New insights on *Laminaria digitata* ultrastructure through combined conventional chemical fixation and cryofixation

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Running Title

Laminaria ultrastructure

Abstract

The objective of the present study is to examine the fine structure of vegetative cells of *Laminaria digitata* using both chemical fixation and cryofixation. *Laminaria digitata* was chosen due to its importance as a model organism in a wide range of biological studies, as a keystone species on rocky shores of the North Atlantic, its use of iodide as a unique inorganic antioxidant, and its significance as a raw material for the production of alginate. Details of the fine structural features of vegetative cells are described, with particular emphasis on the differences between the two methods used, i.e. conventional chemical fixation and freeze-fixation. The general structure of the cells was similar to that already described, with minor differences between the different cell types. An intense activity of the Golgi system was found associated with the thick external cell wall, with large dictyosomes from which numerous vesicles and cisternae are released. An interesting type of cisternae was found in the cryofixed material, which was not visible with the chemical fixation. These are elongated structures, in sections appearing tubule-like, close to the external cell wall or to young internal walls. An increased number of these structures was observed near the plasmodesmata of the pit fields. They are similar to the “flat cisternae” found associated with the forming cytokinetic diaphragm of brown algae. Their possible role is discussed. The new findings of this work underline the importance of such combined studies which reveal new data not known until now using the old conventional methods. The main conclusion of the present study is that cryofixation is the method of choice for studying *Laminaria* cytology by transmission electron microscopy.

Key words

Flat cisternae - *Laminaria* - Ochrophyta - Phaeophyceae - TEM

Abbreviations

TEM – transmission electron microscopy; CCF – Conventional Chemical Fixation; CF-FS – Cryofixation - Freeze substitution

Dedication

This paper is dedicated to Susan Loiseaux-de Goër in Roscoff, one of the pioneers of brown algal cell biology and the techniques in electron microscopy for its study.

Introduction

Brown algae (Phaeophyceae) belong to a lineage that has evolved independently of other major photosynthetic lineages, such as green algae (Chlorophyta) and red algae (Rhodophyta) (Bringloe et al. 2020). Instead, they are classified within the Stramenopiles and Chromalveolates together with Bacillariophyceae, Chrysophyceae and Oomycota (Baldauf 2008, Baldauf 2003). They also represent one of the few eukaryotic lineages that have developed multicellularity. As a consequence of this singular evolutionary history, brown algae exhibit many unusual, and often unique, features. These features are adaptations to the potentially harsh marine coastal environments in which brown algae are often the dominant organisms in terms of biomass (Bartsch et al. 2008, Küpper and Kamenos 2018). The key role of kelp forests, effectively constituting an interface between the ocean, the atmosphere and land masses, in the biogeochemical cycle of halogens is well established (Küpper et al. 2008, Küpper et al. 2011). Their role in marine benthic carbon sequestration is the subject of ongoing research (Krause-Jensen et al. 2018) and there is concern about their regression or changes in keystone composition and ecosystem functioning in the context of climate change (Küpper and Kamenos 2018) (Teagle and Smale 2018).

Since the introduction of electron microscopy for the study of cell structure, many papers have been published on the fine structure of brown algal cells. Among them, we should mention the pioneering publications of Manton (1957, 1959) and Bouck (1965). Regarding the order Laminariales, a number of TEM-based studies have explored the ultrastructure of chloroplasts, the cells of haptera, the adhesive “plaques”, as well as gametogenesis, zoosporogenesis, fertilization, and sperms, in different *Laminaria* species (Bercaloff 1961, Motomura and Sakai 1984, Davies et al. 1973, Oliveira et al. 1980, Henry and Cole 1982, Motomura 1989, Motomura 1990, Motomura 1993). Early reports on Laminarialean species have also studied the ultrastructure of sieve tubes, sieve plates, and plasmodesmata (Bisalputra 1966, Parker and Philpot 1961, van Went et al. 1973a, van Went et al. 1973b, van Went and Tammes 1973, van Went and Tammes 1972, Schmitz and Srivastava 1976, Schmitz and Srivastava 1975, Schmitz and Srivastava 1974, Ziegler and Ruck

1967). The localization of iodine and bromine in stipes of *Laminaria hyperborea*, *Saccharina latissima* and *L. digitata* was also reported by Pedersen and Roomans (Pedersen and Roomans 1983). More recently, Holzinger et al. (2011) studied the sensitivity of sporogenic and vegetative cells of *Saccharina latissima* to ultraviolet radiation.

