Microsc. Microanal. 13, 365–371, 2007
DOI: 10.1017/S1431927607070791

Microscopy AND Microanalysis

An Improved Holey Carbon Film for Cryo-Electron Microscopy

Joel Quispe,[1,*] John Damiano,[2] Stephen E. Mick,[2] David P. Nackashi,[2] Denis Fellmann,[1] Teddy G. Ajero,[1] Bridget Carragher,[1] and Clinton S. Potter[1]

[1]*The National Resource for Automated Molecular Microscopy, The Scripps Research Institute, La Jolla, CA 92037, USA*
[2]*Protochips, Inc., Raleigh, NC 27603, USA*

Abstract: Two issues that often impact the cryo-electron microscopy (cryoEM) specimen preparation process are agglomeration of particles near hole edges in holey carbon films and variations in vitreous ice thickness. In many cases, the source of these issues was identified to be the residues and topography often seen in commercially available films. To study and minimize their impact during specimen preparation, an improved holey carbon film has been developed. Rather than using a consumable template based on soft materials that must be removed prior to grid assembly, a method was developed that uses a hard template and a water-soluble release layer to replicate the template pattern into the carbon films. The advantages of this method are the improved purity and flatness of the carbon films, and these attributes are shown to have a dramatic improvement on the distribution of single particles embedded in vitreous ice suspended across the holes. Improving particle distribution is an enabling factor toward increasing the throughput of data collection for cryoEM.

Key words: TEM, cryo-electron microscopy, holey carbon films, automation

Introduction

In cryo-electron microscopy (cryoEM), macromolecular structures are preserved in a layer of vitreous ice and imaged using a transmission electron microscope (Dubochet et al., 1988; Taylor & Glaeser, 1974). The EM images are projections of the individual particles, and a three dimensional (3D) electron density map of the macromolecule can be reconstructed from these projections if their relative orientations can be determined. To avoid radiation damage to the specimen, very low electron doses are used while acquiring the images, and this results in very low signal-to-noise ratios. The signal can be improved by averaging, but this in turn requires a large number of images. Typically, 100,000 copies of an asymmetric unit must contribute to the average in order to reconstruct a 3D map with a resolution better than 1 nm, the point at which secondary structure starts to be discernable. Extrapolating from these results implies that on the order of 1,000,000 copies will be required to achieve resolutions sufficient to trace the atomic chain (Henderson, 1995). This clearly places very high demands on the throughput of data collection and analysis in cryoEM.

Received March 22, 2007; Accepted May 15, 2007.
*Corresponding author. E-mail: joelq@scripps.edu

Specimens preserved in vitreous ice are most often prepared on a substrate consisting of either a continuous or holey carbon film supported on a copper or molybdenum EM grid. After placing a small droplet (~4 μl) of the specimen onto the grid, the grid is blotted using filter paper to reduce the droplet to a thin film and then plunged rapidly into a cryogen (e.g., liquid ethane or propane), freezing the thin film into a layer of vitrified ice. There are both advantages and disadvantages to preparing specimens on holey carbon foils. One of the principal advantages is that the background carbon does not contribute to images of the specimen acquired over holes and the resulting improvement in signal can be critical when imaging macromolecules of low molecular weight. Imaging over holes is also preferable when examining specimens that can be distorted by interacting with the carbon (e.g., large helical tubes). However, one potential major disadvantage of using holey carbon substrates is that it can sometimes be quite challenging to get the individual particles to disperse across the holes in an even distribution. This is particularly critical when considering the very high throughputs of data acquisition that are required in order to improve the resolution of the reconstructed maps.

