Vitrification of cryoelectron microscopy specimens revealed by high-speed photographic imaging

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Summary
Cryoelectron microscopy is a widely used technique to observe biological material in an almost physiological, fully hydrated state. The sample is prepared for electron microscopy observation by quickly reducing its temperature to −180 °C. The high-speed cooling induces the formation of vitreous water, which preserves the sample conformation. However, the way vitrification occurs is still poorly understood. In order to better understand the phenomenon, we have used a stroboscopic device to visualize the interaction between the electron microscopy grid and the cryogen. By blocking the free fall of the plunger once the grid has penetrated the coolant by half its diameter, we have elucidated the way in which vitrification propagates. The findings were confirmed by numerical simulation. In addition, according to our observations, we now present an alternative way to prepare vitreous specimens. This new method, with the grid parallel to the liquid cryogen surface, decreases evaporation from the sample during its free fall towards the coolant and at the same time achieves a more uniform vitrification over the entire surface of the specimen.

Introduction
Cryoelectron microscopy was developed in the early 1980s (Adrian et al., 1984; Dubochet et al., 1988). It is widely used today for observing biological materials with high resolution in an almost physiological environment (Harris, 1997; Harris & Adrian, 1999). The main advantage of this method comes from the fact that the sample conserves its native fully hydrated physiological state during electron microscopic observation. To achieve this, the sample is deposited onto a grid and cooled at such a high speed and to such a low temperature that the water molecules contained in the sample pass from a liquid to a vitreous state without crystallizing. The method has been successfully applied to observe many kinds of fully hydrated materials that are unfixed and unstained. The vitrification is obtained by rapidly immersing the sample into a liquid cryogen, such as propane or ethane. The cooling speed and the evaporation rate of the liquid before the grid touches the cryogen are believed to be the most sensitive parameters determining the quality of the specimen preservation (Cyrklaff et al., 1990; Battesby et al. 1994; Trinick & Cooper, 1990). In order to optimize these parameters, several technical solutions have been proposed (Ballare et al., 1988, 1998; Trachtenberg, 1993; Talmon, 1996; Egelhaaf et al., 2000). However, the exact influence of these parameters is still poorly known.

In order to better understand the way in which the sample vitrifies, we have used a stroboscopic system to observe in greater detail the penetration of the grid into the coolant. An apparatus was designed to take pictures of the falling grid using a light flash of a few microseconds duration. Images of the grids touching the surface of the cryogen produced by this apparatus reveal the way the cryogen behaves during immersion. According to these results, we propose an explanation of the way vitrification progresses through the specimen and we suggest a new procedure to achieve a more efficient and homogenous vitrification of the sample.
Materials and methods

Vitrification

For vitrification, 4–5 μL of water was deposited on a perforated carbon film supported (average hole size 2 μm) by a 3-mm-diameter copper electron microscopy grid. Excess fluid was removed by blotting with filter paper. The grid was then immediately plunged into liquid ethane by the use of a gravity plunge freezing apparatus described elsewhere (Harris, 1997; Harris & Adrian, 1999).

Blotting for a horizontal grid

Blotting for horizontal grids was performed in the same way as for the ‘classical’ vertically mounted grids.

High-speed imaging

The stroboscopic imaging set-up consists of a light barrier connected to a specially designed electronic circuit that drives two large LED panels and a digital camera (Fig. 1). The set-up has to be operated in darkness with the digital camera shutter open (Nikon®, Coolpix 990). When the grid crosses the light barrier during its approach, it starts a digital timer whose value is compared to a user-defined delay. When the two numbers are equal, the electronic circuit turns on two LED panels over a period of 5 μs and illuminates the scene, which is captured by the camera. The electronic circuit can also trigger a user-defined number of light flashes with a predefined delay. A detailed description of the apparatus can be found in Kasas et al. (2003).

Falling speed determination

The velocity of the grid before its contact with the cryogen was calculated using images recorded by the high-speed imaging system. In these images, the sample has been illuminated by two flashes separated by 2 ms just before entering the coolant. The distance between the two grids appearing on these pictures allowed us to calculate the speed of the plunger at the moment the sample touches the cryogen surface.

Electron microscopy

The perforated carbon film on Cu grids were transferred into a Philips® CM12 electron microscope equipped with a Gatan® cold trap and observed with a Gatan® cryo-specimen holder model no. 626. The extent of vitrification was checked over the entire grid by observing the frozen sample by electron diffraction.

