*Journal of Microscopy, Vol.* 144, *Pt* 2, *November* 1986, *pp.* 215–221.
*Received* 7 *April* 1986; *accepted* 10 *July* 1986

*Short Technical Note*

# Cryo-transfer revised

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KEY WORDS. Cryo-transfer, contamination rate, evaporation rate, coolant protection, mechanical shielding.

SUMMARY

Vapour deposition of water is a major problem in cryo-tranfer and cryo-observation of frozen specimens. The use of (sliding) shields to protect the specimen during transfer has been evaluated and was found to be a fail-safe procedure. Shielding the specimen with solidified coolant is proposed as an alternative or additional protection. The protective layer (ethane, Freon 22) can be evaporated in the high vacuum of the microscope. The evaporating coolant will carry away water films deposited on top. The temperature of coolant evaporation is low enough to prevent possible devitrification of water in the specimen itself.

For the observation of frozen hydrated specimens in the electron microscope, the transfer of a frozen specimen to a cryo-holder and the introduction of the cryo-holder in the electron microscope are crucial steps. During these steps the specimen is liable to contamination, in particular by the vapour deposition of water. Frosting during mounting and transfer is not restricted to the specimen, but will occur on all cold parts of the cryo-holder. In the high vacuum of the microscope, parts of the cryo-holder will warm up (by the necessary contact with the goniometer stage) and when these frosted parts reach temperatures in the range 173–193 K, the hoar-frost will evaporate resulting in the deposition of amorphous ice on all cold surfaces (e.g. cold traps, specimen) in the vicinity. The establishment of a thermal equilibrium with the cryo-holder in the high vacuum of the electron microscope is therefore potentially the second attack by water vapour on the specimen, considering that the first attack will occur during mounting and cryo-transfer of the specimen. The two stages of vapour deposition can be illustrated by measuring the transmissivity of a specimen (using the reading of the photographic exposure timer) as a function of time (see Fig. 1). The generation of water vapour when the cryo-holder is in the microscope (second attack) is illustrated by mass spectrometric analysis (Fig. 2). For our experiments a Philips cryo-holder (PW 6361/00) was used, incorporating double shielding; an inner shield at (controlled) specimen temperature and an outer shield at environmental temperature (liquid nitrogen temperature during mounting and transfer, heating up with the outside of the

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holder). The cryo-holder was loaded with a specimen under liquid nitrogen (Dubochet *et al.*, 1982) or with a transfer device working in an atmosphere of cold and dry nitrogen gas (Lichtenegger & Hax, 1980). The microscope was equipped with a cold trap around the objective pole-pieces and this trap was cooled at least half an hour before experiments were started. The specimen used consisted of latex spheres in a free spanning film (see Fig. 4).

