Title page

Title: Simple plunge freezing applied to plant tissues for capturing ultrastructure close to the living state

Contributors:

1) Koji Nitta,

Department of Regulation-Biology, Faculty of Science, Saitama University

2) Yasuko Kaneko,

Department of Regulation-Biology, Faculty of Science, Saitama University,

Saitama 338-8570, Japan, Tel: 048-858-3411, Fax: 048-858-3698,

E-mail: yakaneko@post.saitama-u.ac.jp

Corresponding contributor: Yasuko Kaneko

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Abstract

Simple plunge freezing in liquid propane was applied to plant tissues to observe ultrastructure close to the living state. With young pea leaves, the method yielded well preserved ultrastructure to a depth of up to 40 μ m from the surface of the specimen. Within the well frozen area, all the membranes appeared smooth and ultrastructural details of each organelle were similar to those obtained by high pressure freezing. Many physical connections between membranes were visualized. The simplicity and satisfactory freezing performance of the method make it suitable for routine ultrastructural studies to capture features of actively functioning cells.

Main text

In order to observe ultrastructure close to the living state, cryofixation is indispensable since biological activities can be fixed instantly by freezing. Several cryofixation methods such as plunging, slamming, propane jet freezing have been practiced widely [1]. A limitation, however, of these methods is that the depth of optimally frozen area without visible freezing damage is restricted to 10-20 μ m from the surface [2]. Few attempts to apply these methods to plant tissues have been reported [3-7]. To overcome the depth limitation of cryofixation, high-pressure freezing was introduced [8], which increased the optimally frozen area dramatically to up to 200 μ m of thickness. Although high-pressure freezing has been

applied successfully to many botanical specimens [9-14], there are some drawbacks compared to other freezing methods: it takes more time between excision and freezing; high pressure freezing machines are not always available; the influence of high pressure on the ultrastructure is difficult to assess. In order to investigate the applicability of a simple plunge freezing method to plant tissues, and further to compare the result with high pressure freezing, young pea leaves were rapidly frozen by plunging them into liquid propane and then freeze substituted.

Seeds of *Pisum sativum* L. cv. Denko 30 days were imbibed in water for 16 h at room temperature. The plumules were excised with a razor blade and stuck on the tip of a stainless steel needle encased in a narrow stainless tube [15]. The sample was rapidly frozen by plunging it into liquid propane precooled with liquid nitrogen and released into the substitution media by pulling the needle. Frozen specimens were freeze substituted in 2% OsO_4 in anhydrous acetone at -85℃ for 3 days, at -30℃ overnight, 4℃ for 2h, then brought up to room temperature. After rinsing with anhydrous acetone, the specimens were gradually infiltrated with Spurr's resin. The ultrathin sections were cut with a diamond knife on a Sorvall MT-2B ultramicrotome and stained with 2% uranyl acetate for 15 min followed by lead citrate for 5 min. The sections were observed with a Hitachi H-7500 electron microscope at an accelerating voltage of 100 kV.

The plunge freezing method with liquid propane yielded well preserved ultrastructure to

a depth of up to 40 μ m from the surface of the young pea leaves (Fig 1). The depth of the area devoid of visible freezing damage was much more than expected. This may be partially because of the nature of the specimens, since the young pea leaves used in this study consisted of tightly packed small cells with dense cytoplasm surrounded by cell walls. Within the well frozen area, all the membranes appeared smooth and the matrices of cytoplasm and organelles were dense (Fig 2-9), which is very close to the ultrastructure obtained by high pressure freezing [10]. Freezing performance was quite reproducible and cracks in cytoplasm and cell wall, which occur occasionally in high pressure frozen material [10], were rarely observed. Membrane stainability varied somewhat with each sample. In most samples, cells at the surface of the tissue possessed membranes with the least contrast and cells at depth with slight freezing damage exhibited intensely stained membranes. Cells between these areas, which contained well preserved ultrastructure with stained membranes, were optimal for observation. The membrane stainability in freeze substituted samples may be affected by water availability during freeze substitution [16].

Unlike chemical fixation, in which membranes are often undulated, plasma membrane appeared as straight lines tightly appressed to the cell wall (Fig 2). Bundles of microfilaments occasionally appeared in the cytoplasm (Fig 3), which is an observation known from high pressure frozen samples [10, 11]. Membranes of each Golgi cisterna were also clearly visualized (Fig 4), exhibiting numerous connections between cisternae.