The application of electron microscopy was a revolution for the study of biological materials. Since the time of the first electron microscope picture of eukaryotic cells by Porter et al. (1945), many papers have been published using the conventional chemical fixation. However, these methods have problems in the preservation of biological material. A continuous effort has been made since that time, for the improvement of the chemical fixation as well as for the development of alternative preservation techniques (Mielańczyk et al. 2015, Kuo 2014).

Polge et al. (1949) were the first to examine the preservation of biological material using low temperature. After that time many papers have been published analysing the details and the advantages of physical vs chemical fixation (Franks 1977, Robards and Sleytr 1985, Gilkey and Staehelin 1986, McDonald 1994, Giddings 2003). Cryo-specimen preparation followed by freeze-substitution (CF-FS) can minimize artefact formation which was caused by the CCF. In addition, a number of "new" structures have been found by this technique, which were not visible after CCF, like the "flat cisternae" found in brown algal cells ((Katsaros et al. 2009).

The application of CF-FS in large brown algae was relatively delayed due to the problem of sample size. That is why the first reports were on small-sized forms, like fucoid zygotes of *Phyllospora comosa* and *Hormosira banksii* (Schoenwaelder and Clayton 2000) and zygotes of *Scytosiphon lomentaria* (Nagasato and Motomura 2002) and *Silvetia babingtonii* (Nagasato et al. 2010). Later, ultrastructural studies using this method expanded to apical cells of *Halopteris congesta*, *Sphacelaria rigidula*, and *Dictyota dichotoma* (Terauchi et al. 2012, Katsaros et al. 2009); and plurilocular sporangia of *Ectocarpus siliculosus* (Fu et al. 2013). All the above studies were based on the plunging method of cryofixation, which gives good preservation up to a depth of 10-20

μm. A detailed review of this cryofixation technique in brown algae was provided by Nagasato et al. (2018). For larger samples, high-pressure cryofixation is necessary.

Laminaria digitata is a major kelp species on North Atlantic rocky shores, including Maine and the Canadian Maritimes, Newfoundland and the European Atlantic coast from Brittany (France) to Svalbard (Bartsch et al. 2008). It is the strongest accumulator of iodine among all living systems investigated so far (Küpper et al. 1998). It was recently discovered that this kelp accumulates iodide as a unique inorganic antioxidant in its apoplast in order to protect its surface against several aqueous and gaseous oxidants (Küpper et al. 2008). Upon reaction with ozone, volatile molecular iodine is released, resulting in aerosol formation and impacting atmospheric processes (Küpper et al. 2008, Palmer et al. 2005). The subject has been extensively reviewed (Küpper and Carrano 2019, Küpper et al. 2011, Küpper 2015, Küpper and Kroneck 2015).

A major open question in this context is the exact localisation of iodide in the cellular and tissue context – which has major implications for a better understanding of this unique antioxidant system, but also its mechanisms of uptake, storage and efflux. A recent study (Ender et al. 2019) employed nano-secondary ion mass spectrometry (nano-SIMS) for the ultrastructural localization of arsenic in *L. digitata*. However, for a better understanding of such mechanisms, good knowledge of the cytology of this important model organism is essential – which prompted us to conduct the present study.

Despite numerous publications on the fine structure of brown algae using conventional chemical fixation, studies on the cytology of *L. digitata* seem to be missing (also the review by Bartsch et al. 2008 does not list any), which is surprising given the ecological and economic importance of this species and also as a model organism.

Therefore, the aim of the present study was to conduct transmission electron microscopy (TEM) of both chemically fixed and cryofixed tissues of *L. digitata* from the coasts of northern Scotland and Brittany, in order to develop a suitable protocol for the study of kelp ultrastructure

which is useful for other studies as well. Particular attention was given to the differences found between the two methods used, i.e. Conventional Chemical Fixation (CCF) and the CF-FS – Cryofixation - Freeze substitution (CF-FS).

Materials and Methods

For all experiments, two different sporophytes of *Laminaria digitata* (Hudson) Lamouroux approx. 0.5 – 1 m in length were collected by snorkelling at Bullers of Buchan, Cruden Bay, Aberdeenshire, on November 10, 2019 and on February 18, 2020, and in the Chenal de l'Ile de Batz, Roscoff on October 28, 2019.

Conventional chemical fixation (Roscoff laboratory)

Small pieces (2-3 mm) of the three thallus areas, i.e. meristem, stipe and phylloids, were fixed in a 3% glutaraldehyde in 0.4 M cacodylate buffer + 10% NaCl, washed in cacodylate buffer and postfixed in 1% OsO_4 in the same buffer. Dehydration was conducted in a graded alcohol series (from 30% to 100%) and infiltration in alcohol/Spurr's solutions (with gradually increasing concentration of Spurr's). Samples were finally embedded in Spurr's resin (Delta microscopies, France; Spurr 1969). All chemicals were from Delta microscopies (22 bis, route de Saint-Ybars – 31190 Mauressac, France).

