Cryofixation and Cryosubstitution for Routine Work in Transmission Electron Microscopy

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Abstract

After a brief review of the present state of the theory of cryofixation, methods and instruments as well as criteria for the application of cryofixation and cryosubstitution in daily routine work in cell biology and medicine are described. Good results are obtainable using liquid nitrogen for impact freezing on highly polished copper surfaces or by plunging into liquefied propane. Based on these results a versatile and safe system for routine plunging and impact freezing for the majority of biomedical objects has been developed. In order to enable ultramicrotomy at ambient temperature a cryosubstitution system according to the Edelmann principle has been designed and applied.

KEY WORDS: Bare grid method (ice embedding), cryosubstitution, cryotransfer, impact freezing, low temperature embedding in LOWICRYL, plunge freezing, safety precautions.

Introduction

The diversity of methods for physical fixation by rapid freezing is confusing if compared with the well established conventional chemical fixation using buffered aldehyde solutions as a first processing step. This is mainly due to the variety of procedures applied to the objects before freezing. The reaction of chemically fixed and cryoprotected specimens is completely different during the quenching process to objects frozen in their fresh natural state. For example no artifacts due to ice crystal formation are observed in biological objects pretreated with the chemical fixative glutaraldehyde followed by cryoprotection in a nearly saturated sucrose solution even if these are frozen by immersion in LN$_2$ at very low cooling rates [5, 30, 43]: tissue blocks pretreated with these methods are perfectly preserved during freezing up to diameters of 0.5 mm. In contrast to cryoprotected specimens of this type, objects in their natural state can be frozen only in very thin layers with diameters $\leq$ 50 $\mu$m in order to prevent mixed protoplasmic or extracellular phases separating into ice crystals and concentrated mixed phases. It is generally believed that only cooling rates exceeding 10,000°C/s at normal ambient pressure guarantee a quality of preservation suited for subsequent TEM investigation [8, 9, 14, 23, 30, 31]. Attempts have been made to measure the cooling rates during freezing processes with thermocouples [8, 9] and to calculate the thermodynamic processes and parameters which determine the freezing process [3, 4, 23]. Unfortunately it has not proved possible to measure the real cooling rates at different positions within the objects. Miniature thermocouples with wire diameters below 10 $\mu$m are difficult to handle. On the other hand the dimension and thermal capacity of thermocouples with bead diameters exceeding 20 $\mu$m do not permit conclusions to be drawn on the freezing process in the well preserved border layer of tissue blocks or in very thin specimens. Therefore thermocouple measurements in model specimens
The most efficient method of checking the results of cryofixation is by ultramicrotomy after cryosubstitution and resin embedding of the frozen object. Routine cryosubstitution in dry ice (solid CO$_2$) is laborious and cooling systems using LN$_2$, outside Dewar vessels have a relatively high LN$_2$ consumption. Therefore a routine cryosubstitution system based on a principle according to Edelmann (private communication), as described in ref. [36] was designed and applied in this study.

Criteria for Routine Cryofixation

It is difficult to present a generally applicable list of criteria for methods and instruments suited for everyday routine cryofixation in cell biology. However, several conditions should be fulfilled regarding methodology and instrumentation, in particular:

1. The procedure should always provide reproducible results.
2. The agents required (e.g., cryogens) should be readily available to all users.
3. The procedure should be easily implemented, and not require special skills and time consuming training.
4. The method should allow quick operation and rapid repetition without limiting the number of repetitions.
5. The procedure should be as versatile as possible for different objects, covering the range from particle and cell suspensions to tissue blocks.
6. The procedure should also be versatile with respect to different follow-up preparations such as freeze cleavage and freeze etching, cryosubstitution or freeze drying with subsequent resin embedding and ultramicrotomy at ambient temperature, cryo-ultramicrotomy for morphological work in the amorphous frozen state, cryo-ultramicrotomy for subsequent element analysis in the frozen dried or frozen hydrated state, direct investigation of thin films of amorphously frozen suspensions and finally health hazards and risks must be completely excluded.

