Ultramicroscopy 13 (1984) 185–190
North-Holland, Amsterdam

A COLD STAGE FOR THE PHILIPS EM300 ELECTRON MICROSCOPE

K.A. TAYLOR *
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

R.A. MILLIGAN
Department of Structural Biology, Sherman Fairchild Center, Stanford University, Stanford, California 94305, USA

C. RAEBURN
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

and

P.N.T. UNWIN
Department of Structural Biology, Sherman Fairchild Center, Stanford University, Stanford, California 94305, USA

Received 1 November 1983; revised 26 March 1984

A cold stage has been constructed for the Philips EM300 (and EM301) electron microscopes for investigating the structure of frozen-hydrated biological specimens. The stage entails minimal alterations to the instrument and is capable of a resolution better than 10 Å at the normal operating temperature of −120°C. Frozen specimens can be readily exchanged without condensation or warming up, and maintained in the stage over periods of several hours without detectable deterioration.

1. Introduction

The native structure of biological specimens can be investigated with the electron microscope at high resolution if some means is provided for retaining their hydration in the surrounding high vacuum. Attempts have been made to develop hydration chambers, operated by a differential pumping mechanism, for this purpose [1]. However, these devices have not proved amenable to high resolution imaging due to the effects of stage instability and radiation damage. Another way for preserving some structures involves glucose embedding [2]. However, glucose is not a perfect substitute for water; the method does not allow control of ionic composition and the contrast may be weak because the electron scattering densities of glucose and protein are closely matched. A third approach involves freezing the specimen in a thin aqueous film (< 1000 Å thick) and observing it at temperatures low enough to prevent ice sublimation [3]. By freezing rapidly, the damage due to ice crystal formation can be prevented. Furthermore, radiation damage is decreased by a factor of at least 5 at the lower temperatures [4], salt conditions can be varied and the low density of ice leads to good image contrast. This last technique is therefore potentially the most useful; a fact which is just beginning to be realized [4,6].

We describe here a cold stage for the Philips EM300 (and EM301) electron microscopes which enables them to be used for high resolution structural studies of frozen-hydrated specimens. The design is based upon the standard high resolution stage and involves minimal modifications to the

* Present address: Anatomy Department, Duke University Medical Center, Durham, North Carolina 27710, USA.

instrument. This stage has features which overcome some of the limitations of the cooling holder accessory to the Philips goniometer. It produces a more thermally and vibrationally stable system and has a long operating period between coolant refills. Transfer of precooled specimens into the microscope column can be accomplished very effectively via the standard airlock assembly.

2. Description

2.1. Stage assembly

Modifications were made to the lower specimen translation plate and specimen cartridge support frame with the object of retaining basic features already present and allowing the microscope to be operated by essentially standard procedures.

The new lower specimen translation plate has the same overall dimensions as the original one (fig. 1). The separate cartridge support frame is attached to the lower translation plate on opposite sides. Two plastic (Perspex) blocks insulate the support frame, which is cooled (see below), from the translation plate. The core of the support frame has a 2° taper to match that of the specimen cartridge and hence achieve a good fit between the two components. The specimen is further stabilized by a locator pin soldered to the frame at the end opposite the cartridge entrance. The frame support and the tip of the specimen cartridge are cut to a thickness of only 1.25 mm to leave space for the objective aperture holder and the liquid-nitrogen-cooled anticontamination device. The anticontamination device used is twin-bladed with a 2 mm gap and 1.1 mm holes for the electron beam. The standard horseshoe design does not provide adequate protection.

2.2. Specimen cartridge

The new specimen cartridge has a 2°-tapered copper body with a stainless steel sleeve and pin, shaped as for the original cartridge to permit exchange using the Philips injector rod. It is held in the support frame by means of a phosphor-bronze spring attached to one of the plastic blocks. The specimen grid is mounted in a recess near the tip of the cartridge and held there

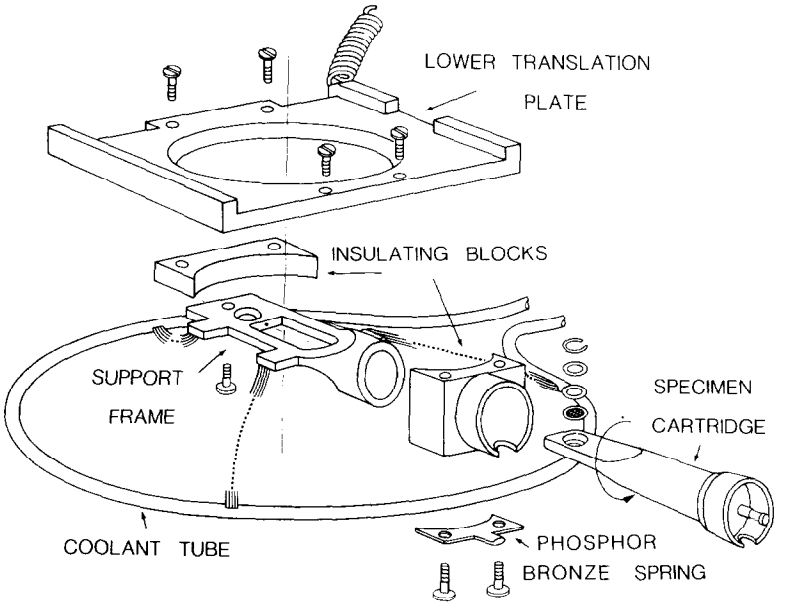

Fig. 1. Cold stage assembly and specimen cartridge (exploded view). This drawing shows only those pieces which have been modified from the standard Philips object stage and specimen cartridge.

tightly by a slightly bent phosphor-bronze washer, compressed by a platinum ring and circlip. This combination allows easy mounting of a precooled specimen under liquid nitrogen. To reduce radiative heat transfer, both the cartridge and the support frame are gold-plated.

