cryoWrite

Reproducible grid preparation with a cold-writing protocol

Abstract

The cryoWriter uses a blotting-free protocol for cryo-EM sample preparation. With this protocol, small amounts of sample (1-2nL) are reproducibly written on a grid using a micro-capillary. With such small volumes, evaporation is not negligible and must be carefully controlled. This note describes a cold-writing protocol that provides reproducible sample deposition under relative humidity and temperature control in the cryoWriter.

Key points

- Sample application at temperatures close to the dew point gives best grid quality
- Reproducible writing conditions are obtained with a multilevel humidity and temperature control system
- With the cold-writing protocol only 1-2 nL of sample are required per grid

Introduction

The structure of proteins is of fundamental interest to understand their function and plays an important role in drug design. Cryogenic electron microscopy (cryo-EM) is a fast- growing technique to determine protein structures. Hardware and software developments over the last decade have made this growth possible. As a result, single particle analysis by cryo-EM has now become one of the main methods for protein structure determination, particularly for larger complexes or biomolecules that are difficult to crystallize. For sample preparation, small amounts of sample are deposited in thin films on grids and are rapidly cooled to vitrify the sample without getting ice crystals, which would damage the proteins [1]. The grids contains a thin carbon film with holes that are spanned by the water layer upon sample application. It is, however, challenging to obtain a reproducible quality of grids. This application note focuses on a cold-writing method resulting in reliable and consistent sample preparation quality. The method is illustrated by examples of apoferritin and liposome deposition.

Grid preparation

The cryoWriter vitrifies the sample by plunging the grid into a cryogenic liquid (plunge freezing), in this case liquid ethane at approximately -180°C. To obtain ice-free vitrification the thickness of the applied water film is limited to a few hundred nanometers. A thin film also improves the electron transmission during data acquisition afterwards. On the other hand, at too low film thickness the coverage of the holes is jeopardized as films may burst open. Finally, the thickness influences the impact of the air-water interface, potentially causing a preferred orientation of amphophilic particles and may even hamper their integrity on the grid: When the film is thick, a significant fraction of the particles can orient freely, but when too thin, proteins are forced to the air-water interface.

The film and its coverage of the holes is influenced by the interaction between the aqueous solution and the membrane on the metal grid. In order to improve wetting of the grids, they are generally hydrophilized prior to the writing process. Furthermore, the addition of surfactants can be used to promote the spreading of the water over the holey grid [2].

Conventionally, the thin film is created by adding a few microliters of sample on the grid. Most of this is removed with paper in a subsequent blotting step. A thin film of liquid is thus created, which is then vitrified. The blotting step has limited reproducibility and can additionally cause damage to the proteins.

Newer techniques, that deposit around thousand times smaller amounts of sample on the grid, and thus omit the blotting step, have been developed. Tiny amounts can be deposited by piezo droplet deposition [3], dip pen technology [4] or writing with a capillary [5, 6].

After deposition or blotting, the film thickness further decreases through evaporation, creating a further irreproducible variability in the thickness. This hampers the sample quality, causing extra screening time at the electron microscope.

Minimizing the exposure time to air before vitrification helps to reduce the effect of evaporation and get more reproducible grids. In the cryoWriter, the time delay between writing and vitrification can be as small as 200ms. However, this can still

be sufficient to allow evaporation of thin films to take place and during the writing process evaporation can also occur. Therefore, it is advantageous to further reduce the evaporation rate.

Humidity control

Multiple factors of the cryoWriter technology [5] reduce evaporation of the sample. Firstly, by using a capillary for deposition, evaporation is largely prevented until the sample is applied on the grid. To further reduce evaporation, the liquid is slightly retracted into the pipette, thus creating a small air cavity. In this cavity, the relative humidity (RH) rapidly saturates. Without cavity, the RH at the apex does not saturate. As a result, evaporation continues and the salt concentration at the apex increases. A further positive side effect of the pipette is a minimized air water interface while the liquid is inside the capillary.