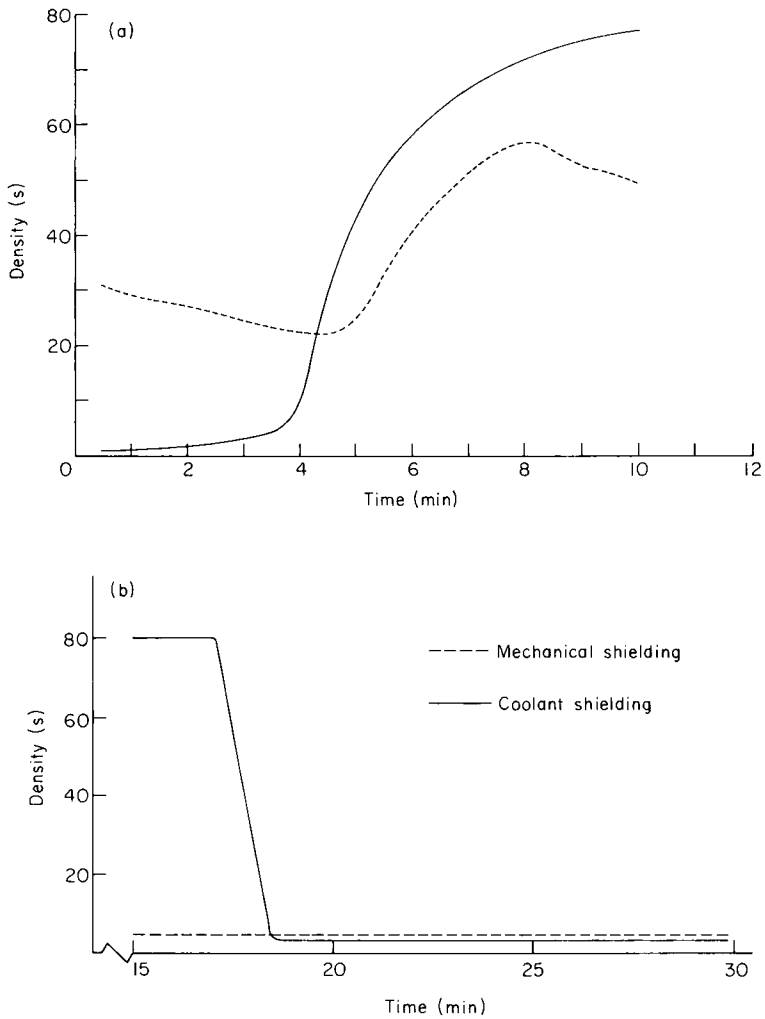


**Fig. 1.** Contamination after cryo-transfer as measured by recording the transmissivity (reading of the exposure timer of the microscope) of a specimen consisting of a thin unsupported latex film (see Fig. 4). The graphs connect the estimated values taken at 30 s intervals. See Fig. 3 for an outline of the position of the shields. (a) Contamination after cryo-transfer with mechanical shielding only. The specimen was mounted under liquid nitrogen in the cryo-holder and transferred to the microscope. Solid line: both shields of the cryo-holder in operation during the transfer; dotted line: only outer shield in operation during transfer. Note the effect of defective shielding; initially a high density when the specimen is not covered by the inner shielding. (b) Contamination free cryo-observation. A specimen was mounted with adhering coolant in the cryo-holder and transferred to the microscope (transfer temperature about 80 K). After 15 min in high vacuum the temperature of the specimen, still covered with coolant (Freon 22, solid line), was raised to about 113 K to evaporate the coolant. Hatched line: mechanical shielding only (as in Fig. 1a) but observation was started after 15 min of thermal equilibration (cryo-holder in intermediate position).

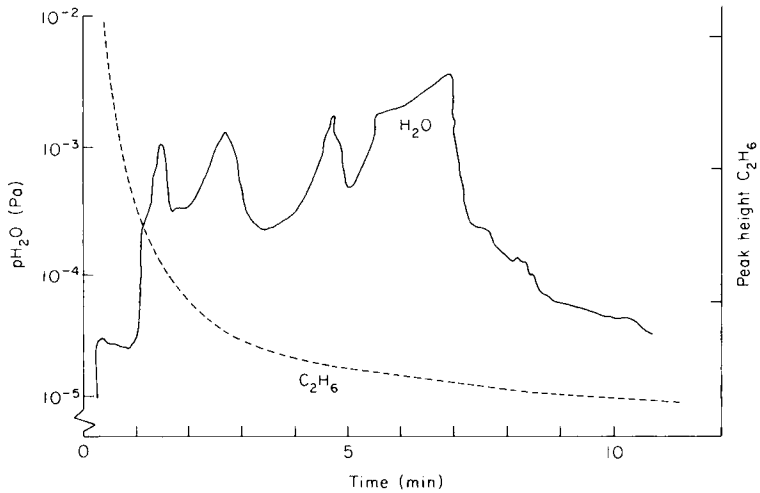


**Fig. 2.** Mass-spectrometer analysis of the vacuum conditions during cryo-transfer. The spectrometer head of the Balzer's QMG 064 was flanged to the microscope column at the level of the object space. In this position the sensor was facing the specimen through one of the openings of the cold-trap. Measurements were started when the cold specimen (initially covered with solidified ethane) entered the high vacuum of the microscope column. The partial vapour pressure of water was continuously monitored (left scale) and after a lag period a transient rise in $pH_2O$ was observed. The evaporation of ethane was estimated in a parallel experiment by scanning (scan time 21 s) between mass 28 and 32. The peak heights of mass 30 are given in arbitrary units (right scale). The first peak was out of range (too high) and excluded from the graph. The graphs shows the high rate of evaporation of ethane from the cold specimen as soon as specimen enters the high vacuum.