Vitrification propagation front detection

The way vitrification occurs on the grid was studied by stopping the plunger during its free fall from a height of 8 cm, once the grid was immersed into the cryogen by half its diameter. The grid was then rapidly immersed in liquid nitrogen following standard cryoelectron microscopy procedures. The specimen was finally introduced into the electron microscope. The structure of the ice was determined by observing the diffraction pattern from the ice layer in different areas of the grid. The orientation of the sample with respect to the cryogen was determined by punching a reference hole at the edge of the grid before its immersion.

In another experiment we let the grid fall from a height of 4 cm (half of the usual distance) and completely penetrate the coolant. The structure of the ice over the entire grid was determined by electron diffraction.

Numerical simulations

The cooling of a falling 3-mm-diameter copper disk in liquid ethane was simulated by the finite element method using specially dedicated software (ANSYS® 5.3, Canonsburg, PA, U.S.A.), which has been programmed to solve the heat conduction equation for a body of given shape, size and material properties. In order to test our numerical procedure, the results of ANSYS were compared with exact solutions for
simple geometries. A copper disc was modelled in ANSYS with 157 two-dimensional thermal conduction capability elements with the material properties corresponding to those of copper. Here we did not simulate the holey carbon–Pt–Pd film, assuming that this layer behaves as a good thermal conductor. A transient-type analysis was performed to simulate the dynamics of the cooling process during the immersion in ethane. The initial temperature of the disc was set at 20 °C. During the simulation, the temperature of the nodes on the circumference of the disc was dropped successively to −180 °C, starting from the lowest point, thereby allowing us to simulate the immersion as a function of time. The speed at which these node temperatures was set was determined from the falling speed. In this calculation it was assumed that only the nodes on the circumference are in contact with the coolant. This assumption was made on the basis of the high-speed images showing the immersion of the grid in the liquid ethane. Different immersion speeds were considered and the nodal results of the simulation were displayed in false colours.

Results

Grid entry into cryogen

First, the falling speed of the plunger at the point of contact with the cryogen was determined in order to check if a friction effect between the plunger and its guiding tube might influence the fall. The calculated falling speed for a falling height of 8 cm is 1.2 m s\(^{-1}\), while the measured speed is 1.1 m s\(^{-1}\), indicating that friction might slow the plunger by 10%. Therefore, at this speed the time necessary for the grid (diameter 3 mm) to penetrate the cryogen fully is about 3 ms.

The next question is the way in which the grid penetrates the cryogen. There are a few possibilities: (a) the grid is completely wetted by the cryogen, (b) the grid is not wetted and is in contact with the coolant only along the circumference, and (c) through capillary action the coolant rises through the grid during immersion.

With the stroboscopic system, images of the grid at different penetration depth into ethane were recorded (Fig. 2). In Fig. 2(a) the grid has just reached the surface of the cryogen, but it is already clear that the liquid cryogen is displaced. In Fig. 2(b,c) this effect is even more evident for deeper penetrations, where the cryogen is in contact with the grid only through the circumference. In Fig. 2(c) it is possible to see through the grid the cavity formed by the grid into the coolant surface. This phenomenon continues for penetrations at least equal or greater than the diameter of the grid and it was present on every image we recorded during these experiments.

Propagation of vitrification

To determine the way in which the vitrification front propagates, three different types of experiments were carried out.

1 In the first set of experiments, the grid was blocked during its free fall as soon as it was immersed halfway into the liquid ethane. The experiment was carried out to test how heat conduction through the copper grid and perforated carbon membrane affects vitrification. By means of electron diffraction, the areas where the water was in a vitreous state were mapped over the whole grid surface. In Fig. 3(a) the dark area above the ethane was still in a vitreous state, while the white area had a hexagonal crystal structure. The corresponding diffraction patterns are depicted in Fig. 3(b,c). In Fig. 3(d) we observe how, under these conditions, the crystalline structure can abruptly change in the carbon membrane from one hole to the next. It should also be noted that the cubic phase was never observed in this transition zone: it could be argued that a heating effect by the electron beam might quickly destroy...
this phase, thus making it impossible to observe. The vitreous area covered a bell-like shape, as shown in Fig. 3(a), and numerical calculations of the temperature as a function of time (Fig. 4c) show a similar pattern (see below).

2 To test the effect of plunge speed on vitrification, the grid was dropped from half the usual distance (4 cm instead of 8 cm) and was completely immersed in the coolant. These experimental conditions reduced the falling speed by about 30%. In this case, the diffraction pattern of the sample corresponded to a poor quality vitreous ice: as soon it was illuminated by the electron beam it changed into crystalline hexagonal ice. Therefore, we conclude that there is a minimum height (i.e. falling speed) needed to achieve a good quality of vitrification.

