Scanning Microscopy

Volume 7 | Number 4 Article 19

12-27-1993

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Scanning Microscopy, Vol. 7, No. 4, 1993 (Pages 1295-1310) 0891-7035/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

OPTIMIZATION AND APPLICATION OF JET-FREEZING

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(Received for publication September 8, 1993, and in revised form December 27, 1993)

Abstract

Cryofixation is considered to be the best method for immobilizing biological material in its natural state. In jet-freezing, the specimen typically is sandwiched between two carriers and kept in place while a coolant is moved very rapidly against the opposite surfaces. The JFD 030 jet-freezing device has been used to optimize the operating parameters. The course of the temperature in place of a specimen was measured with thermocouples and recorded by an IBM-compatible personal computer using a specifically developed software program. Mean cooling rates, over the temperature range of 273K to 173K, achievable with different cryogens, including the non-flammable HCFC 124 (SUVA 124-$CHClFCF_3$), were measured under a variety of conditions. The freezing capability of the JFD 030 was evaluated by analyzing transmission electron microscopic results obtained from freeze-substituted plant cells and freeze-fractured cosmetic emulsions. Jet-freezing, despite its limits in freezing thick specimens, can be applied to cell suspensions as well as to semi-thin sections by the use of thin Ti supports, a higher pressure of the nitrogen gas or by a brief pretreatment with a cryoprotectant (e.g., sucrose). In addition, with the non-flammable HCFC 124 in combination with the thin Ti supports, freezing rates similar to those achievable with propane and standard copper supports can be reached giving researchers the chance to use jet-freezing without the dangerous propane.

Key Words: Jet-freezing, cryogens, HCFC 124, cooling rate, freeze-fracture, freeze-substitution, titanium, tungsten, transmission electron microscopy, plant cells, emulsions.

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Introduction

Rapid freezing techniques are known today as the best methods for the immobilization of biological and aqueous specimens. When compared to chemical fixation, cryofixation can physically stabilize cellular components much faster (milliseconds versus minutes) and numerous artifacts, as reported in the literature, can be avoided. However, during rapid freezing, the temperature drop can introduce segregation artifacts due to ice crystal formation. The aim of rapid freezing techniques is to eliminate such segregation by the withdrawal of the specimen heat in the shortest possible time. The ideal goal would be to achieve a "life-like" state of freezing through true vitrification (amorphous, glassy state) of the specimen water so that no ice crystals exist. Cooling rates for vitrification have been estimated by several authors, not calculated, however, for lack of data. The estimates range from 10^7 to 10^{12} K/s [1, 3; for review see ref. 2]. So far, specimens with a thickness of only a few hundred nanometers can be vitrified by rapid-freezing [8]. To check the state of the frozen water, electron diffraction on low temperature specimens was applied in a transmission electron microscope (TEM). The method used there was plunge-freezing [4]. The plunging speed, the depth of the coolant bath, as well as the choice of the cryogen, are crucial in determining the extent of vitrification (for a review see [25]). The worst shape of a specimen for rapid freezing was found to be spherical (smallest surface for a given volume!), which resulted in the development of spray-freezing of micro droplet suspensions. The technique was further refined by Bachmann and Schmitt [1]. The need to understand both the structure and the function in biological material lead to the development of the rapid-mixing / spray-freezing technique [15]. With this technique, the intermediate stages of biological reactions to a chemical agent can be studied on cell suspensions down to a lag time of about 30 ms. Another approach to rapid freezing is to accelerate a specimen onto a polished copper block which is cooled by liquid nitrogen or helium. This "slamming", "impact cryofixation" or "metal-mirror cryofixation" method [27] (for a review see [24]) is capable of freezing tissue up to a depth of about 15 μm without

detectable ice crystal damage [26]. With all the techniques mentioned so far, the specimen is moved and the heat sink is held stationary. A very high relative speed between the specimen and coolant can be achieved by the jet-freezing technique [17]. A very thin specimen (thickness up to 20 μm) is placed between two thin metal carriers. The specimen thickness can be reproduced easily by the use of a gold grid "spacer". The heat content of the specimen is withdrawn very rapidly from one or two surfaces (depending on one-sided [14] or double-sided [19] jet-freezing) by the fast moving cryogen. Jet-freezing is ideal for most liquid samples, such as suspensions and emulsions. After the freeze-fixation step, the specimens can be processed by freeze-substitution [6, 7, 13] or freeze-fracturing [21].

High pressure freezing [18] is the only method which provides true vitrification of specimens with a thickness of more than 200 μm [16]. However, this is a special case where the high pressure alters the physical characteristics of the water in the specimen so that vitrification is achieved using relatively low cooling rates. It is, in effect, a double-jet cooling method, working with liquid nitrogen as coolant, which will not be discussed further here.

Among the prerequisites for "ultra rapid freezing" at normal pressure [10], is a high cooling rate which is very important in order to minimize the formation of artifacts like segregation of specimen molecules. Traditionally, rapid freezing techniques are characterized by the achievable cooling rate: with the help of thermocouples, the trace of the temperature drop in location which the specimen would normally occupy is recorded, and the time required to pass from 273K to 173K is measured. Based on this time the cooling rate is calculated. With the first laboratory prototype, jet-freezing with propane was found to be about 30 times faster than plunge-freezing in Freon 12. The high cooling rates of propane jet-freezing resulted in an elimination of segregation of aqueous and non-aqueous material within samples containing suspensions of ferritin molecules, tobacco mosaic virus particles, liposomes, and whole cells [19].

Some of the first commercially available instruments for propane jet-freezing were QFD 101 (Balzers), QFD 020 (Balzers Union) and the MF7200 Gilkey-Staehelin jet-freezer (RMC, Tucson, AZ). Later, jet-freezing with propane (modified QFD 020) was compared with plunge freezing in melting (slushy) nitrogen, Freon 22 and liquid propane [21]. Propane jet-freezing was found to be 30 times faster than manual plunge-freezing in Freon 22 and 7 times faster than manual plunge-freezing in liquid propane. The segregation pattern in Freon-frozen cellulose gels could be explained by ice crystallization during freezing [21]. Numerous researchers built their own devices and a variety of ideas, including a thermostatically controlled specimen chamber (temperature up to 333K) for the preservation of temperature sensitive specimens [28], a one-sided jet for cells grown on thermally insulating substrates [23, 14], and an additional exhaust valve for better refilling with propane [11], were verified.

The use of cryoprotectants like DMSO, sucrose [6], and others were proposed to increase the depth of well frozen material over that normally achieved with untreated samples (20 μm). A preferable, non-invasive alternative is to improve cooling efficiency. Propane is the most widely used coolant for freezing because of the large difference in its boiling (231K) and melting points (85.6K). To our knowledge, cryogens, other than liquid nitrogen [22] were not investigated in jet-freezing. Recently, a new Freon replacement product, HCFC 124 ($CHClFCF_3$), was discussed as a possible cryogen for plunge freezing [25]. HCFC 124 has an extremely low melting point (74K), and therefore, it could be of use in jet-freezing as well. Furthermore, propane is well known for its potential for explosion and Freon can contribute to the damage of the Earth's ozone layer. Therefore, we wanted to explore the possibility of using HCFC 124, which is non-flammable and relatively less polluting to the atmosphere than Freon, as a cryogen for jet-freezing.

An interesting discussion on heat transfer was reopened by Zasadinski [29; compare also to ref. 2]: stating that convection from the cryogen to the specimen is the limiting step in rapid freezing of small samples. When applying the "Biot" or "Nusselt number"

$$Nu = (h \cdot d) / k_s,$$

where h is the effective heat transfer coefficient, d is the effective thickness, and k_s is the heat conductance of the specimen [2]; better cooling rates should be expected when raising h and reducing d. We wanted to test this model by using thinner specimen carriers than those currently available commercially and by applying higher nitrogen gas pressure, that is coolant speed.

It is very difficult to draw conclusions from the results of cooling rate measurements in the literature, because in almost every case, the equipment and procedures were different. Also, the variations in specimen carriers, or even the use of uncovered thermocouples, (resulting in extremely high cooling rates) make direct comparisons difficult if not impossible.

In this article, we present results produced with the latest model of the JFD 030 jet-freezing device (BAL-TEC, Liechtenstein). All cooling curves and rates have been recorded using the same equipment and procedures.

Materials and Methods

The cryogens used in our experiments were commercial propane (Shell Switzerland; or Agway, Ithaca, NY, USA), ethane (purity > 98%, GC, Fluka AG, Buchs, Switzerland; or Matheson Gas Products, Twinsburg, Ohio), and HCFC 124 (DuPont De Nemours, Dordrecht, Netherlands, or Wilmington, DE, USA). The physical data of the coolants are listed in Table 1.

Table 1. Physical data of the coolants.