The list of requirements does not include the cost of instrumentation and of agents. It is a recognized fact that electron microscopy, ultramicrotomy and freeze etching have become routine methods without investment costs and costs for the agents used being regarded as relevant aspects. It is therefore conceivable that high pressure freezing may become a powerful tool for routine work if all other criteria are fulfilled. The use of LiHe is limited more by the question of availability and the complications involved in the handling of this cryogen than by the expense. It should be emphasized, however, that simple and less expensive procedures are preferable for routine work provided that the results obtained are identical in quality and reproducibility to...
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those obtainable with more sophisticated and expensive equipment. Careful consideration of the above listed points shows that as far as the reproducibility of the results is concerned several parameters influence the freezing process or at least limit its use as a routine procedure. The most important factors for immersion or jet methods using liquid cryogens are the temperature of the cryogen, the relative velocity "specimen versus cryogen" and the geometric shape of the specimen [8-10, 17, 30-33, 36, 37]. Precooling of the specimen before contact with the liquid cryogen must be minimized [33]. In this respect the one-sided jet procedure appears to provide better reproducibility than the double sided jet method [31]. Based on theoretical considerations the latter was expected to be more effective because of the heat exchange on both sides but it requires perfect synchronization of the cryogen extrusion by both nozzles. In our opinion the simplest and most reproducible preparation is provided by immersion of sandwich specimens with velocities of approx. 2 m/s. Although the cooling rates obtainable with this simple immersion are certainly lower than with jet methods, immersion would appear to be the best suited method for routine because of the advantages already mentioned and the exclusion of risks associated with the use of larger volumes of cryogens (especially propane) for jet freezing (point 7).

With reference to point (2) LN₂, pure propane, ethane and halogenated hydrocarbons (FREON or FRIGEN) are readily available and therefore suitable for routine work. On the other hand LHe is not available everywhere and requires special precautions and instrumentation. As the potential advantages of LHe have not been confirmed in comparisons [6, 29] and are not supported by thermodynamical considerations [3, 23], it is not regarded as a particularly recommendable cryogen for routine work. Figs. 1 and 2 show typical pictures of biological material frozen on a copper mirror with a very simple laboratory device, cooled only with LN₂. The depth of the well preserved zone and the quality of preservation appear to be identical to the best results reported for LHe cooled mirrors [14, 18, 44]: in this respect the results on LN₂ cooled mirrors confirm the theoretical considerations. With reference to point (3), LN₂, cooled mirror or immersion systems using LN₂ as primary cryogen and propane, ethane or a halogenated hydrocarbon as secondary coolant are preferable because they enable rapid processing without lengthy precooling cycles. Unlimited repetitions with a repetition period of approx. 2 to 5 minutes are feasible. This is an improvement on systems using LHe, since both O₂ and N₂ precipitate on LHe cooled surfaces and require special precautions.

It is impossible to determine the most versatile method (points 5 and 6). Cryofixation of suspensions [1, 10, 12, 17, 24, 40-42] is easily achieved using liquid coolants, whereas the cryofixation of tissue blocks in their natural state for subsequent cryo-ultramicrotomy appears to be considerably facilitated by impact freezing [5, 14, 18, 29, 34, 44-47]. Therefore one may conclude that at present both methods should be taken into consideration and applied. Both methods enable amorphous vitrification of biomedical systems. High pressure freezing, which is still in an introductory phase [28] appears to cover the whole area effectively as long as only crystalline freezing is concerned, since temperatures < -140°C required for the amorphous solidification of mixed H₂O phases [11, 12, 25] cannot be reached and maintained at present. As mentioned above, high pressure work was excluded from this study because of the experience and instrumentation this requires and bearing in mind the excellent work already performed at the ETH Zürich.

Criteria for Routine Cryosubstitution

In contrast to routine cryosubstitution a generally applicable list of criteria for methods and instruments suited for every day cryosubstitution in cell biology can be easily drawn up. In particular the following requirements should be met: (a) the normal period of one substitution procedure should be possible without refilling the cryogen (LN₂, solid CO₂), (b) loading the instrument with the frozen specimens should be possible without running the risk of ice recrystallisation, (c) loading capacity should be suitable for synchronous processing of at least 10 individual specimens of usual size (diameter ≤ 10 mm, thickness ≤ 2 mm) in separate containers, (d) the system should also be able to manage specimens with larger diameters (metal mirror frozen disks of up to 15 mm in diameter), (e) presetting of the time/temperature-sequence for automatic substitution should be possible, (f) the substitution medium has to be gently moved in order to avoid the formation of enhanced H₂O concentration zones around the specimens, (g) exchange of the substitution medium against an identical fresh mixture or against a different mixture for staining or embedding should be possible without change in temperature or risk of introduction of H₂O traces, (h) the whole substitution or resin incubation procedure should take place in an inert dry gas atmosphere, and (i) total exclusion of health hazards and risks has to be guaranteed.