3 For the final experiment a grid was mounted parallel to the cryogen surface (Fig. 5). The aim was to test if the falling height can be reduced to minimize the evaporation and to achieve a more homogeneous vitrification over the entire grid. The sample film was on the side facing the coolant. The falling height was 4 cm and the preparation of the sample was exactly the same as for the other experiments. The electron diffraction measurements revealed only good-quality vitreous water (i.e. no transformation into...
hexagonal ice during electron beam illumination) over the entire grid.

The high-speed images of the falling grid mounted parallel to the surface of the cryogen demonstrated that in this configuration too, the liquid ethane is displaced by the falling grid (Fig. 6).

**Simulation of vitrification**

In order to understand the vitrification process, we have performed numerical simulation of the heat conduction over the grid surface and calculated the temperature profile as a function of time during immersion in the cryogen. Different simulations were conducted in order to determine the shape of the isotherms as a function of the cooling speed. The shape of the isotherms 3 ms after the grid came into contact with the ethane was found to be highly dependent on the speed at which the coolant touches the lower edge of the grid. Three different profiles have been obtained. If the speed at which the lower edge of the grid comes in contact with the coolant is lower than $0.01 \text{ m s}^{-1}$, we obtain inverted U-shaped isotherms, as depicted in Fig. 4(a). If the speed is higher than $1 \text{ m s}^{-1}$, the edges of the grid are at a lower temperature than its centre. As a consequence the isotherms take a U shape, as depicted in Fig. 4(c). Between these two extreme cases, there is a speed for which the isotherms are flat ($0.1 \text{ m s}^{-1}$), as it can be seen in Fig. 4(b).

**Discussion**

To achieve vitrification, it is well known that there is a threshold for the aqueous sample thickness (it must be less than 200 nm) and for its immersion speed (Dubochet et al., 1988). Immersing a liquid at low speed into a cryogen produces hexagonal ice (Dubochet et al., 1988). It was therefore suspected that vitrification is obtained by direct contact of the sample with the liquid ethane (rather than gaseous ethane). However, such a conclusion could be inaccurate without any information about the way liquid ethane behaves when it is impacted at high speed by a high-temperature sample. Referring to the images (Fig. 2), which show that the liquid ethane is displaced by the grid during the free fall, it is clear that this hypothesis is incorrect. Since the liquid ethane does not come into direct contact with all the surface of the sample, the only way to achieve vitrification is by heat conduction through the grid and/or the holey carbon. We suggest that as the sample penetrates the liquid ethane, the coolant is displaced and only touches a fraction of the circumference of the grid. As the grid continues its descent a larger part of the circumference comes into contact with the cryogen, permitting rapid propagation of the heat from the edges toward the centre of the specimen, as shown in Fig. 7.

This hypothesis is supported by the other experiments, which have shown that when the immersion speed is reduced (free fall of only 4 cm), only the lower parts of the aqueous sample are vitrified. The hexagonal ice present on the upper parts of the grid was probably produced by other slow processes, such as cooling through the cold gaseous ethane/air gas.

A more homogeneous vitrification, with less time dependency, can be achieved by mounting the grid parallel to the ethane surface, as shown in Fig. 5, instead of perpendicular. This grid orientation reduces the falling distance from 8 to 4 cm. An additional advantage is a reduction in the aqueous evaporation rate from the sample. This is due to the proximity of the grid to the cryogens (ethane and liquid nitrogen) and to a reduction in fall time. Our apparatus does not allow us to detect if the grid is flapping after entering the cryogen. However, since the sample is not damaged during the vitrification, if present, flapping does not seem to represent a problem.

We suggest that this modified method will preserve aqueous biological samples in a more physiological state, since vitrification is almost instantaneous all over the grid and the evaporation prior to vitrification is minimized.

Fig. 6. Time lapse high-speed images showing the penetration of a horizontally mounted grid into the cryogen. Note that also in this configuration the liquid ethane is being displaced by the grid.
VITRIFICATION DYNAMICS

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References


Fig. 7. From our results, we suggest the following time sequence for the formation of vitreous ice: in panel (b), the lower edge touches the coolant and the vitrification edge (in violet) starts to move towards the top of the grid. The resolution of our set-up did not permit us at the time to exclude the formation of a small capillary meniscus between the grid and the coolant (represented by a dashed line in the b). In panels (c–f) the vitrification front progresses with a Gaussian front as the grid penetrates the liquid coolant.