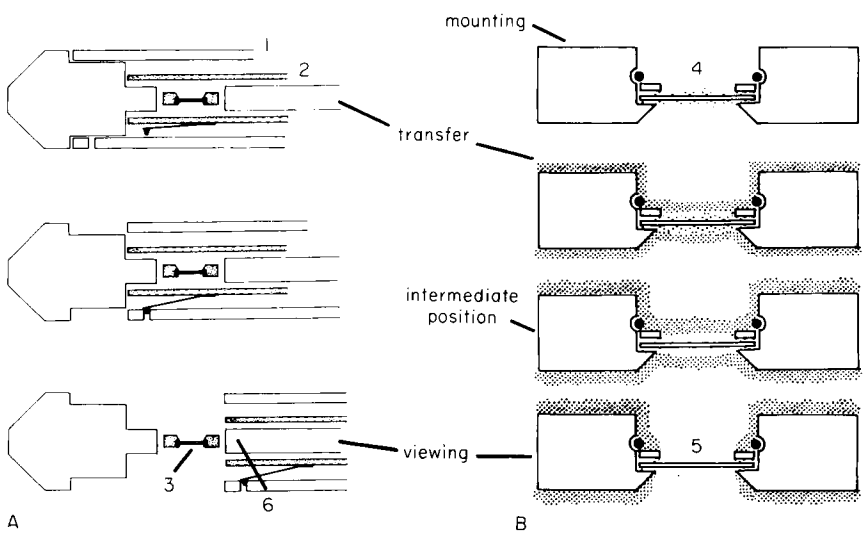


**Fig. 3.** Position of shields (schematic drawing not to scale) during mechanical shielding (A). Coolant shielding (B) is illustrated by enlarging the thermally insulated specimen area of (A). 1=Outer shield (at holder temperature); 2=inner shield (at specimen temperature); 3=specimen; 4=coolant layer; 5='cleaned' specimen; 6=position of thermocouple indicating the specimen temperature. The insulated specimen area (3) is thermally connected to a heat sink and the thermocouple is placed at the thermal junction.

Mounting the specimen under liquid nitrogen (Dubochet *et al.*, 1982) and closing both shields during transfer, resulted in some 4 min of unimpaired vision of the specimen. After this period, the rate of vapour deposition increased and practically impaired observation (Figs. 1 and 3A). The contamination under these circumstances is entirely due to the 'second attack' of water vapour, when frosted parts of the holder warm up in the viewing position. It should be mentioned that the transmissivity of the specimen upon cryo-transfer is initially the same (exposure meter reading) as after freeze drying in the microscope (decontamination by heating to 193 K).

This 'second attack' could be prevented by conditioning the cryo-holder in the high-vacuum (at least 15 min) with the cryo-holder in an intermediate position (Fig. 3). In this intermediate position the outer shields are partly opened but the inner shields (at specimen temperature) are still covering the specimen. Bringing the cryo-holder from its intermediate position to the viewing position in a shorter period (1–10 min instead of 15 min) resulted in an immediate build up of vitrified water on the specimen (see Fig. 1). From mass spectrometric determinations of the vapour pressure of water (a typical example is shown in Fig. 2) the duration and extent of this second attack can be followed. Variations between experiments were small, the shape of the curves in consecutive experiments was the same, variations were found in the lag-time (the period between introducing the specimen in high vacuum and the rise in the vapour pressure of water) and slightly in the peak heights involved.

Mounting the specimen under liquid nitrogen and transferring without protection (inner-shield disengaged), resulted in a significant contamination by vitrified water directly from the beginning of observation (Fig. 1). This contamination was collected during transfer of the cryo-holder from the liquid nitrogen reservoir to the airlock of the microscope (the 'first attack' by water vapour). With this procedure the 'second attack' came again after some 4–5 min. The protection of the specimen during transfer is thus essential and water deposition from atmospheric humidity cannot be prevented by the rapidity of the manipulation or by an additional anticontaminator inside the microscope column (Homo *et al.*, 1984).

Protecting the specimen by solidified coolant can be used in addition or partly as an alternative to the protection offered by sliding mechanical shields. A grid plunged in liquid coolant (e.g. ethane, propane, Freon 22) is transferred to liquid nitrogen. The coolant will solidify and the grid with the adherent coolant is mounted under liquid nitrogen in the cryo-holder. Without using mechanical shielding the cryo-holder is transferred from the liquid nitrogen container through the air to the airlock of the microscope and sluiced to high vacuum. The coolant, solidified on both sides of the specimen (Figs. 1 and 3B), may collect contaminating water during transfer. Ethane will remain solid in the pre-vacuum airlock but will sublime as soon as the specimen enters the high vacuum of the microscope (mass spectrometer data, Fig. 2). From this moment on the specimen is liable to the 'second attack' by water vapour and has to be protected by mechanical shields (in our experiments the inner-shields of the cryo-holder, the anticontaminator described by Homo *et al.*, 1984, may serve the same purpose).