Similar to the considerations in the preceding chapter, the cost of instrumentation and agents is not of primary importance. It is evident, however, that the daily costs (e.g. cryogen consumption, amount of substitution medium required for each specimen), the efficiency and the flexibility of the system are of more relevance in cryosubstitution than in cryofixation if compared to the investment
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for instrumentation. Here the fact that a cryosubstitution normally runs for 1 to 3 days has to be taken into account. For routine work or if apolar substitution media are used for subsequent analyses, instruments for cryosubstitution may be operated over extended periods or even all year round. Under these conditions the difference between 21 LN2 and, for example, 20 l LN2 per day is certainly noticeable. The same applies to the minimum amount of substitution medium required for one substitution process for a single specimen. Experience has shown that often only a few specimens or even a single specimen are processed at any one time. In addition to the cryogen consumed during processing the amount of substitution medium needed for one specimen becomes an important factor in routine work when the expenses of OsO4 used to stabilize structures before resin impregnation and polymerization[20-22,39,45] are considered. From the practical point of view this question may be more important than the number of specimens which can be processed synchronously (point c). Besides these trivial facts one substitution cycle (point a) lasts between 1 and 3 days. Recrystallisation of ice has to be expected at temperatures >-80°C (point b); therefore the loading chamber must guarantee temperatures ≤-80°C during insertion of the frozen specimens. Our experience shows that it is rare for 10 specimens (point c) to be handled during one cycle. In contrast to chemical fixation followed by dehydration at ambient temperature, each cryofixation is an individual process and frozen specimens can be divided only after substitution, mostly after resin embedding, as accurate and reproducible splitting of brittle, frozen specimens is very difficult. The time/temperature-curve seems to be important if delicate or larger specimens (volume ≥ 5 mm3) are to be processed (point e). Accordingly [39], a continuous, slow temperature increase is probably the most gentle method and better suited than a step by step rise in temperature[26,45] or a rapid increase as observed in the low temperature range when warming containers of any size to ambient temperature. During substitution temperature has to be kept constant with an accuracy of less than ±5°C in order to guarantee good and reproducible results.

The convection of the substitution medium (point f) is as important as in standard tissue processing at ambient temperature. During the whole procedure, including the exchange of media, the substitution medium has to be carefully kept separate from humid room atmosphere, since traces of H2O are immediately taken up by the cold media. During low temperature embedding in LOWICRYL [7,20-22] contact with O2 should also be avoided, as this inhibits polymerization. Covering the substitution container with inert dry gaseous N2 (GN2) or CO2 excludes all harmful effects of rehydration or oxygenation (point h). Not only the known risks of working with cold liquids, especially LN2, have to be excluded but also the danger of being poisoned by OsO4 in the substitution media.

With respect to the sublimation temperature of solid CO2 (-78,5°C) LN2 has proved to be superior as coolant since temperatures of approx.-80°C are easily obtainable. There is also an advantage compared to deep-freeze units using compressor systems, which often do not reach the required standard temperature of -80°C reproducibly and therefore create difficulties if the ambient temperature reaches values ≥ +30°C. For these reasons we considered a small container in the neck of a Dewar vessel [36, 37] to be the simplest and most suitable solution: without any other special precautions or constructions the continuously evaporating GN2 prevents the substitution or embedding media coming into contact with the humid ambient atmosphere. Substitution in apolar media and embedding by low temperature media as LOWICRYL and subsequent element analysis can be carried out in the inert dry GN2 without risk.

Materials and Methods

In the course of this study different specimens were frozen by immersion in liquefied propane at -190°C or in liquefied ethane at -170°C or by impact on a LN2-cooled metal mirror. The following tissues were investigated: liver, kidney and muscle from mice and frogs; leaves of green plants and petals. Suspensions were investigated using fresh blood and centrifuged blood or blood fractions (e.g., thrombocyte suspensions). All objects were prepared as quickly as possible with a minimum of mechanical and thermal influence. In order to avoid rapid water evaporation free surfaces were carefully maintained in a humid state either with physiological solutions, which were removed immediately before quenching, or in a humid chamber. All objects were quenched without chemical fixation and/or cryoprotection. Different quenching procedures were used for different follow-up preparations.

**Plunging:** As can be seen in Fig. 3a, plunging of small tissue blocks 0.5 to 1.0 mm in diameter was effected on small aluminium pins. The

Fig. 1 Frog kidney tissue slammed against LN2 cooled copper mirror. TEM magnifications 5,000 : 1 (a) and 9, 200 : 1 (b). Substitution in acetone containing 2% OsO4. Embedded in SPURR. Sectioned exactly transversally to the contact surface between specimen and mirror plane. Contact surface marked with arrow. Distance from the contact surface calibrated in μm along dashed line.