A variety of methods have been described for the preparation of holey carbon films (Murray, 1987). However, these methods do not provide arrays of holes with a regular size and spacing. Regular arrays of holes are preferred when

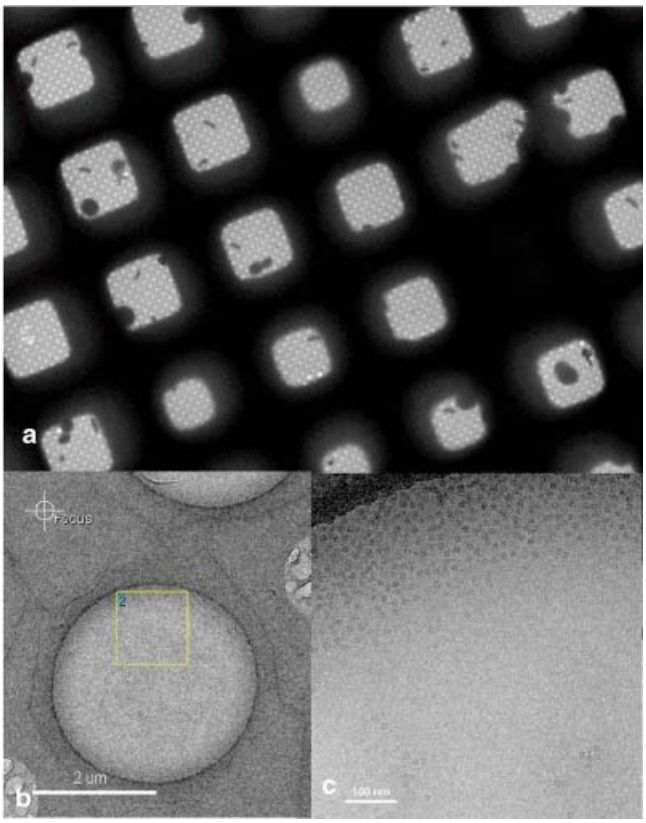

Figure 1. Quantifoil R2/2 design. **a:** Low magnification image of the grid, taken at 120×. **b:** Image of a hole taken at 5000×. A gradient of ice thickness can be seen across the hole. **c:** High magnification image of a Quantifoil hole showing that the particles, GroEl in this case, are crowded into the ice near the edge of the hole.

imaging, particularly when automated procedures are used to identify the holes and the intermediate carbon film used for focusing (Suloway et al., 2005). Commercially available grids known as Quantifoils (Ermantraut et al., 1998) contain perforated carbon support films with holes of a defined size, shape, and arrangement. One geometric arrangement typically used for cryoEM consists of 2-μm-diameter holes with a pitch spacing of 4 μm, as illustrated in Figure 1. Quantifoil support films are manufactured using semiconductor lithographic techniques, using a layer of glutaraldehyde cross-linked gelatin as a sacrificial layer between the photoresist and the silicon wafer. The sacrificial layer is later digested by an enzyme, allowing the photoresist layer to be detached from the wafer and floated off on an air–water interface. It is then picked up on EM grids, which are coated with carbon using a vacuum evaporator. The photoresist may then be dissolved away in ethanol to provide a grid coated only in the holey carbon foil. The array of regularly spaced 2-μm holes provides important advantages in automating the procedures of acquiring low dose images of specimens suspended over holes, and many researchers have used Quantifoil grids almost exclusively for the past 5 years.

Despite the many advantages of commercially available carbon films with hole arrays, we have consistently encountered several problems when using these substrates that may be related to residual contaminants on the carbon surface and a lip or collar around each hole. An ideal layer of vitrified ice will spread with uniform thickness across most of the grid surface and provide large areas of ice that have the appropriate thickness required for imaging the embedded specimen. However, if the surface of the carbon foil is not hydrophilic, the specimen droplet tends to bead up during blotting, and the resulting ice layer is very nonuniform, consisting of small patches of very thick ice surrounded by dry areas. Such grids are unsuitable for high throughput imaging. A variety of techniques can be used to mitigate the hydrophobic nature of the grid surface, including glow discharging or plasma cleaning, treating them with ethanol, acetone, or chloroform, or recoating them with a fresh layer of carbon. Experimenting with various combinations of these methods met with partial success; however, each new batch of grids required reoptimization of the sample preparation methods. Another critical problem was achieving a uniform distribution of particles across the holes in the grids. Figure 1c shows images of a specimen of GroEL embedded in vitreous ice and suspended over a Quantifoil grid. As shown in Figure 1b, the single particles of GroEL are all clustered in the ice layer near the edge of the hole. As a result, much of the acquired image contains no particles at all, and most of the particles that are present are too closely crowded together to be useful for further analysis. We have observed that this particle distribution is fairly typical when using Quantifoil grids, particularly when examining small particles that require a thinner layer of ice. It is believed that this distribution arises because of a fairly large variation of the ice thickness across the hole, and that this variation is compounded by a lip or collar around the edge of each hole.