Evaporation of sample on the grid is reduced by increasing the RH in the cryoWriter hood. In other vitrification methods, a RH >80 % is used in the setup to limit evaporation. This creates high risk for condensation and can cause icing problems around the vitrification bath. As explained later, the cryoWriter can work with moderate values in the hood, in the range of 50 % < RH < 70 %, to get good grids. This reduces the risk of condensation and icing occurring at higher humidity. In addition, it allows short RH settling times of only few minutes after closing the hood, as illustrated in figure 1. This graph shows the RH as function of time after closing the hood. The RH rapidly increases and reaches the setpoint value within 4 minutes. A closed-loop control algorithm keeps the RH stable with a peak-to-peak variation ΔRH < 4 % around its setpoint value (figure 1, inset).

Cold-writing

The moderate humidity in the box by itself is not sufficient to reduce evaporation at the grid to a satisfactory level. Therefore, the cryoWriter uses a so-called cold-writing protocol (patent pending) in addition to the humidity control of the hood. For this protocol, the temperatures of the grid, the writing pipette, the sample and the handling tweezers are independently controlled. By cooling the grid, the local RH is increased. Preferably, the temperature of the grid is cooled to values slightly above the dew point: At ΔT = 2-3 °C above dew point, an RH of 80-90 % is obtained locally around the grid (figure 2). At such high RH, evaporation is sufficiently limited, and condensation from water vapor onto the grid does not occur.

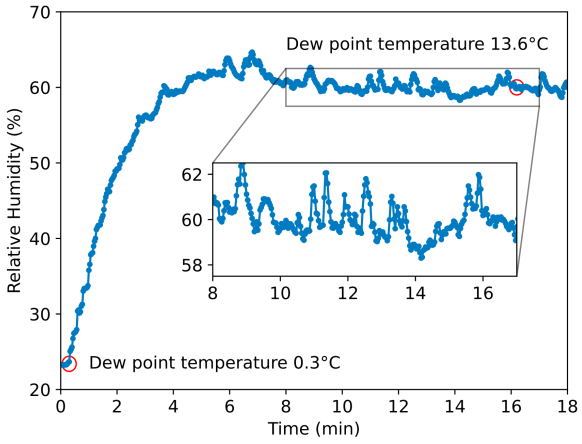

Figure 1. *Settling of the RH in the cryoWriter after closing the hood. Inset: Stability of the set RH after reaching the setpoint.*

The pipette, sample and tweezers are additionally cooled, because the heat capacity and thermal conductivity of the carbon film on the grid are small: Applying a “warm” sample solution and gripping the grid with warm tweezers increase the evaporation rate of the thin film. Therefore, for reproducible sample preparation, the temperature of grid, sample solution and tweezers need to be controlled.

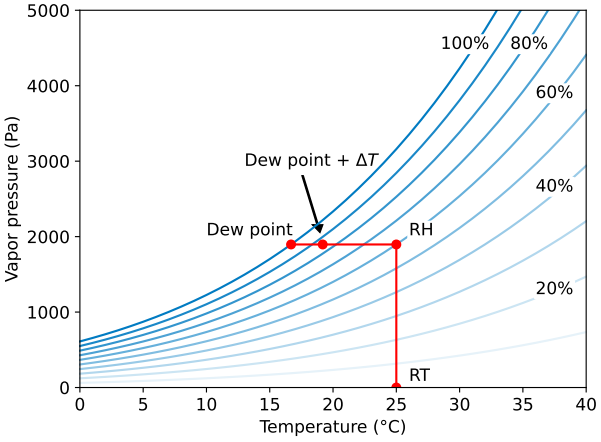

Figure 2. *The grid temperature is precisely controlled in the cryoWriter: The desired relative humidity (RH) in the cryoWriter hood at room temperature (RT) is set by the operator. Next, the dew point is automatically calculated. The grid temperature is controlled to ΔT = 2-3 °C (also set by the operator) above the dew point to obtain a local RH around the grid in the 80-90 % range. Only the RH in the hood and ΔT values for the grid are set by the operator, the rest is automatically regulated by the system.*