Cryofixation – Freeze substitution (Aberdeen laboratory)

High Pressure Freezing was carried out using a Leica EM PACT2 device (Leica Microsystems, Milton Keynes, UK). Since the main problem of cryofixation is the thickness of the samples, in order to make them thinner, we first cut small thallus pieces (about 1 x 1 mm), which were split into two halves parallel to the thallus surface. Samples were then transferred for freeze substitution

using the Leica AFS 2 with the freeze substitution used initially being a standard Leica protocol (Table 1). The time in the 2% osmium tetroxide (TAAB, UK; 2% mix prepared at the University of Aberdeen) / acetone mix was increased for the 2nd run with the 2nd step adjusted to 48 h (Nagasato et al. 2018). Subsequently, the following infiltration steps were carried out in a Pelco Biowave Pro+ (TED PELLA, Inc., Redding, California, USA) using both microwave and vacuum treatment. Samples were then removed and placed in 10% Spurr's (TAAB, UK): Acetone, then 30% Spurr's, then 50% Spurr's, then 70% Spurr's, then 90% Spurr's and finally embedded in Spurr's resin at 60°C for 24-48 h

Thin sections from both methods were cut using a diamond knife on a Leica ultracut UCT™ ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a JEOL JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan). Micrographs were taken using a Gatan Orius camera (Gatan, Pleasanton, California, USA).

Results

The method used for the CCF was successful and, despite the usually faced difficulties due to the large size of the thallus, gave clear images with well-preserved cells. The shape of the cells depends on their type, i.e. the epidermal cells, in transverse thallus sections, appear usually orthogonal, while the medullary ones are more rectangular (Fig. 1). In the meristematic region the cell shape was similar to non-meristematic phylloid tissue but the cells were smaller. The internal structure of the cells was found to be similar to that already described for other brown algae. A large nucleus, with variable shape, was usually at a central position in epidermal cells, while in medullary cells it was more peripherally located (Fig. 1). Large dictyosomes were observed in the perinuclear cytoplasm, occasionally associated with the nuclear envelope (Fig. 2). Active dictyosomes were also found in other areas, particularly in the peripheral cytoplasm (Fig. 7). Most of the cell space was covered by large vacuoles, sometimes containing masses of electron-dense

material (Fig. 1). The vacuole membrane in cells fixed with CCF shows an undulating shape (Fig. 1). Numerous chloroplasts and mitochondria were mainly located in the cortical cytoplasm (Figs 1, 3). The chloroplasts were large in relation to the cell size, mainly elongated (Fig. 3). Their internal membrane system consisted of the usual bundles of three thylakoids, and the nucleoid localized at the poles (genophores) (Figs 3, 4). Large pit-fields were observed in internal cell walls mainly in the medullary cells (Figs 7, 11). The external cell wall was thick, consisting of three layers, i.e. an internal one with parallel, hardly visible cellulose bundles, a thinner median layer with vesicle-like structure, and a thick amorphous external layer (Fig. 5). It was interesting that darkly stained elongated structures were observed attached to the plasmalemma of the external wall of epidermal cells. This was more obvious in cells which were partially plasmolysed (Figs 1, 5).

Examination of thin sections of material prepared with CF-FS revealed a general structure similar to the above described with CCF (Fig. 6, cf. with Fig. 1). However, a detailed analysis of the CF-FS samples revealed some interesting features, which were different or not found with the CCF, in particular:

1. The activity of the dictyosomes was clearer, since the released vesicles and cisternae were full of electron-dense material (Fig. 7).
2. The dark elongated structures associated with the plasmalemma of the external cell wall of epidermal cells were not found (Fig. 6, cf. with Fig. 1).
3. The structure of the pit-fields and their plasmodesmata was clearer compared to those of the CCF material (Figs. 7, 9).
4. A variable number of elongated membranous structures, resembling short detached ER or dictyosome cisternae was observed close to the wall of both epidermal and medullary cells (Figs. 7-11). These structures were either transparent, showing two parallel membranes, obviously cross sections of cisternae, or filled with electron-dense material (Figs 8-11). They are similar to the “flat cisternae” found associated with the developing cytokinetic diaphragm of a number of brown algae (Katsaros et al. 2009). The flat shape of these

cisternae was confirmed in areas where these structures were sectioned parallel to their surface, where the cisternae appear round (Fig. 11).