Pillar-like structures between Golgi cisternae were reported previously in the rat pituitary gland [17] and it has been suggested that they might be a kind of cytoskeleton maintaining constant distance between membranes. Nuclear envelopes appeared as straight lines (Fig 5, arrowhead) and plastid envelopes and ER membranes were more clearly visible (Figs 5-9) than in high pressure frozen specimens [10,11], where they are often hard to discern. In mitochondria (Fig 6), outer and inner envelope membranes were closely situated and difficult to distinguish. This phenomenon was also observed in high pressure frozen samples [10]. In contrast, outer and inner envelope membranes of plastids (Fig 8, 9) kept constant distance and structures connecting the two membranes (Fig 8, arrowheads) were observed frequently. Small lipid droplets were found lining the inside of the smooth plasma membrane (Fig 2) and also around the plastid membranes (Fig 7, 9). Connecting structures between lipid bodies and plastid envelope (Fig 9) could be observed and appeared very similar to those in high pressure frozen specimens [11].

Abundant ER membranes were associated with various organelles. Rough ER with ribosomes was often surrounding protein bodies (Fig 5) and smooth ER was frequently very closely associated with plastids (Fig 7, 8). Physical connections between rough ER and protein bodies were clearly recognized (Fig 5). Numerous physical connections between ER and the associated plastid membranes were observed as well (Fig 8). The connections between ER and plastid membranes have been reported in various plant materials [11], but the

nature and function of the connections is as yet unidentified. Recently, close contacts between ER and mitochondria in living HeLa cells were reported, suggesting the involvement of these structures in regulating Ca^{2+} signaling [18]. The numerous connections between various neighboring membranes visualized in the frozen samples may reflect constant interactions and communication between the membranes.

By the simple plunge freezing method described, dynamic aspects of actively functioning cellular ultrastructure can be observed very easily without any costly device. The results indicate that the limitation of the depth of the area devoid of freeze injury may be overcome to a certain extent by the nature of the specimens investigated. We have applied the method to various plant tissues, such as cotyledons, young root and leaf tissues of pea and bean, cotyledons of soybean, and young leaves of the aquatic carnivorous plant, *Aldrovanda*. The depth of the well frozen area varied, but most of the time, more than 20 μm of good ultrastructural preservation was obtained and revealed novel features which could not be obtained with chemical fixation. In gland cells of *Aldrovanda*, in addition to a variety of membrane structures, abundant microtubules were excellently preserved. The simple plunge freezing method should be adopted as one of the routine specimen preparation methods and applied more frequently for electron microscopic observation of plant tissues. The whole procedure does not necessarily take longer than conventional chemical fixation and the resulting ultrastructure is far closer to the living state of the cells, and thus will provide

indispensable information about how cellular organelles actually function.

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Figure Legends

Fig 1. Electron micrograph of a mid transverse section of a young pea leaf prepared by plunge freezing in liquid propane. Well preserved structures were observed up to about 40 μ m from the epidermis (left). Some freezing damage was seen at asterisk (right). N: nucleus; PB: protein bodies; S: starch grain.

Fig 2-9. Portions of cells in well frozen area of a young pea leaf.

Fig 2. Plasma membrane and lipid droplets. CW: cell wall; L: lipid droplets; PM: plasma membrane.

Fig 3. A bundle of microfilaments (MF).

Fig 4. Golgi apparatus (G). Connecting structures (arrows) between cisternae are discerned.

Fig 5. Nuclear envelope (arrowhead) and protein bodies (PB) with associated rough ER. Connections between ER and protein bodies are indicated by arrows. N: nucleus.

Fig 6. Mitochondria (M).

Fig 7. Plastids (P) with associated ER. PB: protein bodies; double arrows: lipid droplets.

Fig 6. Physical connections (arrows) between ER and a plastid (P). Connections between outer and inner envelopes of plastid (arrowheads) are also observed.

Fig 7. Physical connections (arrows) between lipid droplets (L) and a plastid.

1
PB
N
S
ER
*
N
10 µm
2
PM
L
CW
0.1 µm
3
MF
0.1 µm
4
G
0.1 µm
5
PB
ER
N
0.1 µm
6
M
ER
0.1 µm
7
ER
P
PB
ER
P
1 µm
8
ER
P
0.1 µm
9
L
P
0.1 µm