	HCFC 124 (SUVA 124)[1]	Propane[2]	Ethane[2]
Formula	$CHClFCF_3$	$CH_3CH_2CH_3$	CH_3CH_3
Molecular Weight	136.5	44.1	30.07
Melting Point	74K	85.6K	90K
Boiling Point	262K	231.2K	184.7K
Thermal Conductivity (298K)	0.072 W/mK	0.19 W/mK	0.24 W/mK
Critical Temperature	151.1K	176.5K	241.0K
Critical Pressure	35.7 bar	42.0 bar	48.2 bar
Vapor Pressure at:			
203K	0.03 bar	0.58 bar	3.0 bar
223K	0.15 bar	0.68 bar	5.0 bar
243K	0.40 bar	1.82 bar	11.0 bar
263K	1.00 bar	3.21 bar	18.0 bar
283K	2.50 bar	6.45 bar	30.7 bar
303K	4.40 bar	11.0 bar	49.0 bar
Heat capacity (J/gK)	1.12 (298K)	1.92 (93K)	2.27 (98K)
Flammable limits in air (vol%)	none	2.2-9.5%	3.0-12.5%

HCFC 124 special data:

Ozone depletion potential (reference: Freon 11 = 1.0)	0.02
Green house effect (reference: Freon 12 = 3.0)	0.10
Photochemical reactivity (≤ 36 means "no-smog-activity")	1.00
Toxicity (allowable exposure limit, AEL)	500 ppm (v/v)

[1]2-chloro-1.1.1.2-tetrafluoroethane; data from: the DuPont leaflet ER-8D.
[2]Data from two sources: (1) Handbook of Chemistry and Physics, 67th Edition, 1986-1987 (CRC Press, Boca Raton, FL), 10.91; and (2) The Matheson Gas Data Book, Matheson of Canada Limited, Whitby, Ontario, Canada.

For producing specimen carriers that were thinner than the standard 0.1 mm copper carriers (BAL-TEC BU 012 056-T), a hand-operated punch and dye device was fabricated at Cornell University. Electronic grade 0.05 mm copper foil was obtained from Busby Metals, Inc., Hauppauge, NY, USA. 0.05 and 0.025 mm titanium and 0.05 mm tungsten foils were obtained from Alfa/Johnson Mathey Co., Ward Hill, MA, USA.

The function of the jet-freezer

Figures 1 and 2 illustrate the operation principle of the JFD 030 jet-freezer. After baking out the system to about 333K with the two heaters (see Fig. 1), the chamber wall is cooled to the operating temperature (93K for propane, 103K for ethane, 118K for HCFC 124). The cryogen temperature is controlled by an automatically driven liquid N_2 valve regulated by the wall temperature. The chamber is filled with the cryogen and as soon as the desired cryogen temperature is reached and the N_2 pressure is set: (6 bar for propane and ethane, 9 bar for HCFC 124), the JFD 030 is ready for freezing specimens.

Cooling rate measurements

Cooling rates describe well the relative efficiency of an applied technique or coolant, although the data is obtained in absence of a biological specimen while using a full metal test-sandwich. They clearly point out the importance of the jet speed or other parameters. We placed a pair of 20 μm copper and constantan wires between a sandwich made of two specimen support platelets. By point welding with high current, the thermocouple (Cu/Const) was produced and the sandwich stabilized. For cooling-rate measurements, the temperature-dependent voltage was amplified 1000 times in an amplifier with 273K compensation (Dogrel). Data acquisition and storage (at 20,000 Hz, providing more than 20 values per rate measurement) were accomplished via a high speed analog to digital (AD)-converter and software (Keithley, Dash 8) incorporated in a COMPAQ Descpro 286 personal computer. Data acquisition, calculations, and displaying of the results on the screen as well as printing were accomplished by a software "JETPROG" developed at BAL-TEC and written in TURBO PASCAL (available on request).

Freezing of specimens for freeze-fracturing

Standard BAL-TEC specimen carriers as well as the gold TEM grids {BAL-TEC B8010 010 19, 400 mesh (3 mm dia., 7 μm thick)} were cleaned by sonication in 1% sulfuric acid, water, and acetone, sequentially, for one minute each. A drop of emulsion was placed with a pipette on one side of the gold grid, which was held

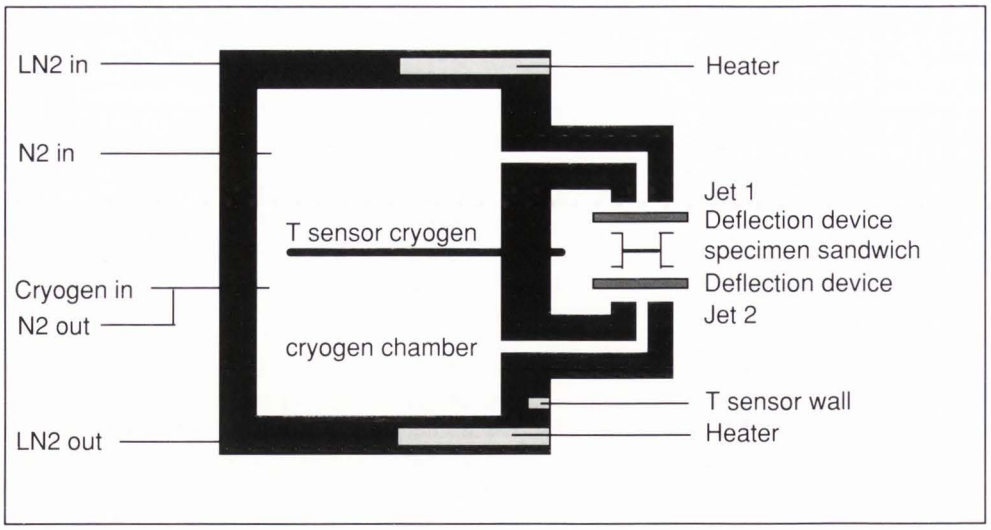


Figure 1. Technical layout A, jet system. The wall of the cryogen chamber is cooled with liquid nitrogen. The jet lines are kept at the same temperature as the wall. The cryogen temperature is measured by a PT 100 sensor (for further description, see text).

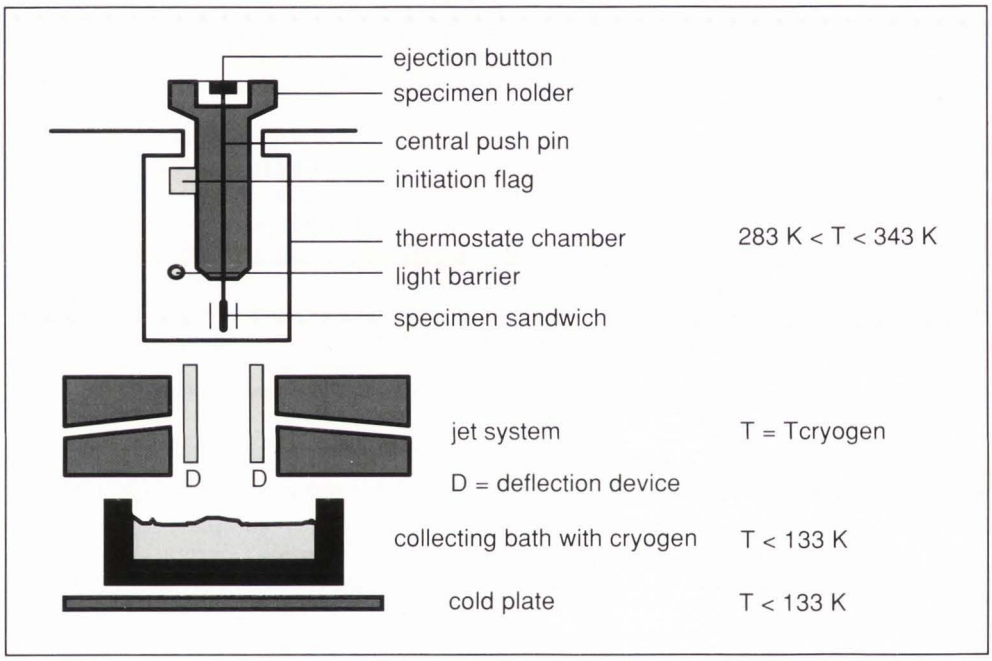
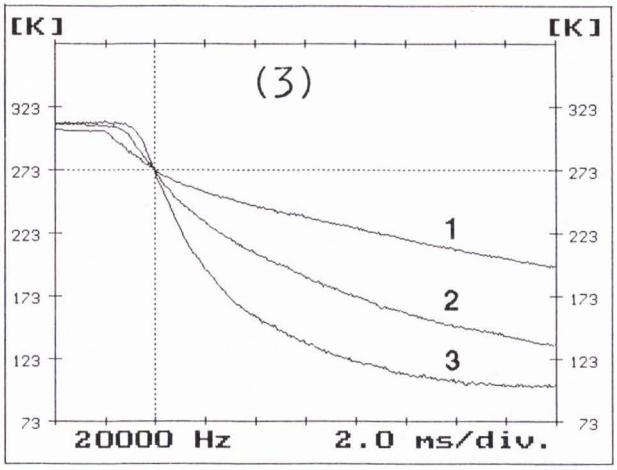