5. In some cases, these flat cisternae were found in parallel doublets, close to the plasmalemma of the external wall of epidermal cells (Fig. 10).
6. An increased number of flat cisternae was found close to the plasmodesmata of the pit-fields (Figs. 7, 11).
7. In areas where flat cisternae are present, invaginations of the plasmalemma were observed, sometimes associated with the flat cisternae (Figs. 12, 13).

Discussion

The use of the high-pressure CF-FS technique in the present study allowed us to examine the thick blades and stipe of *L. digitata* thallus with good results. The combination of the two methods, i.e. CCF and CF-FS, for the first time, was ideal to compare them, in order to examine: a) whether there were artefacts after the application of CCF, i.e. not real structures, e.g. like those found after permanganate fixation (Bradbury and Meek 1960 , Johnson 1966); and b) whether there are important structures which were not observed with the previously used methods of CCF.

As far as the first of the above questions is concerned, it can be underlined that after CCF the general structure of the cells was quite well preserved. However, after a detailed comparison of the two methods applied it was revealed that after CCF the cortical cells of the blades are locally plasmolysed, i.e. the external cell wall is detached from the plasmalemma. This is caused by the method applied here, since it is not observed after CF-FS.

Another interesting finding is the observation of electron-dense material attached to the cell wall of the plasmolysed areas. Of course it is not sure that this is an artefact, but the absence of similar structures in the CF-FS material indicates that some material is not properly preserved, and

therefore the explanation of its presence is not correct. This dark material could be caused by the rupture of physodes during the process of chemical fixation.

The membranous structures, like chloroplast membranes, nuclear membrane, endoplasmic reticulum and Golgi bodies (dictyosomes) are well-preserved after CCF. The external chloroplast membrane and the triplets of the thylakoids, as well as the double nuclear membrane and the nuclear pores are clearly distinguished. However, the plasma membrane and the membranes of the Golgi bodies appear slightly shrunken and not straight, as they are after CF-FS. The shape of the vacuoles appears also shrunken, i.e. their membrane is not round but rather wavy. In some vacuoles amorphous randomly placed darkly stained material is observed. Similar images have been considered as CCF artefacts by Kellenberger (1991), who suggested that chemical fixatives induce leakages, loss of cell turgor and cell shrinkage.

The cisternae and vesicles of the dictyosomes after CCF appear empty, while after CF-FS the cisternae of the maturing face appear to be gradually filled with electron dense material. This is more obvious in dictyosomes close to the cell wall, suggesting the transport of material to the cell wall.

There is a difference in the contrast between the two methods. After CF-FS the cell structure, and particularly the membranes appear more smooth compared to CCF. This was also noted by Hunziker et al. (1984), and Shioda et al. (1990, see also Quintana 1994) and was attributed to the cryofixation. Taking into account the good preservation of the cell structure after high pressure CF-FS, we would suggest that some material is lost during CCF, causing the higher contrast.

A completely new finding of the present study is the flat cisternae, which are visible only after CF-FS. These structures have been found - also after CF-FS - related to the formation of the cytokinetic diaphragm of *Dictyota dichotoma, Sphacelaria rigidula* and *Halopteris congesta* (Katsaros et al. 2009). It was suggested that, together with dictyosome vesicles and ER, they contribute to the formation of the cytokinetic diaphragm. A detailed study, using CF-FS combined with electron tomography, described the formation of plasmodesmata in *D. dichotoma* (Terauchi et

al. 2012). In this work, flat vesicles were present close to the forming pre-plasmodesmata, during cytokinesis.

In the present study, the appearance of flat cisternae close to the young cell walls suggests their participation in the deposition of membranous material in the expanding plasmalemma. The same suggestion can be made for the flat cisternae close to the external cell wall. The observation of flat cisternae close to active dictyosomes localised in the cortical cytoplasm suggests an interaction or collaboration of the two structures, possibly in the synthesis and transport of material to the cell wall and the supply of membranous material to the plasmalemma. The increased number of flat cisternae close to the pit fields is remarkable and deserves further study. One hypothesis is that they participate in the maintenance of the cell wall of pit fields. It can also be speculated that the flat cisternae contribute also to the transportation of some material through the plasmodesmata. Further research – especially applying element-specific tomography methods – will be needed to elucidate whether this has a function in the accumulation and transport of trace elements such as iodine.

Therefore, the main conclusion and novelty of the present study is that cryofixation is the method of choice for studying *Laminaria* cytology by TEM.

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Tables

Table 1. Programme for freeze substitution using the Leica AFS 2.

