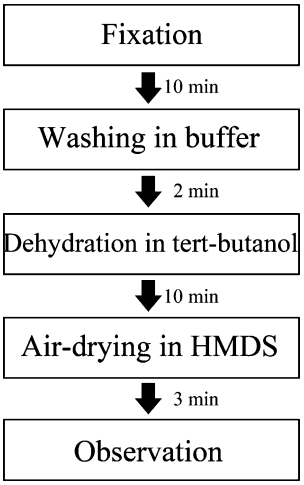

Fig. 1 Schematic diagram of a preparing procedure for SEM with those suggested in this study

South Korea using a capillary method. The organisms were incubated in 300-mL flasks containing 100 mL of f/2 medium (Guillard and Ryther 1962) with shaking (30 rpm) at 25°C and 40 μmol photons $m^{-2}s^{-1}$ under 12:12 light: dark cycles. Cells were grown in log phase to a density greater than 10^3 cells mL^{-1}.

The procedure developed is shown in Fig. 1. All reagents were carefully used in a fume hood because the reagents are hazardous. Live cells were fixed with modified Párducz's fixative (Párducz 1967) at 1:1 *v*/*v* for 10 min at room temperature: the fixative comprised 2% solution of osmium tetroxide (75632, Sigma) in filtered seawater and a saturated solution of mercuric chloride (M1136, Sigma) in distilled water mixed in the ratio of 5:1 *v*/*v*, respectively. Fixed cells were harvested by gravity filtration) onto a 2.0 μm polycarbonate membrane (TTTP, Millipore). To prevent the formation of NaCl crystals, any seawater