Although several data sets of GroEL containing more than 100,000 particles were acquired from a single Quantifoil grid, it was clear that many more particles could be acquired if the particles were evenly distributed across the holes. This prompted interest in exploring alternate methods for manufacturing holey carbon foils that culminated in the development of the grids described in this article.

Materials and Methods

Preparation of Grids

To address the issues identified with current, commercially available support films for cryoEM, a new manufacturing process was developed that is based on a robust pattern transfer technique. A key component to manufacturing any patterned carbon support film is the pattern transfer process itself. Using photoresist and other soft materials as a template requires their removal from the carbon film prior

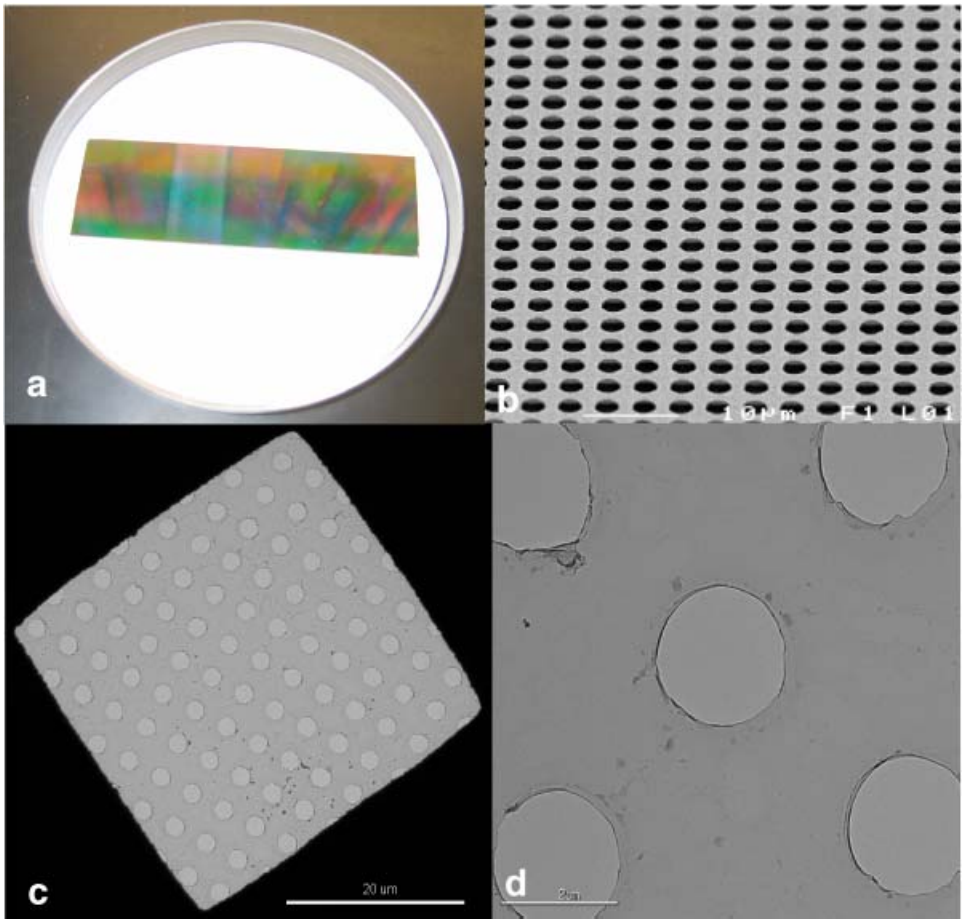

Figure 2. a: Microfabricated templates from Protochips, Inc. (patent pending). **b:** SEM image of microfabricated template. **c,d:** TEM images of a carbon grid before cryo-freezing.

to assembling the grid. As described above, this removal process can leave residues and other materials on the surface of the carbon, contributing to variations in surface properties as well as nonuniform ice formation. In addition, the thickness of many photoresists as well as the difficultly in defining very sharp edges can lead to topography such as collars around the holes of the replicated carbon film.