Step	**Start temp**	**End temp**	**Time (hours:mins)**	**Reagent**
1	-95	-90	30	2% OsO4 in acetone
2	-90	-90	10:00*	2% OsO4 in acetone
3	-90	-30	08:00	2% OsO4 in acetone
4	-30	-10	01:00	Acetone
5	-10	4	01:00	Acetone
6	4	20	01:00	Acetone

*this is the time for 1st run. In the 2nd run this was extended to 64 hours.

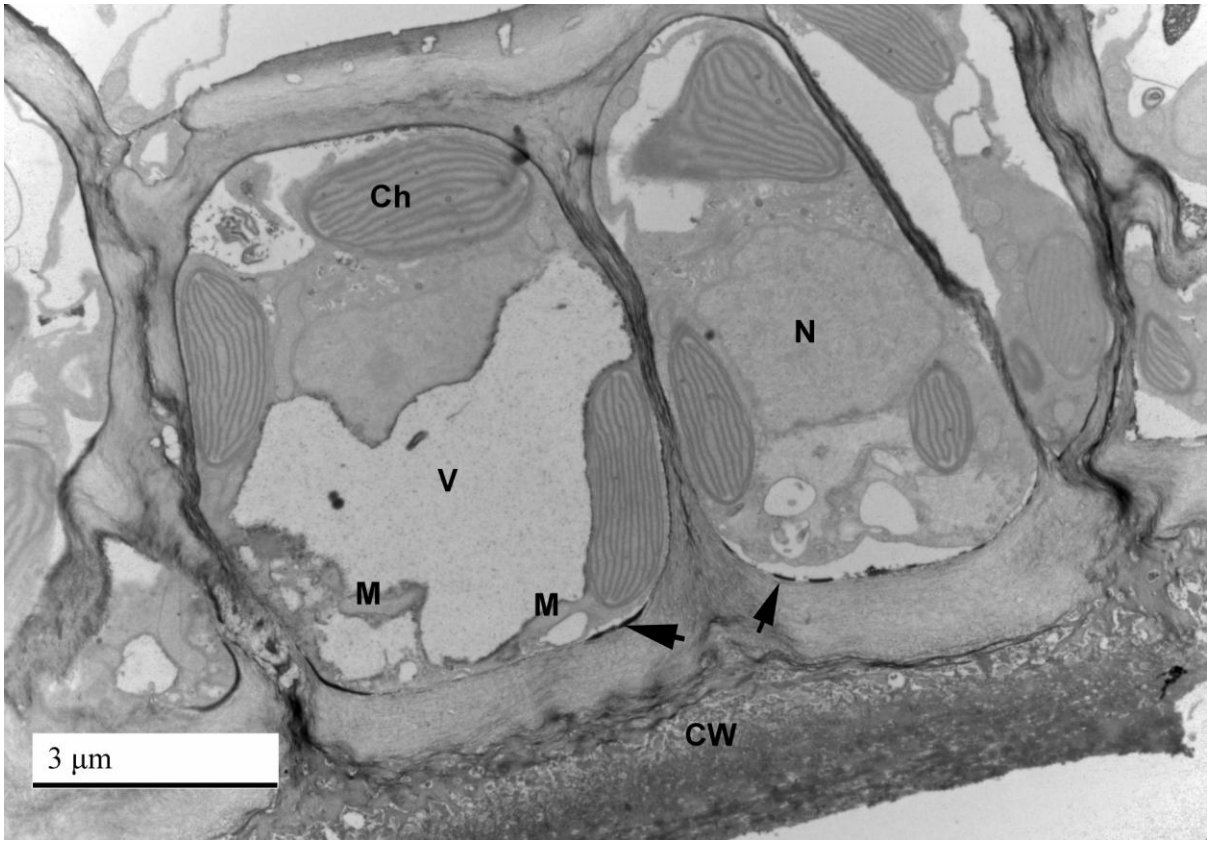