Figure 2. Technical layout B, jet and specimen. When the specimen holder is pushed down, the sandwich is placed between the two jet orifices. The jet direction (diameter 1.5 mm) is inclined by 5° relative to the horizontal, thus driving the sandwich into, rather then out of, the specimen holder (for further description, see text).

with a pair of tweezers. From the other side, the drop was drained through the grid meshes several times to ensure that the grid meshes became filled with the emulsion. Most of the emulsion was removed leaving only a small amount on the grid, forming a slight meniscus. The grid (which served as a spacer) was placed on top of a specimen carrier held by a second pair of tweezers. A second specimen carrier was then placed on the gold grid: the sandwich was complete. Excess emulsion that extruded to the spaces between the bases of the copper carriers was removed by a blotting paper. By the time the desired temperature of the cryogen was reached, the sandwich was inserted into the specimen holder leaving a narrow space between the sandwich and a central push-pin. The specimen holder, which is located at the end of a rod, was then inserted into the thermostatically controlled specimen chamber of the jet freezer (compare to Fig. 2). The specimen temperature could be held at any desired temperature between 283K and 343K.

By pushing the specimen holder rod down to the end position, a light barrier-activated nitrogen gas valve is triggered, the cryogen chamber is pressurized and the cryogen forced rapidly through two jet lines onto both sides of a deflection device. The device is a movable flap which prevents the leading portion of the cryogen jet from reaching the specimen. However, due to the force of the jet, the deflection device is quickly pushed aside exposing the two surfaces of the specimen sandwich at exactly the same time to the remaining portion of the cryogen jet. The time delay for the jet to reach the specimen surface is estimated to be 50 to 100 ms. Each jet lasts 350-400 ms and consumes approximately 20 ml of cryogen.



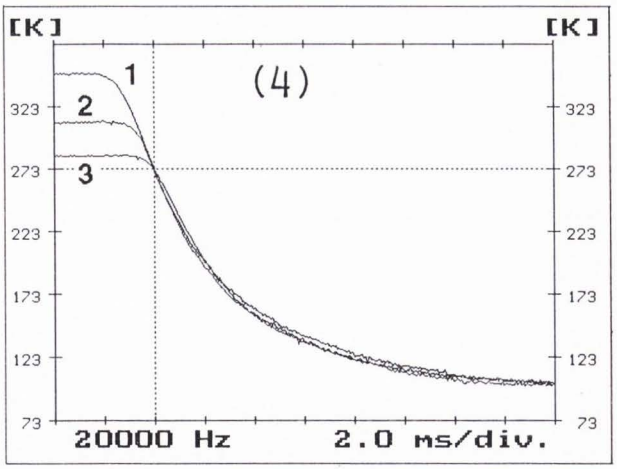


Figure 3 (at left). Conditions of the jet system. **1**: one-sided jet (4,400 K/s); **2**: double-sided jet (12,579 K/s); **3**: double-sided jet with deflection-device (33,898 K/s). The speed of data acquisition was 20,000 Hz.

Figure 4 (at right). Cooling rates versus initial temperature. **1**: 348K (32,258 K/s); **2**: 308K (33,898 K/s); **3**: 283K (31,250 K/s); mean: 32,469 K/s (standard deviation = 4.1%). The speed of data acquisition was 20,000 Hz.

Immediately after the jet ended, the central push-pin of the specimen holder was pressed down to eject the frozen specimen into an underneath collector, containing cryogen held at a temperature ≤ 133K. As many specimens as required were collected. The specimens were retrieved using forceps, and excess coolant was removed by knocking the forceps-holding hand against the other hand whilst en route to liquid nitrogen for storage.

Freeze-fracturing

Up to four jet-frozen specimen sandwiches were inserted under liquid nitrogen into a double replica specimen table [20]. This specimen table then was inserted through a nitrogen gas counter flow loading device into the cold stage of a BAL-TEC BAF 400 T freeze-etch unit held at 143K. At high vacuum conditions (pressure ≤ 10^{-6} mbar) and at a temperature of 168K the specimens were fractured by opening the spring loaded double replica device. After a sublimation time of 5 minutes, the specimens were shadowed with 2 nm platinum/carbon at 45° and coated with 20 nm carbon at 90°. After removing them from the vacuum chamber, the replicas were cleaned with bleach (14% sodium hypochlorite), chloroform/methanol (1/1), and sulfuric acid (70%) for 8 to 24 hours each, with intermediate washings on distilled water. As the replicas usually stayed on the gold grids, they could be cleaned on them. After drying, the gold grids served as the electron microscope (EM) grids.

Freezing of specimens for freeze-substitution

Maize (Black Mexican Sweet, BMS) suspension culture cells and *Dunaliella salina* cells (alga) were harvested at their log growth phase and sandwiched between standard copper specimen carriers or 0.025 mm titanium specimen carriers, with a single-slot grid (thickness ~ 55 μm) as a spacer, and were then jet-frozen.

Coleoptile material from five day old oat seedlings was cut into pieces with a thickness of about 50-100 μm and an extension of 1 mm^2. The pieces were either incubated in a buffer/sucrose solution [6] for 15 minutes, or transferred to distilled water for 1 to 2 minutes in preparation for freezing. Prior to freezing, the material was either sandwiched between two flat specimen carriers or between one flat specimen carrier and a dimpled carrier. Both the standard copper specimen carrier and the 0.025 mm titanium carrier were tried. Propane was used to jet-freeze maize suspension culture cells and oat coleoptile tissues at 86K (with a JFD 030 modified to work at higher N_2 pressures). Propane and HCFC 124 were used to jet-freeze *Dunaliella* cells. HCFC 124 was maintained at a temperature of 113K for freezing at a nitrogen pressure of 10 to 12 bar.

Freeze-substitution

All specimens except for maize suspension cells were freeze-substituted using the tannic acid/acetone and uranyl acetate/osmium tetroxide/acetone protocol of Ding *et al.* [6]. Maize suspension cells were freeze-substituted with 2% anhydrous glutaraldehyde and 0.1% tannic acid/acetone and 2% uranyl acetate/2% osmium tetroxide/acetone. The freeze-substitution was carried out at 186K using either a Forma Biofreezer or BAL-TEC FSU 010 freeze-substitution unit. Infiltration and embedding was done with Spurr embedding medium at room temperature; heat polymerization was carried out at 333K.

Results and Discussion

Cooling rate measurements, optimization of parameters

Three experiments were carried out initially with propane as the cryogen (see Fig. 3):

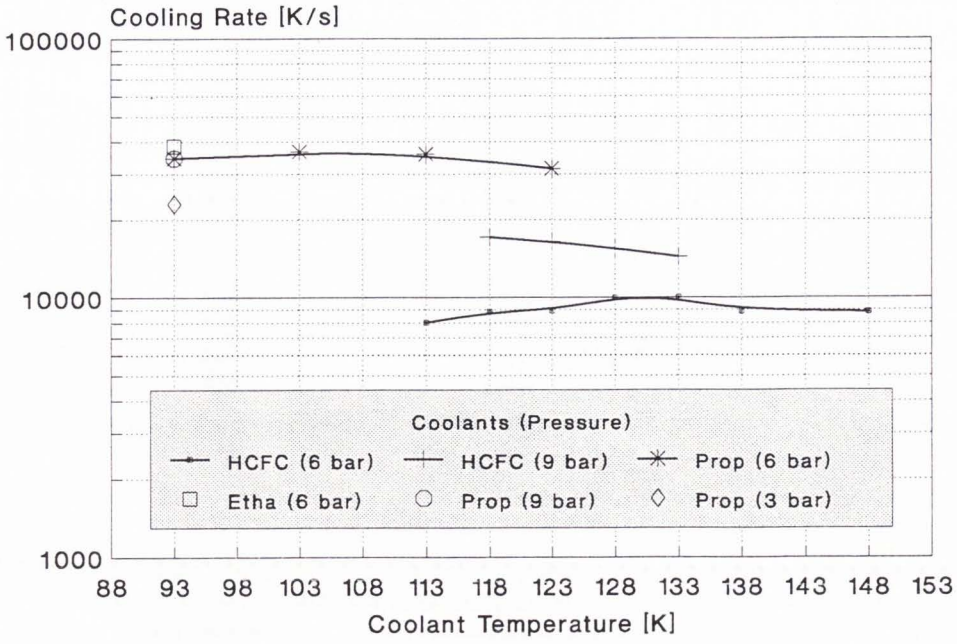


Figure 5. Cooling rates (273K to 173K). The cooling rates resulting from various coolants and N_2 pressure are displayed in order to provide the respective cooling rate ranges.