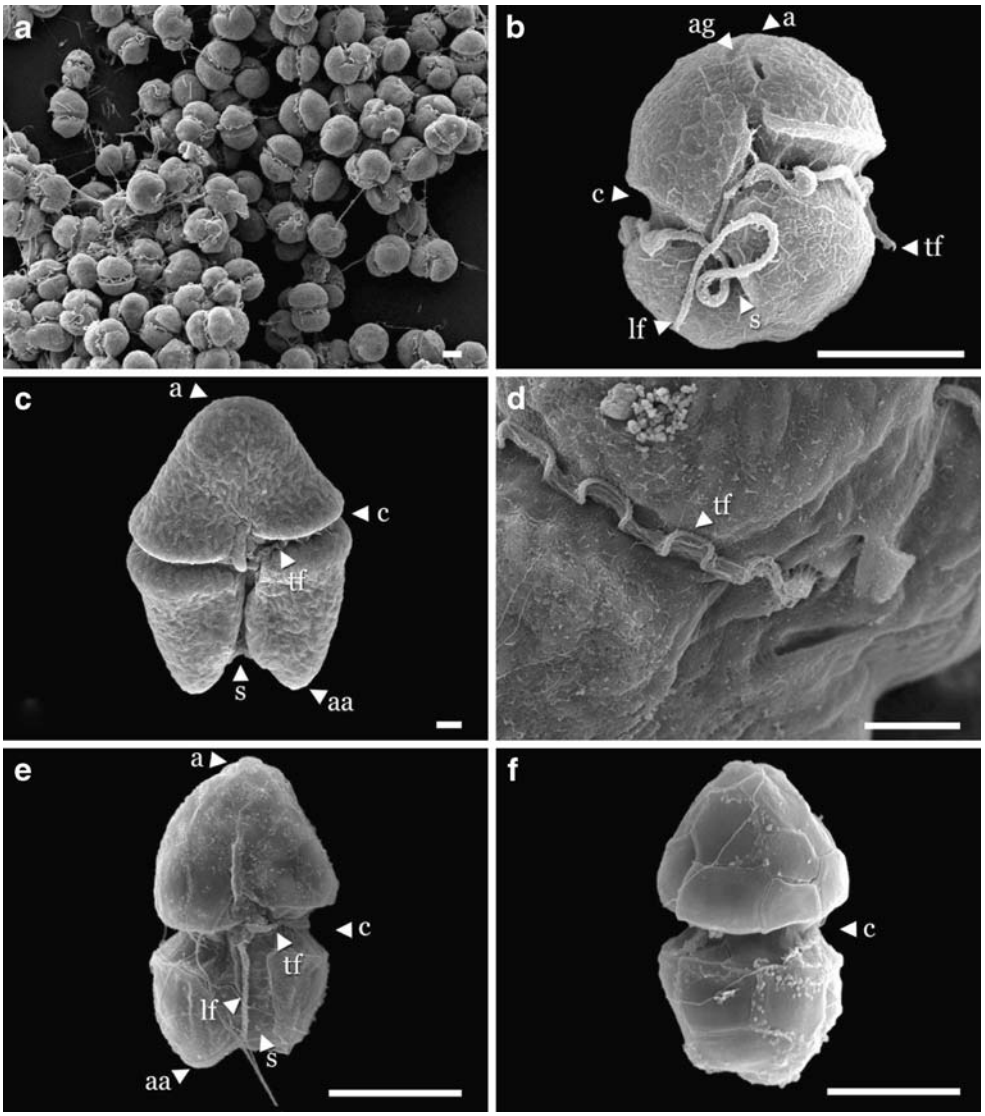

Fig. 2 SEM photographs of three delicate dinoflagellates fixed using the procedure described in this study. **a** and **b** label the taxonomically diagnostic ventral pore on *Karlodinium micrum* showing the apex (*a*), apical groove (*ag*), cingulum (*c*), longitudinal flagellum (*lf*), sulcus (*s*) and transverse flagellum (*tf*). **c**, **d** *Akashiwo sanguinea* showing the apex, antapex, cingulum, sulcus and transverse flagellum. **e** and **f** Label plates on *Heterocapsa niei* showing the apex, antapex, cingulum, longitudinal flagellum, sulcus, and transverse flagellum. *Scale bars* are 5 μm

Table 1 Comparison of preparation procedures and times for scanning electron microscopy

Specimen	Fixative	Washing	Adhesive reagent	Dehydration	Drying reagent	Drying devise	Procedure time	Reference
Gymnodinioid (dinoflagellate)	Párducz[a] (10 min)	D.W[b] and SCD[c] (2 min)	None	TBA[d] (10 min)	HMDS[e] (3 min)	None	Aprox. 25 min	This study
Gymnodinioid (dinoflagellate)	2% OsO_4 (1 h)	D.W (20 min)	Poly-L-lysine (30 min)	Et-OH series (1.5 h)	HMDS (5 min)	None	Aprox. 3 h and 30 min	Botes et al. 2002
Endothelial cells (rat)	2% GLU[f] (12 h) and 1% OsO_4 (1 h)	SCD (1 h)	Collagen (8 hs)	Et-OH series (1 h)	HMDS (3 min)	None	Aprox. 23 h and 30 min	Braet et al. 1997
Nocardia asiatica (actinomycetes)	2% OsO_4 (24 h)	None	None	Et-OH series (1 h)	TBA	None	Aprox. 25 h	Kageyama et al. 2004
Heterocapsa sp. (dinoflagellate)	1% OsO_4 (10 min)	D.W	None	Et-OH series (2 h)	Liquid CO_2 (30 min)	CPD[g]	Aprox. 2 h and 40 min	Iwataki et al. 2003
Karlodinium sp. (dinoflagellate)	4% OsO_4 (1 h)	D.W	None	Me-OH series (2 h)	Liquid CO_2 (30 min)	CPD	Aprox. 3 h and 30 min	de Salas et al. 2008
Karlodinium sp. (dinoflagellate)	2% OsO_4+$HgCl_2$ (2 h)	D.W	None	Et-OH series (3 h)	Liquid CO_2 (30 min)	CPD	Aprox. 5 h	Bergholtz et al. 2005
Gymnodinioid (dinoflagellate)	9.4% GLU+2% OsO_4 (30 min)	SCD	None	Et-OH series (2 h)	Liquid CO_2 (30 min)	CPD	Aprox. 3 h	Steidinger et al. 1996a, b
Tetrahymena sp. (ciliate)	Párducz (1 min)	D.W (1 min)	Poly-lysine hydrobromide	TBA (30 min)	Liquid nitrogen (1 min)	LVC[h] (2 h)	Aprox. 2 h and 30 min	Takahashi et al. 2004
Streptococcus crista (bacteria)	2.4% GLU (2 h)	None	None	Et-OH series (1 h)	Liquid CO_2 (30 min)	CPD	Aprox. 3 h and 45 min	Sutton et al. 1994
Acartia sp. (zooplankton)	3% GLU (4)	D.W (5 min)	None	None	Liquid nitrogen (10 sec)	LVC (20 min)	Aprox. 4 h and 25 min	Suzuki et al. 1995
Meloidogyne incognita (nematode)	2 % GLU—1% FOR[i] and 2% OsO_4 (30 h)	None	None	Et-OH series (1 h)	Liquid CO_2 (30 min)	CPD	Aprox. 30 h and 30 min	Eisenback 1986.