Freon 22 will remain solid when the cryo-holder is operated at liquid nitrogen temperature even in high vacuum. Freon 22 is evaporated after 15–20 min (the duration of the second attack) by raising the temperature to 103–113 K. The evaporating coolant will carry away any contaminating layer deposited on top (Figs. 1 and 3A) without the risk of devitrification of water in the specimen itself (Heide, 1984). The protection provided by solidified coolant is in our hands a reliable procedure for removing the vitrified layers inevitably collected during the transfer procedures using an 'open' cryo-holder. A temperature control in the range 77–133 K (not available on the previous types of cryo-holders manufactured by Philips) is of course a prerequisite to ensure coolant protection of the specimen during the critical stage of the procedure.

Thus far the description of the processes involved in cryo-transfer is rather qualitative. On the basis of the kinetic theory of gases and the estimated pressures a quantitative approximation can be made for a number of processes. For the calculation of the rate of evaporation or condensation ($Q_n$) in vacuum the equation derived by Malmstrom (1951) is used: $Q_n=(P_t-P_a)\sqrt{(M/2\pi RT)}$, where $P_t$=saturated vapour pressure at specimen temperature, $P_a$=partial vapour pressure in ambient vacuum, $M$=molecular weight, $R$=universal gas constant, and $T$=specimen temperature in K. For the calculation of the rate of condensation at atmospheric conditions Fick's law was employed. During the transport of a cold specimen through the air, water, originating from the humidity of the environmental air, will be deposited on cold surfaces ('first attack'). If there is no obstruction to the flow of water molecules the deposition rate on a cold surface would be 0·2 mm/s (assuming a partial vapour pressure of $12\times10^{-2}$ Pa for water in the environmental air at 293 K). In practice, diffusion will limit the deposition rate and a concentration gradient will be established over an undisturbed (no convection of air) layer. If an undisturbed layer of 0·1 mm is assumed, which is most probably too thick for an object that is rapidly moved through the air, the deposition rate would still be 2·7 $\mu$m/s (the deposition rate takes the form of: $Q_n=D.S.\ c/a$

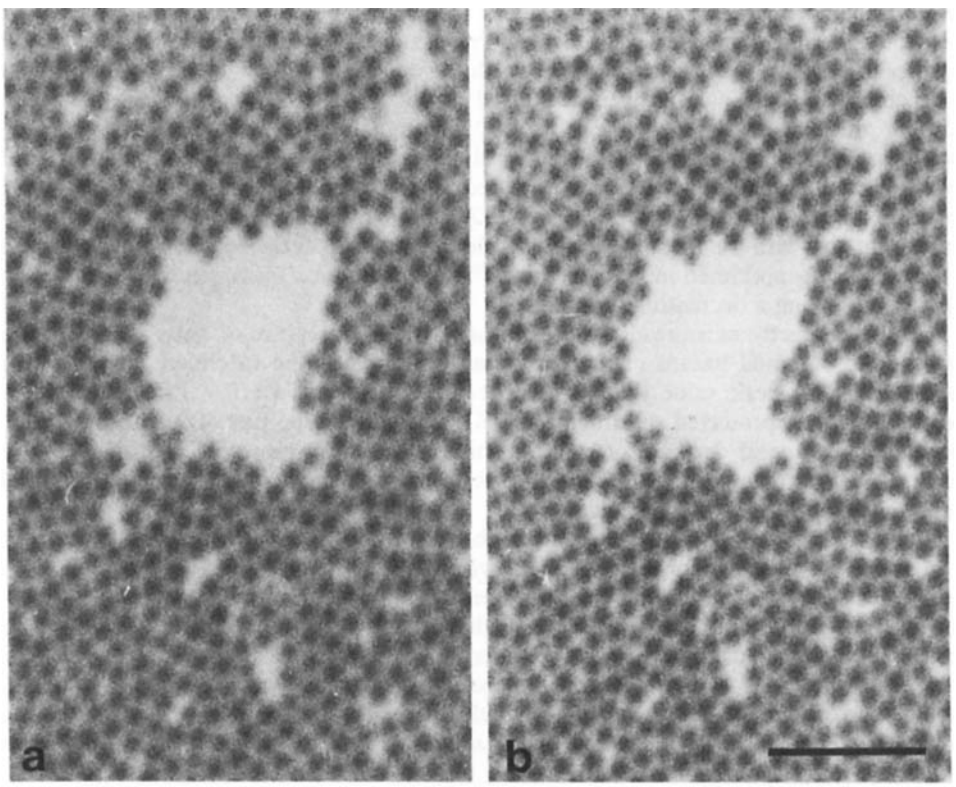


**Fig. 4.** A free-spanning film of latex spheres (∅ 91 nm) in phospholipid imaged upon cryo-transfer and cryo-observation at 77 K (a) and the same area imaged again after freeze-drying and observation at room temperature (b). Latex spheres (polystyrene latex, Polaron) were diluted 1:1 in sonicated vesicles of dimyristoyl–phosphatidyl–choline (final concentration 1 mg/ml). A 600 mesh grid, without supporting film, was drawn from the suspension, allowed to dry at room conditions and quenched in ethane (marker represents 500 nm).