Figure legends

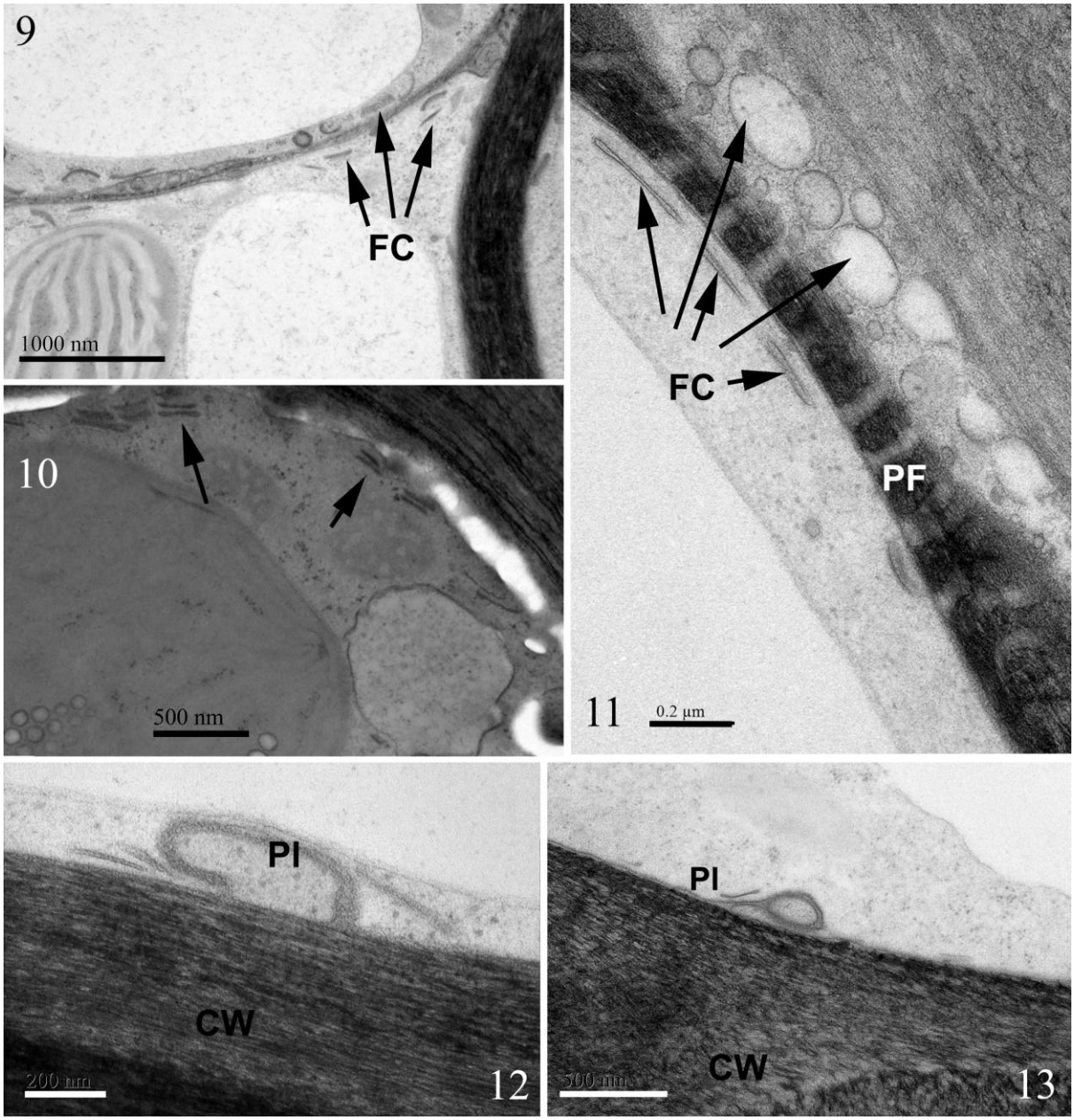
Fig. 1. Cross section of a blade, showing epidermal cells of *Laminaria digitata* after Conventional Chemical Fixation (CCF). N: nucleus; Ch: chloroplast; V: vacuole; CW: cell wall; M: mitochondria. Arrows indicate dark material attached to the plasmalemma of the external cell wall. Scale bar = 200 μm.