Fig. 2 Mouse liver tissue slammed against LN2 cooled copper mirror. All technical data as in Fig. 1.
Fig. 3  Different modes of specimen mounting for routine immersion cryofixation ("plunging").
(a) Object O for subsequent cryosectioning in the frozen hydrated state mounted on a light weight pin P. Sucrose or gelatine solution G is used as glue. The pin is affixed to the holder H on the injector rod I. Plunging of tissue blocks appears to be advantageous only where pretreated specimens (aldehyde fixation and/or cryoprotection) are to be frozen for histochemical work.
(b) Sandwich foils F1/ F2 for subsequent freeze cleavage of suspensions held by tweezers T.
(c) Bare grid B for subsequent "ice embedding" ("bare grid method") and direct transmission electron microscopy held by tweezers T.
(d) Immersion of suspension S in the wire loop W allows freezing for subsequent cryosubstitution, resin embedding and sectioning at ambient temperature.
(e) Application of a microdroplet D (diameter ≤0.1 mm) onto the top of a needle-like extension E of the special pin P' for subsequent cryosectioning of the amorphously vitrified particle suspension.

Tissue blocks were mounted onto a layer of nearly saturated sucrose solution or a fresh 2% gelatine solution maintained at a temperature of 30°C before gluing. The tissue blocks were either curved or cut into tiny pyramids. Plunging was realized with the apparatus described below with an entry velocity of approx. 2 m/s. The depth of cold GN2 penetrated before contact with liquid cryogen was between 10 mm and 40 mm (GN2 temperatures -100°C to -160°C). In most cases pure propane at a temperature near its freezing point was used. Sandwich specimens for subsequent freeze cleavage and freeze etching as set out in Fig. 3b were inserted between BALZERS sandwich elements (BUO 12054 T/056 T) and held by stainless steel tweezers. In this way the specimens were immersed in liquid propane. Bare grids held by standard steel forceps were loaded with suspensions and immersed into liquid ethane at a temperature near its freezing point (Fig. 3c). In these experiments the depth of cold GN2 penetrated before contact with ethane was also either 10 mm or 40 mm. The vitrified suspension layers were directly transferred in a Zeiss cryotransfer chamber to a Zeiss EM 10 CR transmission electron microscope[19, 37]. Transfer and investigation were carried out at temperatures below -140°C. Particle suspensions for subsequent cryosubstitution
Features of a Routine System for Cryofixation

Based on the studies described above a simple and versatile apparatus was designed suitable for both immersion and impact cryofixation. This apparatus is manufactured by the C. REICHERT Optische Werke AG, A-1170 Vienna (Austria) and will be described in detail elsewhere. Some features of this apparatus are of interest with respect to the general confusion mentioned in the Introduction and difficulties encountered in the past.

Some of these difficulties are listed below: one problem often observed in cryofixation by plunging is the maintenance of the correct temperature for the secondary cryogen. In order to obtain optimum cooling rates the temperature of this secondary cryogen should be near its freezing point. At this temperature partial freezing may occur and interfere with the preparation of the object foreseen for cryofixation. Experiments often have to be stopped and begun again after melting the frozen cryogen and renewed cooling to the freezing temperature. A second problem is caused by temperature gradients inside the secondary cryogen. These are due to partial cooling by LN₂ and differences in the temperature of the GN₂ in the vicinity of the cryogen container and the surface of the secondary cryogen. These temperature gradients are minimized by vigorous stirring. The temperature within the cryogen has to be measured continuously as this parameter is of importance for the freezing process. Experimental systems incorporating all the prerequisites mentioned (Fig. 4 a) are fairly complicated both in their assembly and operation and are therefore not well suited for routine use. The first goal was therefore to design an improved system, which is set out in Fig. 4 b. The newly developed system includes an insulated container C for LN₂ as primary cryogen. An aluminium cylinder A is connected to the bottom of C by a thin walled tube T and partially surrounded by a sleeve S of height H. As long as the LN₂ level remains below H there is no direct contact between LN₂ and the surface of A. The heat transfer by GN₂ through the tube T is sufficient to hold temperatures down to -190 °C within the vessel V containing the secondary cryogen (e.g., propane P). Since the heat exchange with LN₂ and GN₂ is fairly small, the temperature of cylinder A may be increased to -100 °C without a noticeable increase in LN₂ consumption. The temperature of cylinder A is thermostatically controlled with an accuracy of ±1 °C by the temperature sensor X and the heating resistor R. Due to the good heat conductivity of aluminium the temperature of the cryogen P at the bottom of vessel V differs at the most by 2 °C from that at the upper surface. Therefore a stirrer is not required and the results obtained with this fully automatic system are absolutely reproducible. Different cryogens with freezing points of between -100 °C and -190 °C can be used without difficulty.