To study the impact of these issues on cryoEM specimen preparation and imaging, a new holey carbon film was developed that uses a robust pattern transfer process. Rather than patterning a consumable template based on soft materials that require removal prior to grid assembly, a new method was developed that uses a hard template and a water-soluble release layer to replicate the template pattern onto the carbon grids. The template is a silicon wafer with an array of holes or wells imprinted into the surface. The templates used in this study were supplied by Protochips, Inc. By removing photoresist and plastic materials from the pattern transfer process, the resulting carbon films have a cleaner and more uniform surface. Edges and topography around the holes are also minimized, and other artifacts such as "pseudo holes" are avoided.

For the results described in this article we have typically used holes 2 μm in diameter with a 4 μm pitch spacing, but a variety of other templates are also available. An optical image of one of the templates is shown in Figure 2a, and a scanning electron microscopy (SEM) image is shown in Figure 2b. A clean template surface is first coated with a water-soluble releasing agent; this technique was developed by Martin Muller and Theo Koller of the ETH, Zurich, and was described by Downing (2003) as a means to manufacture support films as replicas of Nucleopore filters. Downing used a layer of sodium metaphosphate, supplied commercially under the name Victawet, as a releasing agent. After release layer deposition, the template is transferred to a second evaporator, which is capable of achieving high vacuum ($P < 0.5$ mPa) and is reserved exclusively for evaporation of carbon in order to ensure the highest quality carbon films. A layer of carbon of the desired thickness (typically between 15 and 50 nm) is then evaporated onto the Victawet-coated templates.

The EM grids, typically 400 mesh copper grids, are cleaned by sonicating them in acetone followed by deionized water and allowed to dry overnight. The clean grids are

placed on filter paper at the bottom of a petri dish filled with nano-pure water. The template is slowly submerged under the water, which dissolves the releasing layer and results in the carbon lifting off the template and floating on the air–water interface. The water is then siphoned off to lower the carbon gently on top of the grids (Figure 2c,d). The grids are allowed to dry overnight in a covered environment to minimize dust particles and inspected in a phase contrast light microscope.

Preparing Vitreous Ice Grids

Immediately prior to sample preparation, the grids are treated in a plasma cleaner for 20 s. The plasma cleaner is an oil-free vacuum system that utilizes a diaphragm pump and turbo-molecular pump to reduce the pressure in the chamber to ~1.3 mPa and then uses a mixture of 25% O_2 and 75% Ar at a pressure of ~6.6 Pa to create an oxygen plasma that removes carbonaceous material. We have used both the Fischione 1020 and the Gatan Solarus devices. When using the Fischione system, the grids are mounted in a multigrid holder using the shielded specimen holder port. The shield dampens the plasma's effect and limits the amount of carbon removed from the surface. In the Solarus system, the grids are placed in a plastic petri dish placed in the bottom of the chamber, and the power settings are reduced to lower the intensity of the plasma. In our hands, plasma cleaning has proved a more reliable and reproducible method for preparing good, clean hydrophilic surfaces than the more traditional method of glow discharging. We speculate that this is because plasma cleaning provides a cleaner and more controlled environment than regular glow discharging.

Following plasma cleaning, the samples are then blotted and vitrified with a Vitrobot using a standard protocol that results in consistent and reproducible vitreous ice for a wide variety of specimens and buffer conditions (Quispe et al., 2004). In this article, we show results from vitreous ice grids prepared using a test specimen consisting of a mixture of GroEL (small single particles, diameter ~14 nm), TMV (long helical viruses, diameter 18 nm), and SA11-Rotavirus (a small spherical virus, diameter ~75 nm). The GroEl, TMV, and rotavirus were diluted in 50 mM Tris, 50 mM KCl, 1 mM DTT, at pH 7.4, to a final concentration of 3.2 mg/ml, 10 mg/ml, and 0.25 mg/ml, respectively. A volume of 4 μl of this solution was applied to the plasma-cleaned grids and blotted for 4.0 s using the Vitrobot set to 100% humidity and 4°C.