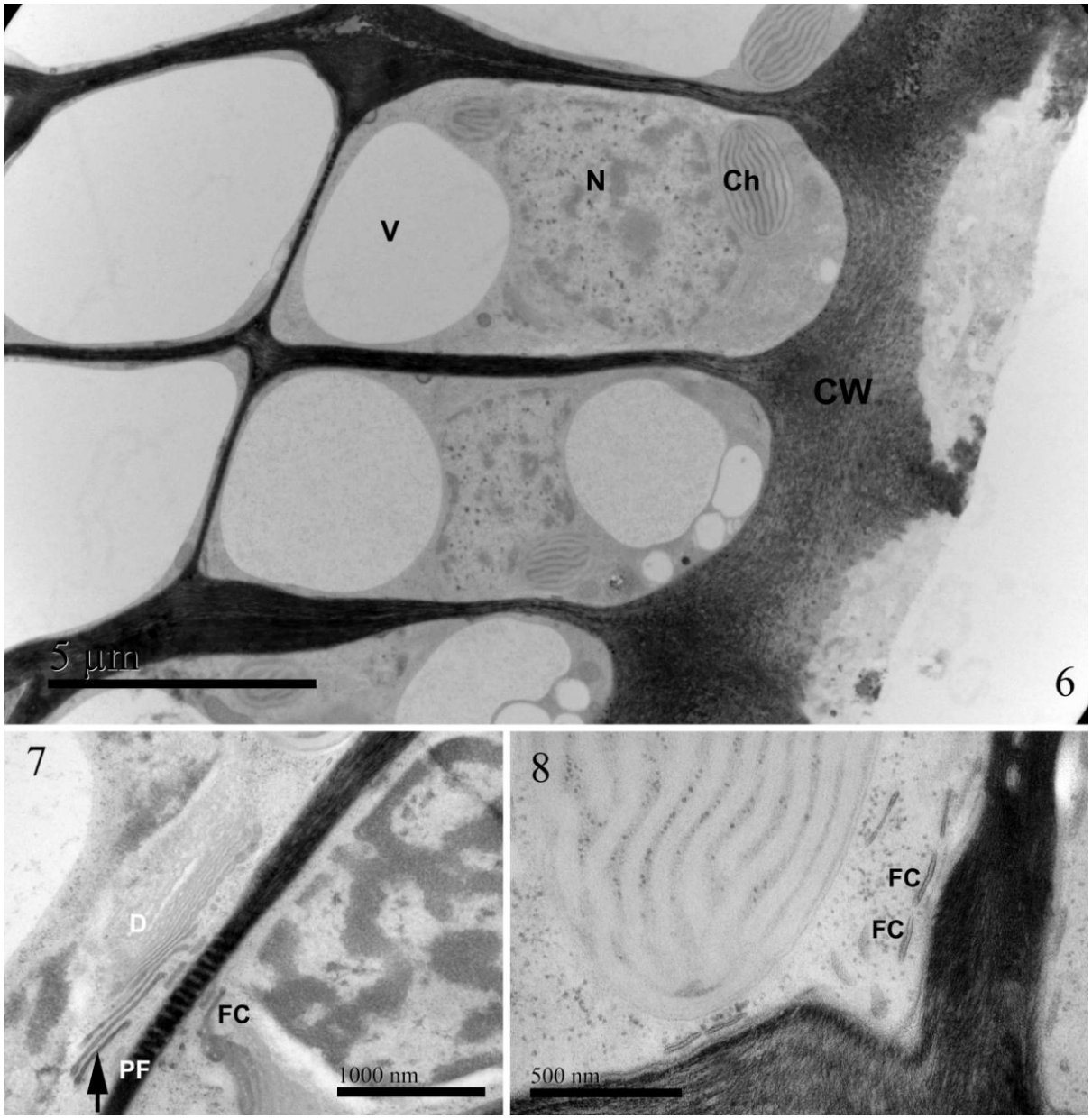
Figs. 2-5. Details of epidermal cells after Conventional Chemical Fixation. Fig. 2. Perinuclear dictyosomes (D) of an epidermal cell. Scale bar = 200 μm.**Fig. 3.** Chloroplast of an epidermal cell. Arrow indicates the nucleoid (genophore). Scale bar = 200 μm. **Fig. 4.** Chloroplast at higher magnification, showing tri-partite structure of the thylakoids. Scale bar = 500 nm. **Fig. 5.** Portion of an epidermal cell showing the external cell wall (CW). Arrows indicate darkly-stained material associated with the plasmalemma. Brackets indicate the layers of the cell wall. Scale bar = 200 μm.