2.3. Cooling

The system can use either liquid nitrogen or liquid-nitrogen-cooled vapor as coolant. Coolant is transported from a large (10.4 litre) external Dewar to the cold stage area via 1/16 inch internal diameter copper tubing (figs. 2 and 3). The Dewar is sealed and pressurized using a resistive heater. When nitrogen vapor is used as coolant, it originates from near the top of the Dewar and first passes down a length of copper tubing submerged in the liquid nitrogen so that the vapor is precooled. The tube then takes an upwards path, entering an evacuated transfer line which connects the Dewar to the specimen stage via the pumping port (fig. 3). Evacuation of the transfer line is provided by the microscope's vacuum system. The evacuated transfer line extends inside the liquid

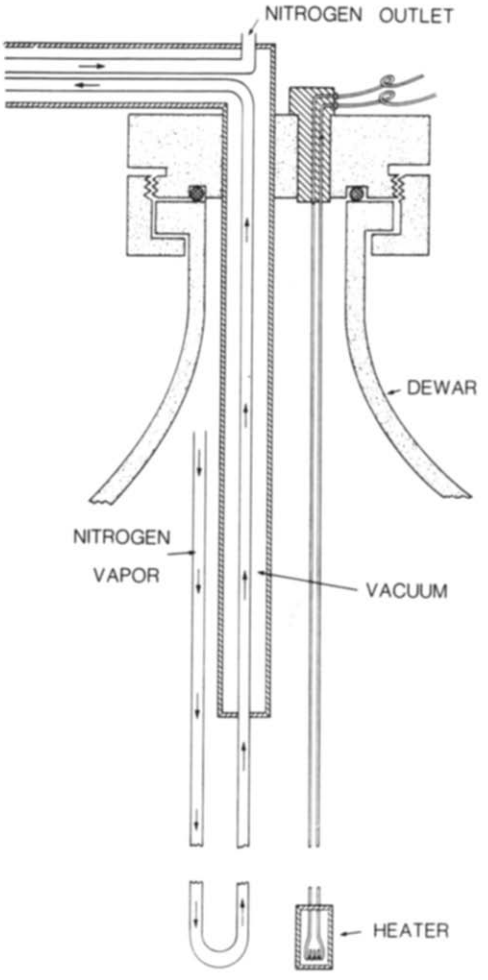

Fig. 2. Internal details of storage Dewar, with nitrogen vapor as coolant (sectional view). The inlet to the coolant tube is located near the top of the Dewar, projecting above the surface of the liquid nitrogen. The "U" turn of the coolant tube and the heater are located near the bottom of the Dewar.

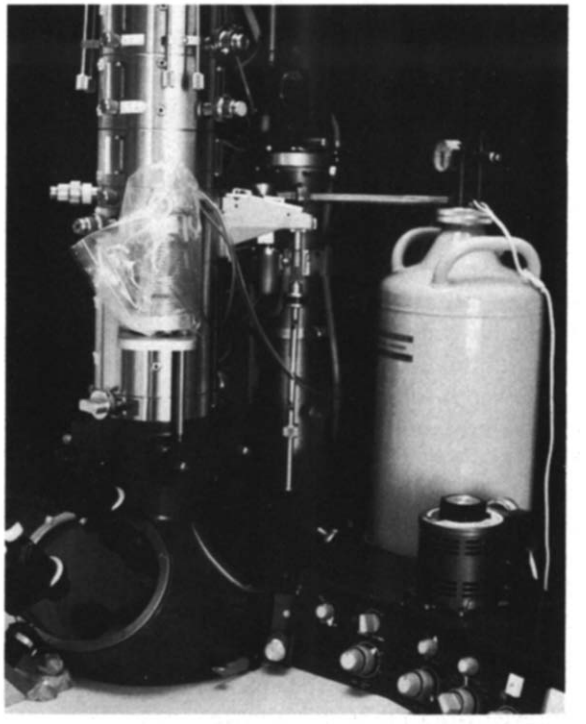

Fig. 3. Photograph of the microscope set up for low dose operation (2), with the cold stage and cooling system in place. The Dewar of liquid nitrogen used for cold specimen transfer is mounted on a shelf just below the specimen airlock, and the two are enclosed by a plastic bag. This bag is flushed with dry nitrogen gas before the actual transfer is carried out. The tubes projecting from the top of the storage Dewar (right), not indicated in fig. 2, are used for refilling. A pressure gauge and safety valve is mounted on one of them. The pressure, and hence the flow of nitrogen vapor, is controlled by the variac shown, which operates the resistive heater inside the Dewar near its base.

nitrogen Dewar with the vacuum seal held below the level of the liquid nitrogen to minimize warming of the coolant prior to entry into the stage. The tube runs along the transfer line, loops around the stage area and passes back along the same line to exit above the Dewar into the room atmosphere. It is held in place where it loops around the stage area using clamps made of teflon. Some material has to be removed from the stage block to accommodate this tube.