Results

An example of the holey carbon films prepared using the described methods is shown in Figure 2c,d. The foil is an accurate replica of the template with ~2-μm-diameter holes with a 4-μm pitch spacing. The carbon appears to be clean, uncontaminated, and flat. A high-resolution image of the carbon film was taken using a scanning electron microscope and is shown in Figure 3. From these images, the flatness and cleanliness of the film across a large area is clearly seen (Fig. 3a), and a magnified image of a single hole (Fig. 3b) displays minimal topography around the edges.

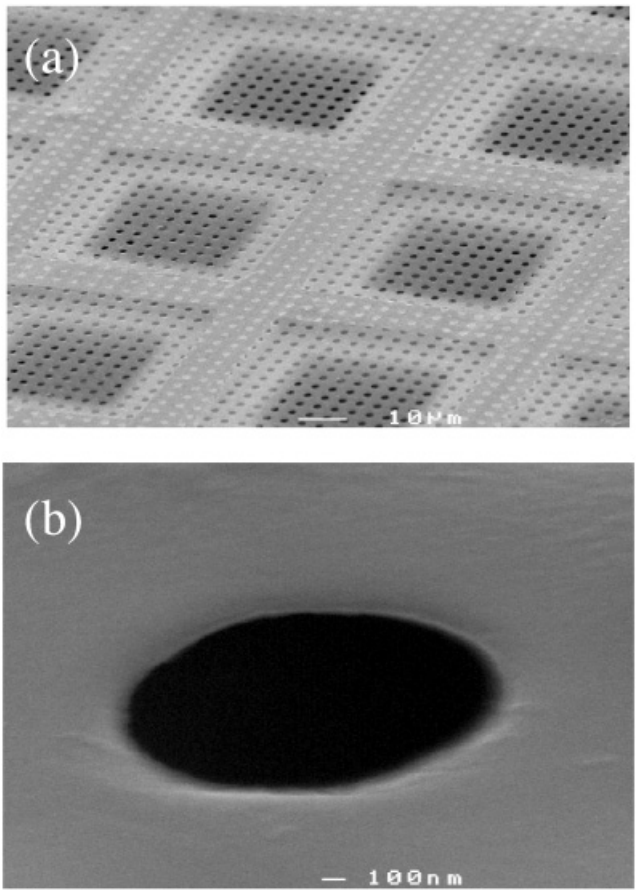

Figure 3. SEM images of the carbon film deposited on a 400 mesh copper grid. The holes are 2 μm in diameter on a 4-μm pitch. **a:** Illustration of the flatness and cleanliness of the carbon surface. **b:** The smoothness and minimal topography of an individual hole edge.

Vitreous ice prepared over these grids using the standard grid preparation technique described above consistently displays large areas of ice with a range of thicknesses suitable for imaging macromolecules. Examples from a test sample containing a mixture of GroEL, TMV, and rotavirus are shown in Figure 4. Figure 4a shows an area of fairly thick vitreous ice suspended over a hole as evidenced by the presence of the spherical virus, which has a diameter of 75 nm. In contrast, Figure 4b shows an area of thinner ice in which only small single particles of GroEL and long filaments of TMV (both of which have a diameter of ~15–18 nm) are embedded. Features on the GroEL and TMV are clearly discernable in this thin ice whereas they are much harder to identify in the areas of thicker ice. In both cases, the particles are uniformly distributed across the holes, with no tendency to aggregate at the edge of the hole.