Another important feature of the new system is the reduction of the distance D between the sharp boundary B of the ambient atmosphere and the GN₂ inside the vessel V and the surface of the cryogen P. Along its path (arrow) an object O mounted onto the injector rod I has to penetrate the distance D through the cold GN₂. If fresh specimens have to be frozen, precooling phenomena within a distance D > 30 mm may occur in thermally sensitive parts (e.g., lipids of cell membranes) of the object O [9, 10, 33, 48]. Therefore in special cases it is advantageous to reduce the distance D to less than 10 mm since it is a known fact that near boundary B the temperature drops from the ambient value (approx., +20 °C) to values ≈ -100 °C within a few millimeters. Fig. 4 b shows that D is easily reduced by raising vessel V for the brief interval required for plunging (interrupted lines in schematic drawing...distance D'). Finally the hazard associated with the use of propane P...
as the most suitable cryogen can be eliminated by the transfer of the vessel V into a container which allows safe burning of the potentially dangerous propane within the laboratory (see Fig. 6).

As discussed above it is not possible to solve all problems by either plunging or impact freezing. Fig. 4c presents a simple solution: replacement of the cryogen vessel V by a metal block M. In the same way the injector I for immersion cryofixation is exchanged against a spring loaded slamming rod K. If Figs. 1 and 2 are compared with pictures in papers from other authors [18, 44], it can be seen that there is no difference to pictures obtained from objects slammed against LHe cooled surfaces. The system works very simply with rod K powered by the spring Y (force approx. 5 kg at the start position and 2 kg at the end position). After mounting object O onto the elastic and thermally insulating foam rubber F, the release element E (arrow) frees the rod, which slams the object O with considerable force against the mirror surface. For rapid repetition of subsequent freezing cycles both the foam rubber F and the copper cylinder M' with the mirror surface can easily be substituted. The system is then ready in less than 5 minutes for the next freezing process. The whole metal block M/M' reaches a temperature of approx. -190 \degree C if the LN₂ level exceeds height H of the sleeve S.

**Operation of the Cryofixation System - Cryotransfer Facilities**

The routine system (Fig. 5) has a modular design for various applications and can be assembled in different arrangements. The basic unit B includes the insulated LN₂ container and a support plate for a variety of injector systems. Fig. 5
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Fig. 5  REICHERT-JUNG universal system KF 80 for routine cryofixation. The picture shows the basic unit B with the injector I for the plunging of bare grids as illustrated in Fig. 3 c or sandwich preparations as depicted in Fig. 3 b. On the left-hand side the automatic LN$_2$ refilling unit U for continuous standby operation is mounted onto the Dewar vessel D. In the center the Zeiss cryotransfer chamber T is mounted onto the entry port on panel P (see text and Fig. 6a).

shows the injector type I equipped with forceps F for the injection of bare grids [1, 37] and sandwiched specimens. While applying the suspension onto the grid the LN$_2$ container is closed by the cover C with the glass window G, which separates the specimen from the cold GN$_2$. Object mounting can be observed through the stereomicroscope S, which can be swung out sideways with its holder H if not required or if the cryotransfer chamber T [19, 37] is mounted onto the entry port. The microscopic observation of the preparation process is considerably facilitated by the illumination L of the specimen through the glass windows G/G’ (see Figs. 4 b and 5).