1. In order to simulate the "one-sided jet", only one jet was directed to the "thermocouple-sandwich" (the other one was closed mechanically), the resulting cooling rate was about 4,000 K/s.

2. Interestingly, the double-sided jet produces cooling rates of about 12,500 K/s which is more than double the value of one-sided jet-freezing.

3. The use of the deflection device gives the highest cooling rates of about 34,000 K/s.

Based on these results, we defined the constant and variable parameters of the JFD 030 jet-freezer:

Constant parameters:

Two jets in synchronous operation
Use of the deflection device
Coolant supply pressure of 1.5 bar
Collecting bath temperature of 133 K.

Variable parameters:

Type of cryogen
Nitrogen gas pressure
Cryogen temperature
Thermostat temperature of specimen chamber

In order to investigate if the initial specimen temperature affects the cooling rates, we carried out measurements with three different thermostat temperatures: 348K, 308K, and 283K (see Fig. 4).

From Figure 4, it is clear that the cooling rate is not influenced by the initial specimen temperature and the high reproducibility (standard deviation < 5%) clearly demonstrates this. Therefore, the cryogen, the nitrogen gas pressure, and the cryogen temperature are the variable parameters. Cooling rate measurements were carried out varying all these parameters and the results are summarized in Figure 5.

Propane: The cryogen temperature was varied from 123K to 93K and the freezing rates ranged from 31,500 to 36,600 K/s with a maximum at 103K. This result seems to be in disagreement with other findings [5] since the maximum cooling rate is not at the lowest possible temperature. However, the standard deviations have to be taken into account: The cooling rate variation at a certain coolant temperature is larger than the difference between the mean cooling rates achieved at different temperatures (Fig. 5). A pressure of 6 bar resulted in an average cooling rate of about 34,000 K/s. At 3 bar, the cryogen velocity seemed to be too low and the cooling rate dropped to about 23,000 K/s. A 9 bar nitrogen pressure yielded high cryogen velocity and cooling rates of about 34,000 K/s. However, such a high pressure also created turbulence that could, in practice, make it difficult to direct the frozen specimen during ejection into the collector. A cryogen temperature of 93K and a nitrogen gas pressure of 6 bar were concluded to be the optimal values for propane.

Ethane: This coolant is widely used in plunge freezing [8] and is reported to be superior to propane. Compared to propane, a slightly higher cooling rate was reached at 6 bar of nitrogen gas pressure and 103K cryogen temperature. The average cooling rate was about 38,400 K/s. Since the reproducibility was quite poor (the standard deviation was over 10%), the yield was rather small, and the cryogen is expensive, and since only a few interested researchers can be expected, experiments with different pressures of the nitrogen gas or different cryogen temperatures were not carried out.

HCFC 124: This Freon-replacement product has an unusual property which is not obvious from its disclosed physical data. The cryogen's viscosity changes dramatically at about 110K. Actually, at this tempera-

Table 2. Mean cooling rates (K/s) in the range of 273K - 173K for different specimen carriers at different N_2 pressures for the three cryogens.

Carrier	N_2 pressure (bar)	Cryogen: Propane (93K)	HCFC 124 (118K)	Ethane (103K)
Copper 0.1 mm	3	23,000 [----]	----	----
standard	6	36,100 [2.0 %][1]	10,100 [5.3 %]	38,500 [> 10%]
	9	37,400 [3.6 %]	15,400 [8.9 %]	----
Copper 0.05 mm	6	51,000 [16 %]	20,300 [2.6 %]	
	9	55,000 [----]	21,300 [----]	
Titanium 0.05 mm	6	52,200 [4.1 %]	26,300 [5.0 %]	
	9	62,700 [6.0 %]	33,600 [1.4 %]	
Titanium 0.025 mm	6	68,000 [7.1 %]	43,300 [4.4 %]	
	9	71,600 [6.4 %]	47,600 [----]	
Tungsten 0.05 mm	6	55,300 [2.6 %]	22,200 [19 %]	
	9	55,800 [3.1 %]	20,400 [5.7 %]	

[1][standard deviation]

ture, the jet lines become blocked and freezing of specimens at or close to this temperature is impossible. At a nitrogen gas pressure of 6 bar, cooling rates ranging from 8,820 to 10,000 K/s were achieved with a maximum at 133K. Obviously, the higher viscosity at lower temperatures influences the cooling rate at this nitrogen gas pressure. At 9 bar, a stringent control of the cryogen temperature resulted in the highest cooling rate of 17,100 K/s at 118K. Since 113K is very close to the temperature where the viscosity changes, a nitrogen gas pressure of 10 to 12 bar was applied for the freezing of plant specimens. For routine use, a nitrogen pressure of 9 bar and a coolant temperature of 118K seem to be optimal. Cooling rates obtained with different support materials, cryogens, and N_2 pressures are listed in Table 2.

From our results, it is clear that a compromise has to be considered. One could procure the most expensive, flammable and difficult-to-handle (under high pressures) cryogen like ethane and attain the highest cooling rates (although with poor reproducibility), or use the cheapest, but also highly flammable, cryogen, commercial propane, to attain highly reproducible results. Alternatively, one can choose to have an environmentally safe, non-explosive coolant that is relatively expensive and yields moderate results under proper conditions. Although the cooling rates obtained with HCFC 124 were lower than that of propane or ethane, the coolant can be used to freeze samples that contain a certain amount of natural cryoprotectants or after careful treatment with proper concentrations of cryoprotectants, [e.g., see ref. 6]. Our results also indicate that it is possible to use HCFC 124 in combination with 0.025 mm titanium specimen carriers and attain higher cooling rates than with standard Cu carriers and propane. Thus, under the above mentioned conditions, it is possible to obtain the quality of freezing with HCFC 124 that is normally obtained with standard copper specimen carriers and propane. It was not clear that we could draw direct conclusions from these measurements (pure metal sandwiches) for the freezing of biological specimens, so we carried out freeze-fracture and freeze-substitution experiments.

Jet-freezing of emulsions for freeze-fracturing

Jet-freezing cannot provide vitreous (amorphous) specimen water, so a fine segregation pattern is present in water-rich compartments of a specimen. By a shallow "etching" (sublimation of specimen water), these compartments can easily be identified by the roughness of their fracture face. This "etching" also helps to identify the fractured lipid membranes by their typical fracture step (arrows in Fig. 6A) on the convex fractures of the liposomes. If fat spheres (Fig. 6B) or oil droplets (Fig. 6C) are fractured, they can be identified by their flat fracture face and the convex surface revealed by the sublimation process.

The filamentous network of a gel is completely embedded in ice during the fracturing process (Figs. 6B and 6C). After sublimation of a layer of 10 to 30 nm of specimen water, the filaments are revealed. The apparent mesh size of the gel depends on the segregation by ice crystal formation during freezing [9, 21] and a possible recrystallization by warming the specimen to temperatures higher than 138K. As we cannot assume to be able to achieve real vitrification with jet-freezing, we can only consider the first possibility.