[a] Párducz fixative

[b] Distilled water

[c] Sodium cacodylate buffer

[d] *tert*-Butanol

[e] Hexamethyldisilazane

[f] Glutaraldehyde

[g] Critical point dryer

[h] Low vacuum chamber

[i] Formaldehyde

remaining associated with the specimen was removed by washing for 2 min at room temperature with drops of distilled water followed by drops of 0.05 M sodium cacodylate trihydrate buffer (pH 8.0). For dehydration, drops of *tert*-butanol were continuously dripped on the specimen for 10 min at 30°C. Following this procedure, several drops of hexamethyldisilazane (H4875, Sigma) were immediately dispensed onto the membrane to complete the drying process. Finally, the specimens were coated with gold–palladium for 3 min, and examined using a SEM (JSM-5600 LV; Jeol). The entire fixation procedure could be completed in 25 min.

Results and discussion

SEM images of the three delicate dinoflagellate species are shown in Fig. 2. Cells of *K. micrum* were well preserved with very little morphological distortion (Fig. 2a); key identification characteristics of this species were clearly evident, including the apex, apical groove, longitudinal flagellum, transverse flagellum, and sulcus (Fig. 2b). A cell of *A. sanguinea* had a pentagonal shape with a broadly conical epitheca and a bilobed hypotheca (Fig. 2c), and a transverse flagellum in a transverse cingulum (Fig. 2d). *H. niei*, a delicate armored dinoflagellate, is shown in ventral and dorsal views in Fig. 2e and f, respectively. The cell had almost equal-sized rounded to conical epitheca and rounded to attenuated hypotheca, and was slightly compressed dorsoventrally; thin thecal plates and triangular-shaped scales were also evident.

When comparing the efficacy of the fixatives Párducz and OsO_4 using the same fixation time (10 min), the Párducz fixative caused little distortion of a cell wall of *K. micrum*, while the OsO_4 caused cell wall damage of the species examined (figure not shown). As a primary fixative, OsO_4 causes gross permeabilization of the membrane, with cessation of cytoplasmic movement occurring within seconds to minutes, but this is one of the slowest penetrating fixatives (Dykstra and Reuss 2003). To overcome this problem, Párducz (1967) added $HgCl_2$ to OsO_4 as a fixative, to help reduce the fixation time as well as to increase cell hardness. Takahashi et al. (2004), for example, obtained good results after applying this fixative for approximately 1 min (Table 1).

tert-Butanol can effectively remove water in a cell and reduce the dehydration time. Although ethanol continues to have the widest application as a dehydrating agent, its use does not completely remove water from the cell (Luft and Wood 1963). Moreover, cell shrinkage is frequently associated with ethanol use, and the dehydration time may exceed 1 h (Hanstede and Gerrits 1983). The use of *tert*-butanol has been introduced in freeze drying because of its high freezing point (25.5°C) and vapor pressure (Inoue and Osatake 1987), and it has been recommended as a more effective dehydration agent than ethanol (Handa et al. 1998; Suzuki et al. 1995).

Drying using CPD has been the general approach to biological SEM since its inception and, despite advances in cryopreservation techniques (e.g., Craig and Beaton 1996), continues to dominate the field (Dykstra and Reuss 2003). However, this procedure has the disadvantages of being time-consuming and causing cell shrinkage. HMDS can reduce the collapse and distortion of delicate specimens during drying. The reagent cross-links proteins, reducing the surface tension and giving strength to the sample during air-drying (Nation 1983). In contrast, CPD causes shrinkage due to vigorous solvent exchange with the cells, and temperature and pressure changes (Boyde 1980). The use of HMDS for SEM observations of delicate specimen produced excellent results.

Compared with other preparative techniques (Table 1), the present method has a number of advantages.

1. It is rapid and effective. Our procedure minimized preparation time (approximately 25 min) and cell distortion. To our knowledge, the present study may represent the shortest time consumed for preparing SEM.
2. Air-drying involves HMDS, which reduces the shrinkage of delicate specimens
3. The use of *tert*-butanol is effective in removing water in a cell as well as decreasing the dehydration time.
4. Párducz's fixative can enhance rapidly the rigidness of a soft cell wall.

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