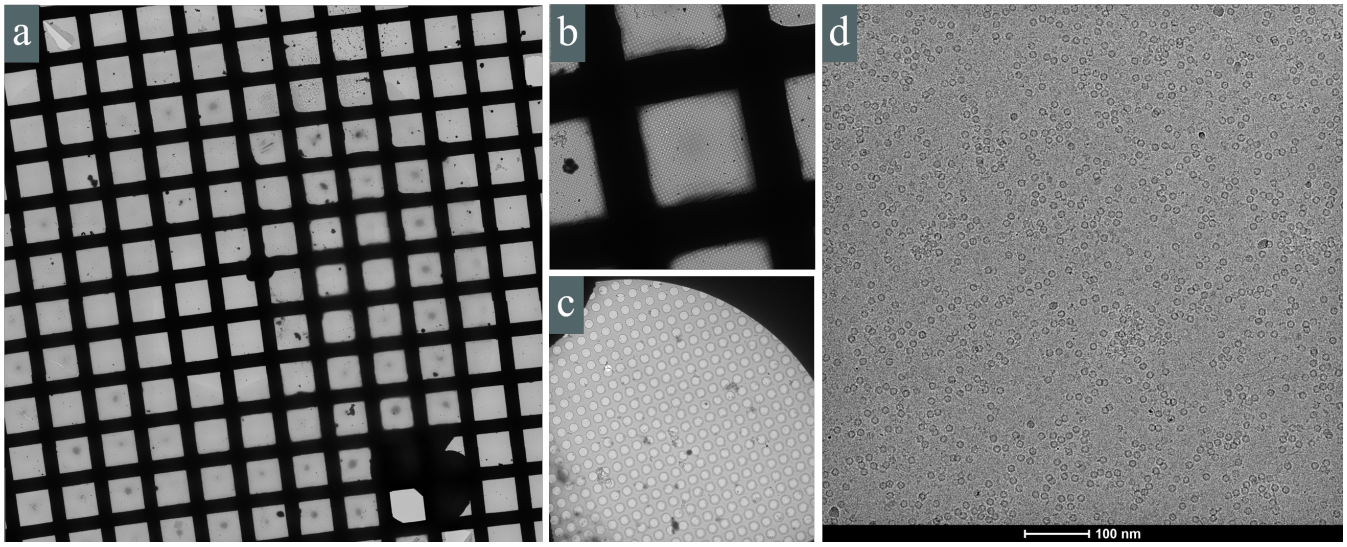

Figure 3. *Apoferritin applied to the grid with a cold-writing protocol. The atlas (a) gives an overview of the grid. The medium resolution images (b, c) show the intact holes inside the squares and high-resolution image (d) shows apoferritin particles in vitrified ice*

Ease of use

First of all, the above mentioned moderate values of the RH in the hood improve handling of the system. Most importantly, this feature reduces the ice formation around the cryogenic bath and thus prolongs the operation time.
To further ensure ease of use, the number of parameters to be set by the operator is minimized, namely the desired RH in the hood and the temperature difference of the grid, pipette, sample and tweezers compared to the dew point temperature. After that, the cryoWriter takes care of the rest: The cryoWriter has a feedback control loop to keep the RH constant; the operating software calculates the dew point temperature, based on the measured temperature and RH in the hood; the temperature of grid, pipette, sample and tweezers each have their feedback loop to maintain the preset values automatically. The result is ease of use with maximum flexibility.

Apoferritin

The first example presented here is apoferritin. Apoferritin is the established sample to characterize and calibrate electron microscopes, due to its rigidity and well-known structure. Figure 3a shows the atlas of a grid written with apoferritin. The combination of moderate RH and writing close to dew point reproducibly yields over 100 good squares on this grid. The vitreous ice in the squares is relatively homogeneous and thin. The images recorded at medium magnification (figure 3b,c) indicate that most holes are intact and at high-magnification (figure 3d), the particles appear with good contrast on the low background signal of the vitreous ice layer.