A
O
X
S
W
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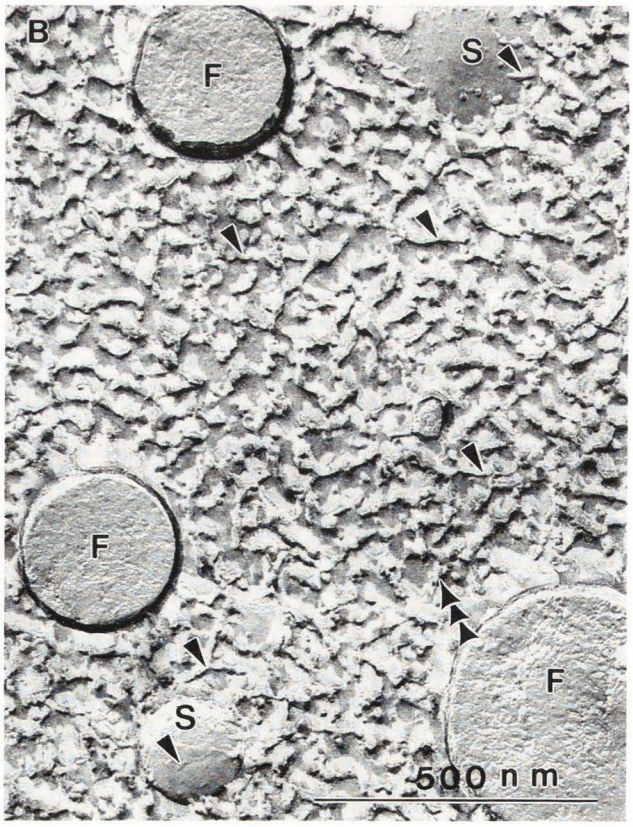
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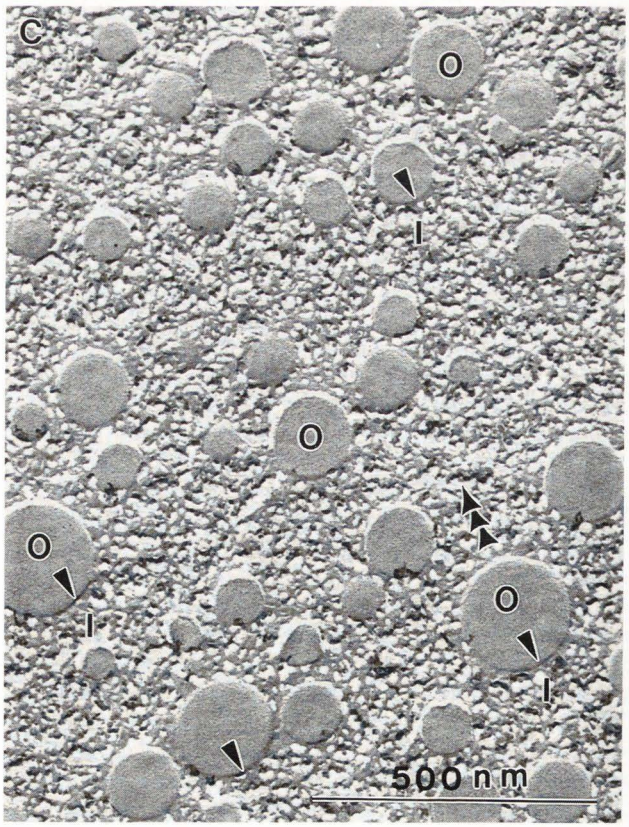
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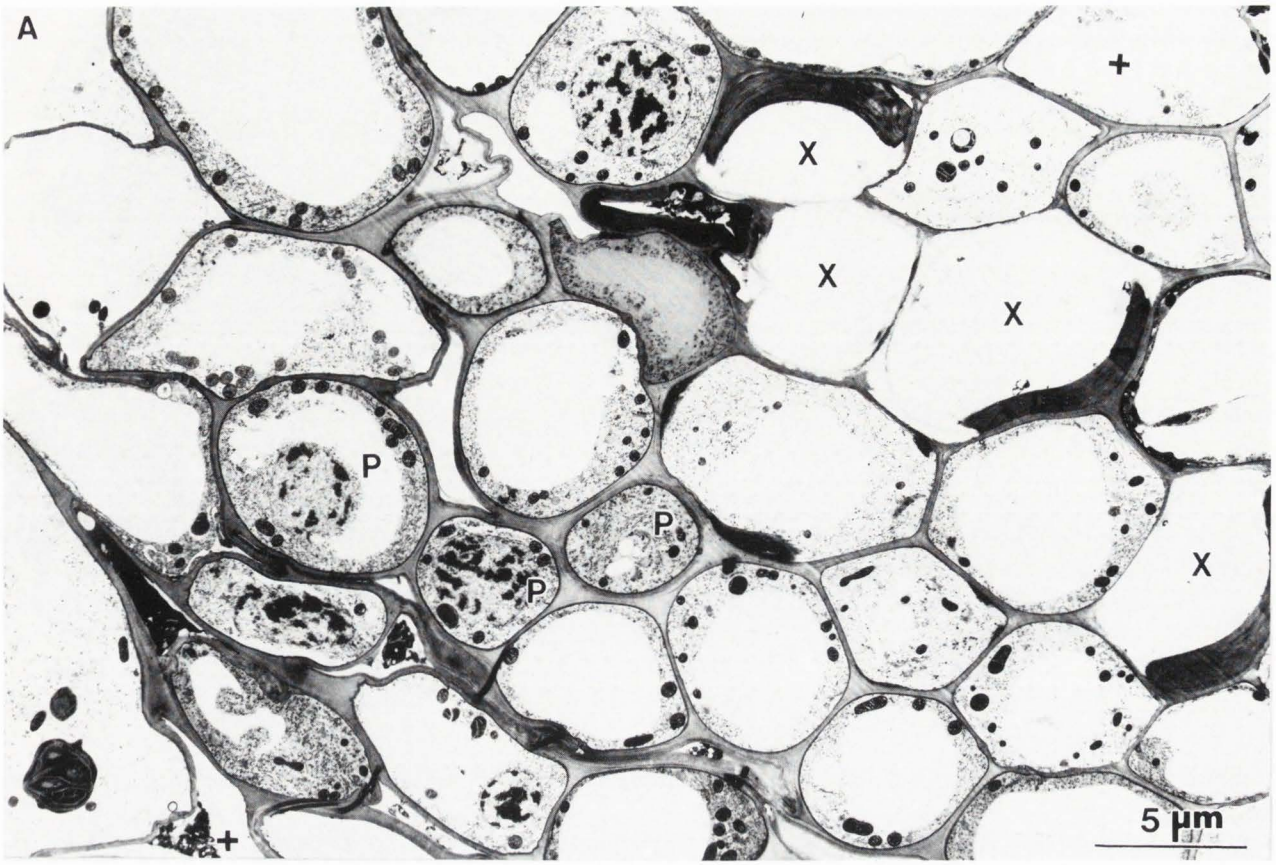

Figure 7 (above). Transverse section of a vascular bundle in oat coleoptile that was treated with buffered 0.2 mM sucrose before jet-freezing with propane. 0.05 mm copper specimen carriers were used. Although the cells were located deep within the tissue, most of them appear well frozen without ice-segregation artifacts. X = xylem cells; P = phloem cells; + = damaged cells.

Figure 6 (on the facing page). Jet-frozen cosmetic emulsions. Jet-freezing cannot provide vitreous (amorphous) specimen water, so a fine segregation pattern is present in water-rich compartments of a specimen. By a shallow "etching" (sublimation of specimen water) these compartments can easily be identified by the roughness of their fracture face (W, in Fig. 6A). The shadowing direction is indicated by a triple arrowhead. **A**: Oil in water emulsion, stabilized with lipids. As the ice (W) level is lowered by sublimation, the typical fracture steps of lipid membranes become visible (arrowheads). A membrane-fracture reveals a convex fracture face (A) or a concave one (B). When fracturing oil droplets, the fracture face is a more or less flat plain (O). In this specimen, many oil droplets are surrounded by a lipid membrane, so combinations of convex and flat fractures can be observed (X). In some cases a space, filled with water, can be observed between the oil droplet and the lipid membrane (S). **B**: Fat spheres in water, dispersed in a gel. The filamentous network of the gel and the fat spheres are completely embedded in ice during the fracturing process. Lowering the ice level reveals the gel structure (arrowheads) as well as non-fractured spheres (S), partly covered with gel filaments. The fractured fat exhibits a granular structure (F). **C**: Oil droplets in water, dispersed in a gel. The oil droplets are statistically distributed. On the individual oil droplets, the level difference of fracture face (O) and ice level (I) can be observed (arrowheads). The fractured oil exhibits a smooth structure.

Jet-freezing of cell suspensions for freeze-substitution

In sucrose treated, propane-jet frozen oat coleoptile material, we were able to obtain good freezing up to a depth of 50 μm from each side of the specimen that was sandwiched between 0.05 mm copper carriers (Fig. 7). This result is comparable with those obtained by Ding *et al.* [6] in sucrose treated tobacco tissue. It was possible to freeze single cells such as *Dunaliella* and maize suspension culture cells without any pretreatment, using the standard 0.1 mm copper specimen carriers and propane as the cryogen (Figs. 8A, 8B, 9A and 9B). However, *Dunaliella* is known to produce glycerol and that might have contributed to good freezing. Similarly, the sugars normally present in the growth medium of