Immediately before injection is started by pushing the release button R, the cover C is pushed aside. In addition to the LN$_2$ container, set out in the schematic drawings in Figs. 4 b and c, the basic unit incorporates the electronic system for automatic LN$_2$ refilling, the LN$_2$ filling level indicator, elements for temperature indication and control and all safety installations. The temperature may be displayed for either the aluminium container with the secondary cryogen or for the GN$_2$ compartment above the cryogen 10 mm below the boundary layer separating the cold GN$_2$ and the room atmosphere (see B in Fig. 4 b). All buttons and indicators are positioned on the front panel P including the LED diagram indicating the LN$_2$ level both in the basic unit and in the 25 l vessel D of the automatic refilling system. The safety installations provide optical and acoustical alarm signals before the LN$_2$ in the container of the basic unit B or in the Dewar vessel D is exhausted and a separate warning if the temperature of the aluminium container for the secondary cryogen rises above -70°C so that unintended cryogen evaporation can be prevented.
Injector I (Fig. 5) is easily exchanged against the injector system for pins or the injector rod for metal mirror cryofixation (see Fig. 4 c). In the same way the standard vessel normally used for the liquid propane (see V and P in Fig. 4 b; volume approx. 20 ml) is exchanged against a vessel with a reduced volume of 3 ml especially designed for the use of pure liquid ethane for the bare grid method [1, 37]. Furthermore the vessels for liquid cryogens are exchangeable against a metal block for impact freezing (see M/M’ in Fig. 4 c). All these manipulations require no special experience and can be carried out within one minute. The rapid changes from plunge freezing to impact freezing and vice versa enable the apparatus to be used alternatingly for these different methods alternatively. LN₂ can be refilled by hand with a small LN₂ container with a volume of approx. 4 l. If standby operation is required, the automatic refilling system with the Dewar vessel D and the refilling unit U (Fig. 5) of the FC 4 D cryosectioning system may be used. This automatic unit permits uninterrupted standby operation over a period of more than 12 h. Cryotransfer of frozen specimens to a TEM or SEM is possible in all transfer chambers [19, 37, 49, 50] compatible with the FC 4 D cryosectioning system (e.g., Zeiss and HEXLAND cryotransfer systems). The cryotransfer of frozen specimens to other instruments (e.g., freeze etch and cryosectioning devices, loading chambers for cryotransfer) is possible within small containers, which can be filled with LN₂ with a special LN₂ lifter incorporated into the basic unit.

Special attention was paid to ensure hazard-free use of the potentially dangerous propane. The system therefore includes a countercurrent liquefier suitable for propane and all other gases used.
Fig. 7 Schematic cross sections of substitution systems according to Edelmann. See text and Fig. 8. - (a) First prototype: the aluminium container A is located inside the neck N of the Dewar vessel V. LN₂ for cooling is not in direct contact with the container A (distance... D). - (b) Second prototype similar to the REICHERT-JUNG system CS-auto: substitution container A₁/A₂ is thermally insulated by polyurethane foam insulation I and connected by the metal tube T with the aluminium rod R, which is always in direct contact with LN₂. - (c) Top view (above) and cross section (below) of the substitution container A₁/A₂ with attachments T/R and surrounding thermal insulation I.

as secondary cryogens (e.g., ethane and halogenated hydrocarbons). To the best of the authors’ knowledge the closed liquefying system meets all safety regulations. This also applies to the elimination of liquefied cold propane illustrated in Fig. 6: the vessel V with the liquid propane P (see also Fig. 4 b) can be lifted out of the aluminium cylinder C with a special manipulator M. It is then transferred into the steel tube T and immediately sealed with the cover element E. A propane burner U allows the propane gas to be safely eliminated in the laboratory. Nevertheless it is recommended that the responsible safety officer be contacted before using propane in the laboratory.
Features of a Routine System for Cryosubstitution

The development of a cryosubstitution unit suited for daily routine work is based on the system shown in Fig. 7 a: the aluminium container A with a cylindrical hole for the substitution medium M is mounted onto a thermally insulating cover C and positioned in the neck N of the Dewar vessel V. Between the bottom of the aluminium cylinder A and the surface of the LN$_2$ in the vessel V a shield S is inserted to avoid additional LN consumption by thermal radiation from the counter heated container A. The LN$_2$ level is always kept at some distance D to the shield S. The container is therefore only cooled by the GN which continuously evaporates from the LN$_2$ and flows through the slit between the neck N of the vessel V and the cylindrical surface of the container A (indicated by arrows). The GN finally escapes through the holes H/H' in container A and the cover C. The frozen objects O are deposited on a metal grid G and are thus freely exposed to the surrounding medium M. Under optimum conditions the container A reaches a temperature of approx. -80°C measured with the thermal sensor X. The temperature may be increased above this steady state temperature by using the electric heater E. Elements X/F thus enable thermostat temperature control as well as controlled temperature increase. The prototype illustrated in Fig. 7 a allows substitution in the inert GN$_2$ with a consumption of 2 L LN$_2$/day even if the container is heated to temperatures of approx. +20°C. However this very simple apparatus presented several problems viz: (1) the minimum temperature of the container A depends on the distance D between container and LN$_2$ level and rises as the LN$_2$ level decreases, (2) the exchange of media M is difficult, (3) it is not possible to process different individual specimens synchronously and (4) the minimum amount of substitution medium M is too much for routine processing of a single object O.