where $D$=diffusion constant of water in air, $S$=surface, $c$=concentration outside the undisturbed layer, and $a$=thickness of the undisturbed layer. Cold surfaces (77 K) will thus collect contamination layers of at least 2 $\mu$m thick when transferred through environmental air. The specimen must be protected (mechanical shielding and/or coolant shielding) against this 'first attack' by water vapour. Parts of the cryo-holder will inevitably collect contamination and this may contribute to the generation of water vapour when the cryo-holder comes to a thermal equilibrium in the high vacuum of the microscope column.

The amount of coolant solidified on a specimen grid was estimated by suspending methylene blue crystals in Freon 22. Specimen grids, rapidly cooled in Freon 22 and immediately stored under liquid nitrogen, were submerged in water and the amount of methylene blue (related to the amount of Freon 22) was determined by measuring the extinction at $\lambda$=668 nm. Thin layers of coolant were found to have a thickness of about 50 $\mu$m (thicker layers may come to a few hundred micrometres). Such a layer of solid coolant will hardly evaporate in the prevacuum airlock. At this pressure an equilibrium between solid and vapour will soon be established without net transport. A thin layer of coolant represents an enormous amount of gas at high vaccuum, 50 $\mu$m solid ethane at 133 K represents 24,000 litres at high vacuum ($6{\cdot}7\times10^{-6}$ Pa). This amount of gas has to be pumped away (by the ion getter pump) or the pressure would rise (to about 0·1 Pa at 293 K in the absence of pumping). The evaporation of a 50 $\mu$m ethane layer will take some time. Assuming conditions of the triple point of ethane 99·73 K, $1{\cdot}1\times10^{-3}$ Pa (Gas Encyclopaedia, 1976), an ethane layer of 50 $\mu$m will evaporate in 11 s. At the vapour pressure of the triple point ethane can counteract the weight of a 122 $\mu$m thick ice layer. There is no evidence that contamination layers are lifted off as a whole or, more likely, that ethane escaping through cracks and pores in the contamination layer carries away pieces of contamination. However, it is clear that a solidified ethane layer leaves no residue behind upon evaporation (Fig. 4b) and the procedure is surprising subtile to the specimen (compare Figs. 4a and 4b). In practice (at 77 K) ethane will evaporate slower than at its triple point and with the mass spectrometer elevated ethane levels were observed during the first 2 min of the specimen in high vacuum (Fig. 2, note that a mass spectrometer has some delay in indicating a decreasing pressure).

The mass spectrometer readings of the partial vapour pressure of water (Fig. 2) clearly point to the potential hazard of the increasing $pH_2O$ when the specimen has entered the high vacuum. A peak value is observed for $pH_2O$ of about $4\times10^{-3}$ Pa and this would contaminate an unprotected specimen at 77 K with a deposition rate of 9·3 nm/s. This was observed as the rapid decrease of the transmissivity of an unprotected specimen (Fig. 1). During the transient rise of the partial vapour pressure it is thus essential to have the specimen in a protective environment.

The partial vapour pressure of water in the microscope (cold trap and ion getter pump in operation, cryo-holder for 30 min in high vacuum) was found to be $1{\cdot}2\times10^{-6}$ Pa. At this pressure deposition of amorphous ice becomes negligible. A rate of 0·16 nm/min at 77 K respectively 0·13 nm/min at 113 K was calculated according to the kinetic theory of gases. (In this example calculations come to molecular dimensions and a statistical approach would be more appropriate.) Without an operating cold trap a partial vapour pressure of water is found in the order of $8\times10^{-5}$ Pa and this would give rise to the deposition of amorphous ice on a cold specimen (77 K) at a rate of 11 nm/min. This rate can hardly be considered compatible with cryo-observation.

The calculations based on experimentally estimated data, as presented above, provide some insight in the magnitude of the processes of evaporation, condensation and sublimation involved in cryo-transfer experiments. The need for specimen protection during cryo-transfer and prior to cryo-observation is clearly illustrated by the examples presented. Mechanical shielding eventually assisted by coolant shielding plays an essential role in the outcome of a clean and thus successful cryo-transfer procedure.

ACKNOWLEDGMENT

We acknowledge the ongoing discussions with W. M. A. Hax (Philips, Eindhoven, The Netherlands) regarding cryo-transfer procedures.

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