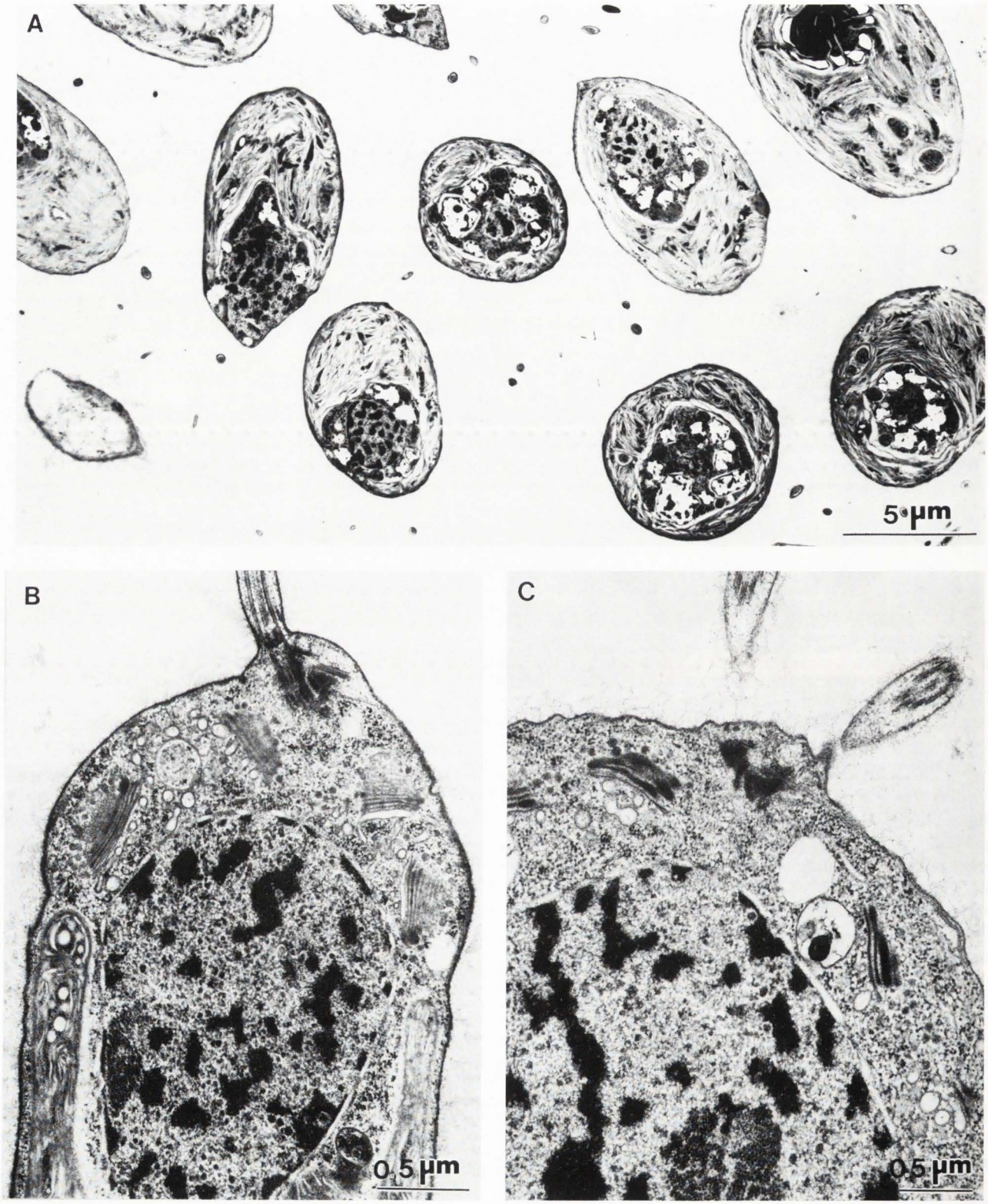

Figure 8. Unicellular alga *Dunaliella salina* frozen without pretreatment. **A**: Low magnification of propane-jet frozen cells using the standard copper specimen carriers. The clear areas near the nuclei represent lipid material that was extracted. **B**: High magnification of a cell jet-frozen with propane (compare to Fig. 8a) showing well preserved organelles. **C**: High magnification of a cell jet-frozen with HCFC 124 using titanium specimen carriers.

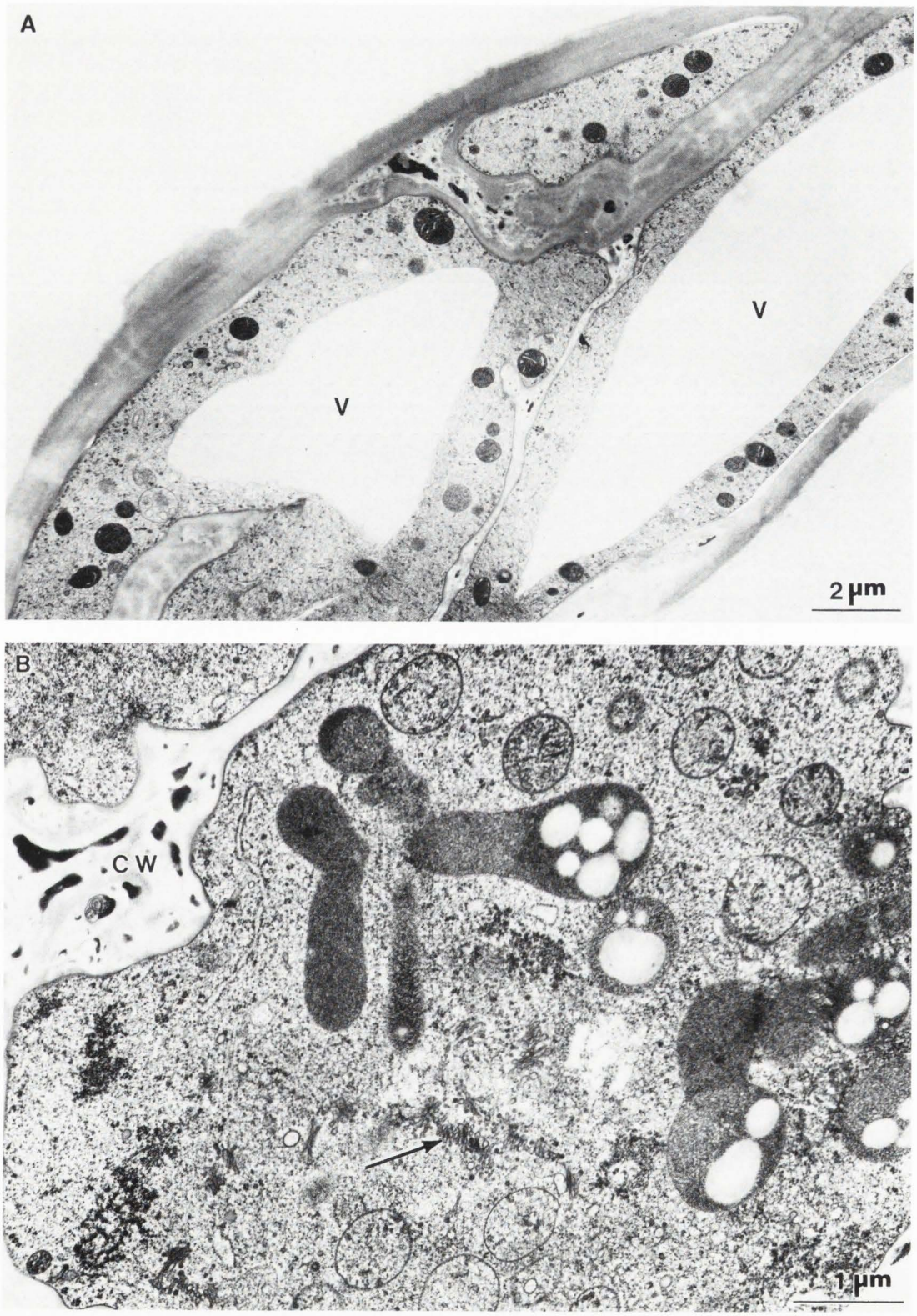

Figure 9. Maize (BMS) suspension culture cells propane-jet frozen using standard copper specimen carriers. **A**: Low magnification of cells with large vacuoles (V). **B**: High magnification showing organelles and a cell plate (arrow). CW = cell wall.