For these reasons a modified second prototype was designed (Figs. 7 b and c). The main differences to the first prototype (Fig. 7 a) are: (a) the substitution container consists of two separate aluminium parts, an outer holder A$_1$ and an insert A$_2$, which are thermally insulated by a polyurethane foam insulation I against the LN$_2$ compartment, (b) heat extraction from the containers A$_1$/A$_2$ takes place only through the tube T and the aluminium rod R (diameter 40 mm), which touches the bottom of the Dewar vessel V and therefore is always in direct contact with LN$_2$, (c) the insert A$_2$ can be turned on its longitudinal axis YY so that either a single hole H$_1$ or 3 communicating holes H$_2$ are ready for operation. These holes correspond to a stirrer wheel W connected to a motor drive and with the holes H$_3$/H$_4$ for filling and emptying the holes H$_2$/H$_4$, (d) the elements A$_1$/A$_2$ are mounted in the thin walled tube B of special steel alloy of low thermal conductivity, (e) heat uptake of the containers A$_1$/A$_2$ is further minimized by the polyurethane foam stopper F connected to cover C which seals the upper half of the neck N of the Dewar vessel, and (f) holes H$_5$ or H$_6$ are all able to take up to 6 individual containers A$_3$ for single specimens or charges of similar specimens which can then be processed simultaneously.

The drawbacks of the first prototype illustrated in Fig. 7 a above have been completely eliminated in the second prototype set out in Figs. 7 b and c. The minimum temperature of the container is approx. -80°C and depends only minimally on the LN$_2$ filling level. Filling and exchange of media is possible without opening the cover C/F. Up to 23 different specimens or charges of similar specimens can be processed synchronously and the minimum amount of substitution medium required for the substitution of a single object is approx. 3 ml.

The REICHERT-JUNG CS-auto (Fig. 8) offers additional features of relevance for daily routine work which will be described in detail elsewhere. For example opening cover C'/F is coupled to additional evaporation of GN$_2$ using heating element E' (Fig. 7 b) and is implemented slowly by a motor driven lifter system. This automatic process ensures that the gap between the foam stopper F and the containers A$_1$/A$_2$ is immediately filled with evaporated GN$_2$ rather than with humid ambient air. A special illumination system provides a good view of the substitution chamber during loading and unloading of the specimens for the final embedding procedure. All electric elements and printed circuits are incorporated into the unit. The time/temperature-programme is easy to preset with knobs on panel P which also contains all other indicators and controls (e.g., functional diagram showing the LN$_2$ level in the incorporated 10 1 Dewar vessel and of the 35 1 vessel for automatic LN$_2$ refilling). A refilling system automatically refills the unit's 10 1 Dewar vessel if cycles longer than 5 days are necessary for substitutions in spolar media or of larger specimens.

Operation of the Cryosubstitution System - Low Temperature Embedding

Cover C' is opened and the unit (Figs. 7 b and c and Fig. 8) is filled either through a funnel connected to a long thin walled metal tube reaching to the bottom of the Dewar vessel D or by an automatic filling system similar to the system used for the REICHERT-JUNG low temperature sectioning system FC 4 D (volume 35 l). The Dewar vessel D takes approx. 8 l of LN$_2$. This is sufficient for at least 4 days automatic operation. After filling the vessel V the container A$_1$/A$_2$ is cooled down by LN$_2$ filled manually from a small LN$_2$ can into the holes H$_2$/H$_3$/H$_4$. After the required temperature is reached (e.g., -80°C) the fro-
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zen objects O are introduced into the open chamber, which can be flushed during the operation with cold dry GN\textsubscript{2}, using the evaporator E'. The temperature of the GN\textsubscript{2} in the chamber always remains below -100 °C during this filling procedure. Any risk of recrystallization of the frozen specimens can therefore be excluded by additional GN\textsubscript{2} flushing. After loading the chamber is closed by the cover C' and the substitution medium is introduced through the filling hole H\textsubscript{o} or H\textsubscript{p}. It initially penetrates a counter-current heat exchanger inside the aluminum insert A\textsubscript{2} and therefore has a low temperature when it reaches the substitution holes H\textsubscript{o} or H\textsubscript{p}/H\textsubscript{p}. This enables the introduction of the substitution medium M at room temperature and further considerably reduces the risk of H\textsubscript{2}O uptake from humid ambient air. The water contact of the substitution medium can be further minimized if the substitution medium is filled in through a column containing water adsorbing granules (e.g., dried molecular sieve). This improves the reproducibility of substitutions with apolar media for subsequent element analysis. In this case all steps from substitution to monomeric resin impregnation and polymerization can be carried out within the inert dry GN\textsubscript{2} inside the chamber. Initial results are encouraging and it appears possible to use conventional media (e.g., epoxides and polyesters) as well as low temperature inclusion media such as LOWICRYL in this unit. Normal substitution processes (e.g., in Van Harreveld's acetonе ОSО\textsubscript{4} mixture) can be carried out without exchanging the medium, since the volume ratio "specimens : substitution medium" is extremely small (<1: 1,000). An increase in the H\textsubscript{2}O concentration during the substitution process is, in these cases, negligible.