The cartridge support frame is cooled through three conducting leads connected to the coolant tube. The leads are copper fibers taken from the cooling brushes of the Philips anticontamination device and are soldered to the frame and to the coolant tube to obtain the most efficient heat transfer. The resultant increased drag on the lower translation plate necessitates replacement of the spring which connects it to the stage block by a slightly stronger one.

2.4. Cold specimen transfer

Transfer of the specimen holder from liquid nitrogen into the microscope is accomplished using the Philips injector rod and airlock assembly. In order to minimize condensation of atmospheric water vapor onto the grid during this process several precautions are taken. Suitable aqueous films are prepared on copper grids [3,5,6] and mounted in the specimen cartridge in a small Dewar of liquid nitrogen. This Dewar is then mounted just below the airlock entrance on a shelf (fig. 3). A polyethylene bag encloses both the Dewar and the airlock. Prior to transfer the bag is sealed and dry nitrogen gas is flushed through it for about 20 s. The specimen cartridge is then retrieved from the liquid nitrogen using the injector rod and inserted quickly into the airlock. The airlock is allowed to pump for 10–15 s and then the cartridge is inserted fully into the precooled support frame.

2.5. System performance

We have operated this cold stage with both liquid and gaseous nitrogen as the coolant. With liquid nitrogen as the coolant, the cold stage reaches a temperature of $-160°C$ in about 30 min. However, it does not operate very well under these conditions due to vibrational instability and a high rate of contamination. Much better performance is obtained with liquid-nitrogen-cooled vapor. With vapor cooling, the equilibrium temperature is not as low as with liquid nitrogen. In our experience, a specimen temperature of about $-120°C$ seems to be optimal in terms of stability and specimen contamination. At this temperature, measured specimen drift is < 5 Å/s, specimen contamination due to ice build-up is not detectable, and sublimation of ice already present is negligible over at least a 2 h period. The capacity of the Dewar is sufficient to give a running time of about 24 h under these conditions.

The cold stage has been used in a number of structural studies utilizing two-dimensional crystalline arrays including gap junction and urothelial bladder membranes [8]. Although this stage has no tilting capability, we have found that three-dimensional data sets can be obtained by bending the grids to obtain the tilts [7]. The optimum operating temperature of $-120°C$ precludes the observation of specimens in amorphous ice films. Such an operating temperature may place a limitation on the type of biological specimen that can be examined, since specimens containing large aqueous cavities appear to be damaged by ice crystal formation (see ref. [8]). The optimum operating temperature is limited primarily by the unacceptably high rate of contamination at lower temperatures. However, this limitation could be overcome by providing a more efficient anticontamination device or by improving the vacuum in the microscope. It would then be possible to operate the stage, using vapor cooling, at sufficiently low temperatures to maintain ice in the amorphous state.

3. Conclusions

The cold stage for the Philips EM 300/EM 301 described here can be built with minimal modification of the instrument and can be used with minimal alteration in microscope operating procedures. Its advantages over the commercially sup-

plied stage are a long running time between coolant refills, easy insertion of pre-cooled specimens, and greater resolution. The best operating temperature appears to be at −120°C, but could be lower if better anticontamination and better microscope vacuum are provided.

Acknowledgements

We wish to thank the Electron Optics Department of N.V. Philips Gloeilampfabrieken for the gift of a high resolution object stage. K.A.T. was supported by a NATO Postdoctoral Fellowship. This work was supported, in part, by grants from the National Institutes of Health (GM27764) and the American Cancer Society (RD121).

References

[1] D.F. Parsons, V.R. Matricardi, R.C. Moretz and J.N. Turner, Advan. Biol. Med. Phys. 15 (1974) 162.
[2] P.N.T. Unwin and R. Henderson, J. Mol. Biol. 94 (1975) 425.
[3] K.A. Taylor and R.M. Glaeser, Science 186 (1974) 1036.
[4] K.A. Taylor and R.M. Glaeser, J. Ultrastruct. Res. 55 (1978) 448.
[5] J. Dubochet, J. Lepault, R. Freeman, J.A. Berriman and J.-C. Homo, J. Microscopy 128 (1982) 219.
[6] J. Jaffe and R.M. Glaeser, in: Proc. 40th Annual EMSA Meeting, Washington, DC, 1982, Ed. G.W. Bailey (Claitor's, Baton Rouge, LA, 1982) p. 72.
[7] P.N.T. Unwin and P.D. Ennis. Nature 307 (1984) 609.
[8] R.A. Milligan, A. Brisson and P.N.T. Unwin. Ultramicroscopy 13 (1984) 1.