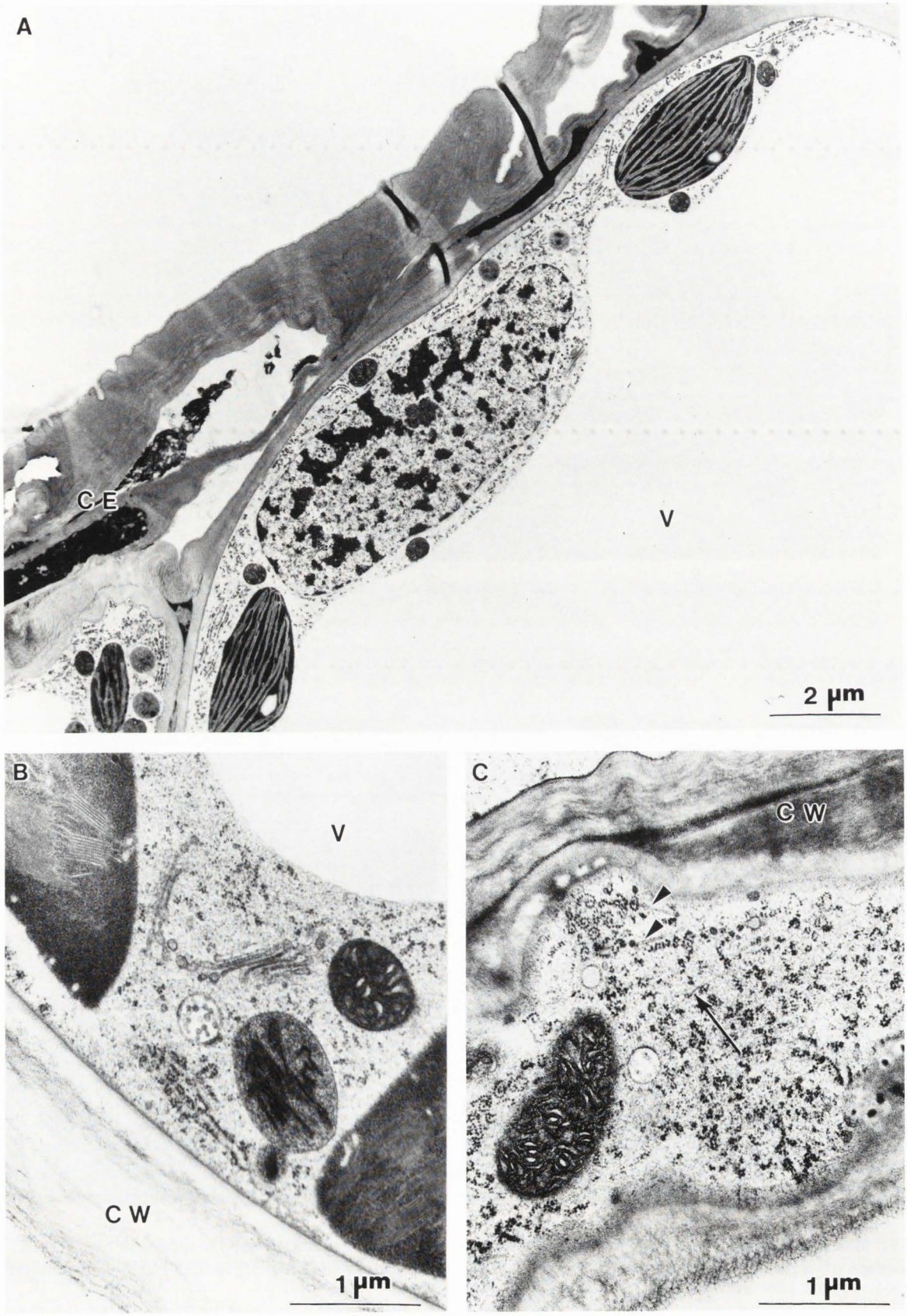
A
C E
V
2 µm
B
V
C W
1 µm
C
C W
1 µm

Figure 10. Oat coleoptile tissue propane-jet frozen without any cryoprotective pretreatment, using titanium 0.025 mm specimen carriers. **A**: Low magnification micrograph showing the crushed exterior cells (CE) when the tissue is sandwiched between flat specimen carriers, but the large, uncrushed mesophyll cells are well frozen. V = vacuole. **B**: High magnification of a mesophyll cell showing good preservation of organelles. CW = cell wall; V = vacuole. **C**: High magnification of a vascular cell showing excellent preservation of organelles including microtubules (arrow) and microfilaments (arrowheads). CW = cell wall.

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maize suspension cells might have acted as cryoprotectants. Using 0.025 mm titanium specimen carriers and HCFC 124 as cryogen, it was possible to get a fair yield of well frozen *Dunaliella* cells (Fig. 8C) at a cryogen pressure of 10 to 12 bar and a temperature of 113K. Experiments to freeze other cell types with HCFC 124 are currently in progress.

When oat coleoptile tissue was frozen without sucrose treatment using the standard copper specimen carriers and propane as the cryogen, it was not possible to obtain good freezing beyond a depth of 10 to 12 μm from either side of the specimen. However, when 0.025 mm titanium flat specimen carriers were used, it was possible to obtain reliable freezing up to a depth of 30 μm from each side of the sandwiched specimen. Good thermal contact (no air-filled spaces) between specimen carriers and the sandwiched specimen is a key requirement for reliable freezing. This may be one of the reasons for the very low yields of well frozen cells (up to 20%) in the case of untreated oat coleoptile tissues. Using flat titanium carriers instead of dimpled ones promotes better thermal contact with the specimen, but also frequently contributes to the crushing of peripheral cells (Fig. 10A). Cells interior to the crushed layer, however, usually exhibit excellent freezing (Figs. 10B and 10C). Devising proper spacers that permit good contact with the specimen carriers without crushing the cells will be a challenge that one will have to address when freezing untreated tissue material.

Conclusions

We have demonstrated that high quality freezing of liposomes, cosmetic emulsions and plant cells can be obtained under proper conditions with jet-freezing. Transmission electron microscopy results of *Dunaliella salina* and corn suspension-culture (BMS) cells after freeze-substitution and ultramicrotomy showed no segregation artifacts and the cell organelles were preserved perfectly. Except for the crushing of the peripheral cells, the same was true of untreated oat coleoptile cells that were well frozen when 0.025 mm titanium specimen carriers were used. In liposome, fat-in-water, and oil-in-water emulsion samples of the cosmetic industry, all the components of the formulation were visualized after freeze-fracturing and replication using standard 0.1 mm copper specimen carriers. Finally, our results seem to support the heat transfer model to predict cooling rates [2, 29]: The cooling rate is increased by the higher jet speed and also by the thinner specimen carrier plate. Although the thermal conductivity of copper is twenty-fold better than titanium, the freezing rates obtained with the 0.025 mm titanium specimen carriers were up to two-fold better than with the thicker (0.1 mm) copper ones. Our results obtained with untreated oat coleoptile tissues also indicate a doubling of the depth of freezing if the thinner titanium specimen carriers are used instead of the standard copper ones that are four times thicker. Further experiments using thin metal foils as specimen carriers might prove useful in increasing the depth of good freezing. It should also be pointed out that our use of thin titanium specimen carriers for rapid freezing of biological specimens is not novel. Handley *et al.* [12] used 4 μm titanium foil as specimen carriers in a spring-augmented plunge-freezing device to obtain good freezing of erythrocytes up to a depth of 30 μm.

Acknowledgements

The contribution of cosmetic emulsions from L'Oréal, Paris is gratefully acknowledged. We are grateful to Dr. Randy Wayne and Rosa Spivey of Cornell University for the supply of *Dunaliella salina* and maize (BMS) tissue culture cells respectively. We also thank Michael Bugl at BAL-TEC for his skillful technical assistance, and A.K. Nelson, Cornell University for fabricating the punch and dye device.

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Discussion with Reviewers

K.P. Ryan: Is there any reason why a gold grid spacer is used? Would a copper spacer be better from the consideration of conductivity? Could a thinner spacer be used, for example, by putting a copper grid through a fine double roller device?

Authors: Gold grids are chemically stable in all cleaning solutions. We think the use of copper as a spacer material will not considerably enhance the freezing quality of the specimen, since only the biological material between the mesh bars is observed in the microscope. Gold grids thinner than 7 μm probably are not mechanically stable enough, in order to serve as TEM-grids.

K.P. Ryan: Following jet-freezing, you state that specimens are freeze-substituted or freeze-fractured. Is there any reason why they could not also be trimmed and cryosectioned, using suitable low temperature adhesives, such as 40% ethanol in isopropanol {Richter K, Gnägi H, Dubochet J (1991) A model for cryosectioning based on the morphology of vitrified ultrathin sections. J. Microsc. **163**, 19-28}; or 30% ethanol, 30% methanol and 40% water {Michel M, Hillmann T, Müller M (1991) Cryosectioning of plant material frozen at high pressure. J. Microsc. **163**: 3-18}? Both of these mixtures can be used at about 135K (-138°C) and set solid at lower sectioning temperatures of 115K (-158°C) to

105K (-168°C). This could extend the usefulness of jet-freezing.
Authors: We generally agree with your comments, but the experiments remain to be done. However, diamond knives are probably not so fond of cutting gold spacers.

M. Müller: What is the rationale behind impregnation with cryoprotectants? Could these cryoprotected samples not equally well be frozen by simple plunging, e.g., into liquid propane?
Authors: As the jet-freezing technique most probably is not able to vitrify specimens, a cryoprotection is necessary. The attempt is to treat specimens with extremely low ("natural") sugar concentrations, in order to reduce sugar related changes. Jet-freezing always will produce higher freezing rates than plunge freezing so the sugar concentration for jet freezing is estimated to be lower.