Conclusions

The study shows that both plunging and impact freezing using LN\textsubscript{2} are necessary and suited for daily routine work if all parameters influencing the quality of the resulting cryofixation are kept at reproducible values. It is particularly important to keep the temperature of the secondary organic cryogen near its freezing point. Thermal gradients inside the cryogen should be excluded by a thermostatically controlled metal envelope of good thermal conductivity. The depth of cold GN\textsubscript{2} penetrated by the injected specimen should be minimized, if sensitive or very thin specimens (e.g., thin films of particle suspensions for direct TEM investigation in the frozen hydrated state or sensitive lipid layers) are cryofixed. The entry velocity of the object into the liquid cryogen should be standardized. Since liquefied propane provides the best results, safety precautions required for daily routine work in the laboratory have been designed and implemented with the special systems and elements described above. For immersion cryofixation the simple spring loaded injector provides entry velocities of up to 2.5 m/s. The development of an injector system for higher velocities of up to 15 m/s presents no severe problems but at present the results of plunging appear to depend more on the geometrical shape of the specimen and the cover layers in sandwich preparations than on the entry velocity \cite{10,17}. This is particularly true for tissue blocks, which are usually easier to freeze and are better frozen by impact. The use of LN\textsubscript{2} and the simplification of the injector system have made impact freezing less complicated. In this respect our own studies agree with the results obtained by

![Fig. 8 REICHERT-JUNG system CS-auto for routine cryosubstitution (see also Figs. 7 b and c).](image)
Phillips and Boyne [6, 29]: neither LHe nor sophisticated systems appear to improve on the results shown in Figs. 1 and 2. Based on these findings an advanced, versatile and simple cryofixation system suitable for daily routine work has been designed. An equivalent system has also been developed for cryosubstitution. This unit not only works on a low budget due to the low LN₂ consumption and the minimum amount of the substitution medium but also guarantees reproducible results due to simple and risk free handling of the specimens, accurate temperature control and continuous operation in dry and inert \( \text{N}_2 \) atmosphere.

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References

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Additional References


Notes Added in Proof

The metal mirror system has been redesigned so that soft specimens are more gently applied onto the mirror surface [51]. A special pneumatic damping element of minimal mass combined with a very soft intermediate layer permits mirror cryofixation of fragile specimens without disturbing distortions and compression artifacts appearing in the structures. All parameters influencing the quality of freeze preservation, especially the application velocity and the final pressure between specimen and mirror plane can be preset independently over a wide range. Special accessories for the KF 80 have been developed both for plunging and slamming in order to minimize H₂O evaporation during mounting of the specimens and movement towards the cryogen or the mirror surface.

The cryosubstitution system has been complemented by an accessory for LOWICRYL embedding in the substitution chamber with UV-light in dry inert GN₂. This accessory [52] consists of an embedding plate manufactured from an antiadhesive plastic material with 50 molds for individual specimens and a capacity of up to 100 small specimen blocks if every mold is used for two specimen blocks. The specimens are transferred under GN₂ from the individual containers of the substitution system into the molds. For radiation polymerisation a UV-light source is mounted replacing the cover which normally closes the substitution chamber. Polymerization is possible at all temperatures ≥ -80°C. Finally all hazards presented by OsO₄ vapours are excluded by a special system of tubes connected to an adsorbing filter element. Following suggestions made by Alan Boyde it has been proved, that all substitution media containing OsO₄ and aldehydes in organic solvents can be easily prepared and mixed inside the substitution chamber. This excludes any risk resulting from an exothermic oxidation reaction.