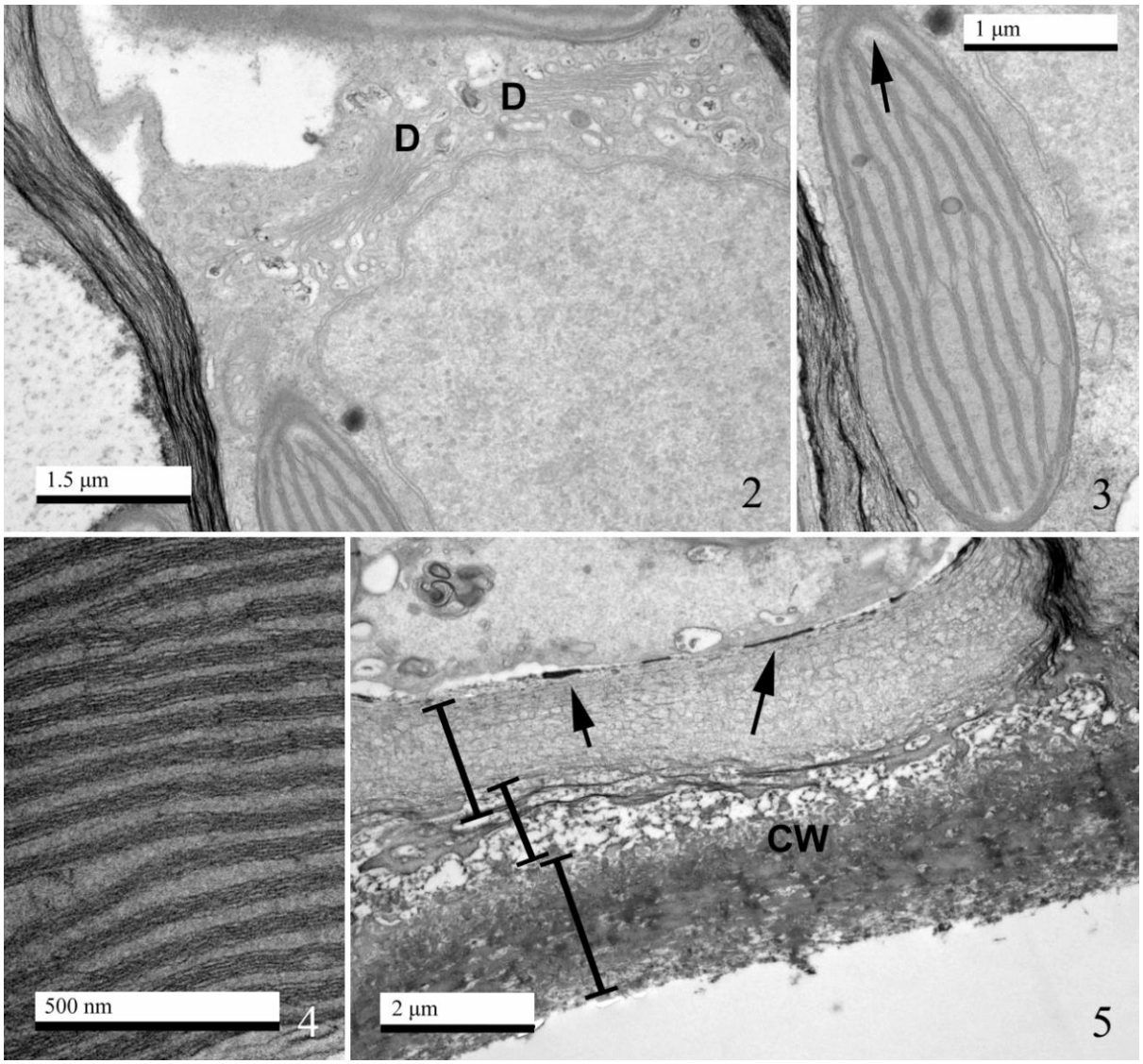
Figs. 6-13. Epidermal cells after Cryofixation - Freeze substitution (CF-FS). **Fig. 6.** Note the difference from similar cells after CCF (Fig. 1). N: nucleus; Ch: chloroplast; V: vacuole; CW: cell wall. Scale bar = 5 μm. **Fig. 7.** Active dictyosome (D) in a cytoplasmic area close to a pit-field (PF), after CF-FS. Arrow indicates dictyosome cisternae filled with darkly-stained material. FC: flat cisternae. Scale bar = 1000 nm. **Fig. 8.** Cortical cytoplasm of an internal cell (2^{nd} cell layer), showing several flat cisternae (FC) arranged parallel to the cell wall. Scale bar = 500 nm. **Fig. 9.** Young cell wall separating internal cells, with flat cisternae (FC) arranged parallel to and on both sides of the wall. Scale bar = 1000 nm. **Fig. 10**. Cytoplasmic

area close to the external cell wall of an epidermal cell. A large number of flat cisternae (arrows) are positioned in doublets, parallel to the wall. Scale bar = 500 nm. **Fig. 11.** Oblique section of a cell wall, showing a pit-field (PF) between two internal cells. The flat cisternae located on one side have the usual form, while on the other they appear round due to the plane of sectioning. Scale bar = 0.2 μm. **Figs 12, 13.** Invaginations of the plasmalemma associated with flat cisternae in internal cells. FC: flat cisterna; Pl: plasmalemma; CW: cell wall. Scale bars = 200 nm (Fig. 12) and 500 nm (Fig. 13).


Ch
N
V
M
M
CW
3 µm


9
FC
1000 nm
10
500 nm
FC
PF
11
0.2 µm
PI
CW
200 nm
12
PI
CW
500 nm
13


N
Ch
V
CW
5 µm
6
7
D
FC
PF
1000 nm
8
FC
FC
500 nm


D
D
1.5 µm
2
1 µm
3
500 nm
4
CW
2 µm
5