M. Müller: We have demonstrated the interdependence of cryogen temperature, streaming velocity of the cryogen, and mass of the carrier system {Müller and Moor (1984) Proc. Electron Microscopy Soc. America, pp. 6-9}. We also showed, that increasing the cooling rate at the surface of the specimen does only marginally increase the cooling rate in the center of an organic specimen with thermal properties similar to water. Optimum conditions cannot be determined with thermocouple measurements using a fully metallic test system, since this is indicative only for the temperature reached at the specimen surface.
Authors: We used a 400 mesh gold grid spacer of 7 μm thickness. So the individual probe size was approximately 40 x 40 x 7 μm. This is considerably smaller (thinner) than the probes in your investigation. Please see part 5 of chapter 1 by Bachmann and Mayer [2] for a possible explanations for better freezing by higher cooling rates of specimens with a thickness < 10 μm {compare also, Mayer E, Brüggeller P (1982) Vitrification of pure liquid water by high pressure jet freezing. Nature (London) **298**: 715-718}.

M.J. Costello: Your results suggest that the temperature of the cryogen (Fig. 4) has little effect on the cooling rate and may have an optimum somewhat warmer than the cryogen melting temperature. It should be noted that your log plot masks the linear trend of increasing cooling rate as a function of the temperature difference (initial temperature minus cryogen temperature), as predicted by theory (see ref. [29]) and as reported previously for plunge cooling of bare thermocouples (see ref. [24]). Can you elaborate on your interpretation of the results presented?
Authors: We think that fixing the cooling rate measurement to the time difference between 273K and 173K is the reason why such an impression is conveyed in Fig. 4. Actually the cooling rates as judged by the steepest parts of the cooling curves fit well into the proposed theories, but, in order to compare with some relevance to biological material we had to deal with the applied temperature interval. However, from the data in Fig. 5, it is obvious that it is not the cryogen temperature but the geometry of the specimen and the chosen parameters that are limiting the cooling rates.

M.J. Costello: The background ice in the freeze-etch images (Fig. 6) shows a granularity suggesting significant ice crystal growth. How could you distinguish between the presence of freezing induced microcrystalline ice and ice formed on recrystallization at the etching temperature? Since the lipid fracture surfaces are very smooth, do you think that these images demonstrate that microcrystalline ice had little or no damaging effect on the specimens being examined?
Authors: The patterns of the ice in the parts of Figure 6 have a different history: Figure 6a shows the microcrystalline (pure?) water, while Figures 6b and 6c exhibit a hydrous gel. We never find water compartments without ice crystals when applying jet-freezing. We also find no difference between samples etched at 168K or at 183K [21]. As we would expect larger ice crystals at higher etching temperature if they had been formed by recrystallization, we think that the microcrystals visible are the product of the freezing process. Etching makes the size of the microcrystals more pronounced due to geometric conditions in the sublimation process. We think that the real size of the ice crystals is smaller than they appear. This may explain why the lipid membranes in Figure 8a are not deformed by ice crystals.

M.J. Costello: Four freeze-substitution protocols were described for examining plant tissues. Were there notable differences in preservation or visibility of membranes or cytoskeleton using different protocols?
Authors: We actually used only two different freeze-substitution protocols. One was mainly that of Ding *et al.* [6] in which the frozen samples were freeze-substituted with 0.1% tannic acid at 186K in acetone for 2 days, followed by 2% uranyl acetate/2% osmium tetroxide/acetone at 255K for 12 hours and at 280K for 2-3 hours. The samples were then substituted with pure acetone at 280K for 2-3 hours and brought to room temperature before plastic infiltration and embedding. The second protocol was very similar to the first except that 2% glutaraldehyde/0.1% tannic acid/acetone was used instead of just 0.1% tannic acid/acetone. We did not find a significant difference between the two protocols as far as cytoskeletal and other organelle preservation was concerned. We now routinely use only the first protocol.

M.J. Costello: What are the prospects for improving the jet-freezer further?
Authors: On the machine side (parameters), a lot has been done already. Although it is difficult, if not impossible, to build a perfect instrument, we believe that the JFD 030 jet freezing device has already incorporated many improvements. However, there is further room for improvement as far as specimen carriers and the proper thermal contact of sample with the specimen carriers is concerned.

G. Knoll: You mention a nice trick for treatment of replicas after freeze fracture, in that you use a 400 mesh gold grid as a spacer which serves as a support during the cleaning procedure and finally as a EM grid. Is it possible to use grids with less meshes (what is the limit); also, have you been confronted with problems during cleaning steps in organic solvents?
Authors: The mesh size, that is possible to use, depends strongly on the thickness of the carbon layer (stability) of the replica. We think making carbon layers of, e.g., 30 nm (instead of 20 nm) would enable the application of 200-300 mesh grids. On one hand, this would be helpful for the microscopist to orientate himself on replicas of tissue material; but, on the other hand, the occurrence of carbon "ghosts", due to the accentuation of steep flanks, could increase the interpretation difficulty. Normally, the gold grids float on the water surface due to the surface tension of the liquid, however, sometimes they sink in water, as they do in organic solvents, because of the lack of surface tension.

In organic solvents we use a "bed" of a stainless steel net (mesh size approx. 0.5 mm) standing on two legs with a slight dimple in the middle (see Figure 11). The grids are placed just under the surface of the liquid in the dimple of the net.

The "bed's" size is 10 mm x 10 mm and fits into the spot plate for cleaning replicas [B 8010 030 (ceramic) or B 8010 003 88 (Teflon)]. The spot plates have to be covered with a glass plate, in order to prevent the organic solvents from fast evaporation.

T.H. Giddings: Did you encounter any problems with the thinnest specimen carriers, such as deformation, loss of sample, or difficulty in handling? Have you reached the lower limit of thickness of these materials that still allows reasonable handling characteristics?
L. Bachmann: Are the specimen carriers made of titanium, tungsten, and very thin copper commercially available?
Authors: The 0.05 mm copper specimen carriers are mechanically unstable, and the 0.05 mm tungsten specimen carriers seem to be brittle and very difficult to machine. Therefore, only the 0.025 mm (for freeze substitution) and the 0.05 mm (for freeze-fracturing) titanium specimen carriers will be commercially available in the future.

T.H. Giddings: The efficiency of HCFC 124 for jet-freezing would appear to be in doubt based on the cooling data and the quality of freezing you obtained. *Dunaliella* should be relatively easy to freeze due to its endogenous glycerol, yet you report a "fair" yield of well frozen cells, even with the very thin specimen supports. Are there other non-flammable alternatives? The importance of safe handling of propane and ethane cannot be overstated, but they do seem to be the best choices in terms of performance. Are you aware of any accidents involving commercially produced jet-freezing devices?

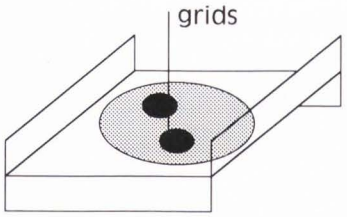

Figure 11. Bed of stainless steel net with a slight dimple in the middle.

Authors: The yield of well frozen cells always is lower than 100%. Using the best coolants (ethane or propane) just increase the possibility of finding well frozen cells. This possibility is considerably higher with the flammable coolants as compared to HCFC 124. However, applying the non-flammable coolant with the cryo-jet will always give better results than, e.g., plunge freezing. To our knowledge, from all the other freon-replacement products no other coolant is applicable to jet-freezing. To the best of our knowledge there has been no accident when a commercially available instrument was used. However, while working with his prototype, Martin Müller encountered a fire ball around his head, as he was not using a closed chamber for the jet. Some portion of the propane could reach a nearby light bulb. The spark of the extinguishing bulb was good enough to ignite the propane/air mixture. Fortunately, he survived without being harmed, he only got short curled hairs (personal communication M. Müller).

G. Knoll: Why are there no error bars in Fig. 5 and why are standard deviations missing from values in Table 2?
Authors: We chose this logarithmic presentation in Fig. 5 for the reason not to pronounce the absolute values, but to give an idea about the range of cooling rates which could be expected. We think that error bars are not necessary for that purpose. We only made two measurements of some parameters so we could not calculate standard deviations of them {displayed in the Table as (---)}.

G. Knoll: What was the cooling rate for HCFC at 12 bar (used to freeze the plant tissue).
Authors: We only modified one instrument to work with this high pressure. We did not measure the cooling rate, but we estimate it to be 10 to 20 % higher than with 9 bar.

G. Knoll: What was the speed of the cryogen, diameter of nozzles and the volume/time of cryogen applied? How do these values compare to the published values?
Authors: We only see from the increasing turbulence during the jet, that the speed must be higher, we did not measure it, so we cannot compare directly with the published data.

T.H. Giddings: What was the sucrose concentration used for freezing the plant tissue?
Authors: We applied a buffered 0 2 mM sucrose solution.