Liposomes

As a second example we show the vitrification of a liposome sample. Liposomes, the spherical shells formed by water-impermeable lipid bilayers, are highly sensitive to osmotic shock and dehydration. Several hurdles had to be overcome for the first preparation of thin, vitrified layers of aqueous liposome solutions for cryo-EM [7]. Therefore, liposomes are critical test samples to verify the cryo-EM sample preparation workflow of the cryoWriter. Figure 4 illustrates the difference between writing liposomes at room temperature (figure 4a, c, e) with a cold-writing protocol (figure 4b, d, f), in which sample, pipette and tweezers are cooled.
The sample written at room temperature shows a heterogeneous distribution of sample thickness over the squares in the atlas (brighter and darker contrast figure 4a). When zooming in on the squares, it can be observed that many of the holes in the carbon film show water films that have burst open (figure 4c). Finally, at high magnification images of single holes show that the liposomes are blown up (figure 4e).
In contrast, the overview images of the cold-written samples show a relatively homogeneous layer of vitrified sample, with many good squares (figure 4b). In the cold-written sample, the majority of holes were spanned by vitreous ice (figure 4d). Inside the holes, the intact vesicles liposomes can be observed at high magnification (figure 4f).

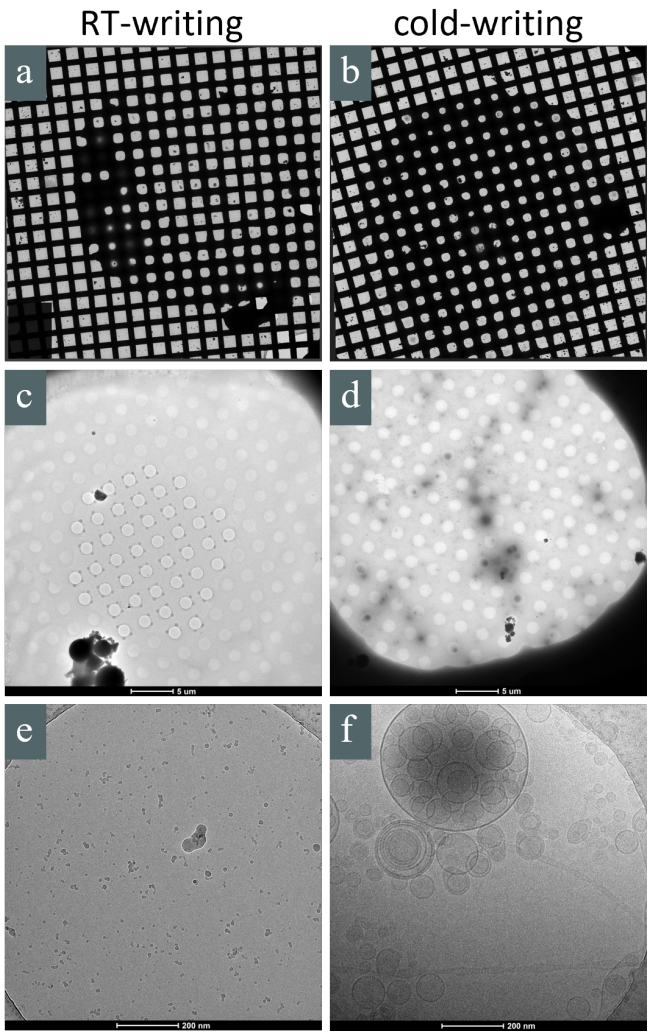

Figure 4. *Liposomes written at room temperature (RT) (a, c, e) and with cold- writing protocol (b, d, f). The cold-writing protocol shows more squares with vitrified water in the atlas (b vs. a), fewer plopped open holes at medium resolution (d vs. c) and intact versus broken vesicles at high magnification (f vs. e).*

Conclusions

In conclusion, the cryoWriter provides a solution to routinely prepare good quality grids for cryo-EM analysis. This is reached by using a pipette to apply the sample to the grid. The robotic pipette enables automation of the grid preparation protocol. Essential for reliable grid preparation is the combination of accurate control of RH and temperature in the system and its components. For single particles this was illustrated with apoferritin, yielding over 100 squares with well vitrified ice on a single grid. The benefits of the cold-writing protocol was additionally demonstrated on a fragile sample: A liposome sample was applied to the grid both at room temperature and with cold writing. Good vesicles that were obtained with the cold-writing protocol could not be obtained when writing at room temperature.

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