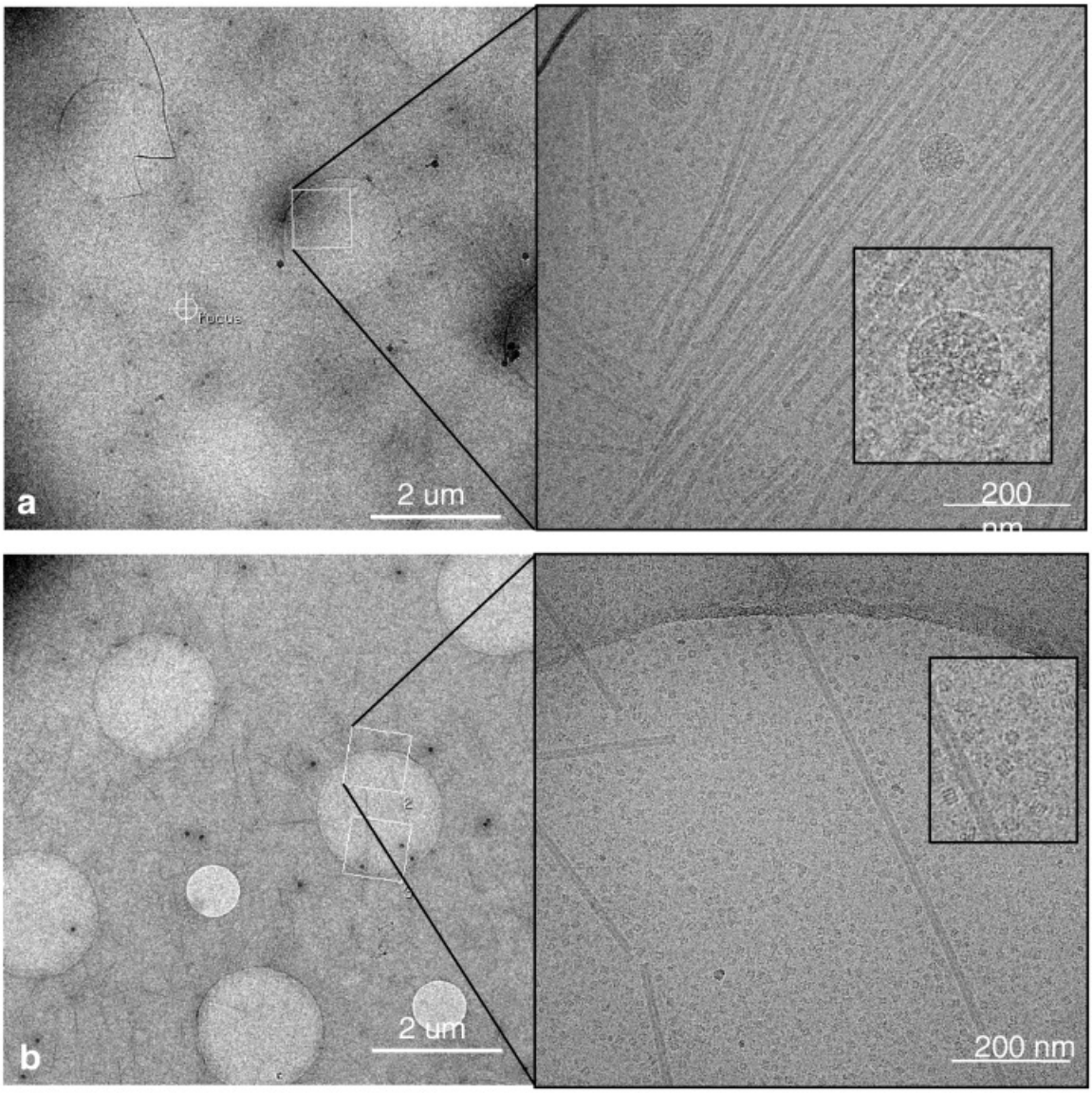

Figure 4. A mixture of GroEL, TMV, and rotavirus frozen on a carbon grid. **a:** The ice is thick enough to accommodate the rotavirus, but it makes it difficult to clearly see the TMV and GroEL. **b:** This is an area with thinner ice and the TMV and GroEL can be clearly seen, and there are few rotavirus particles.

The much more uniform distribution of particles across the holes has a dramatic effect on the throughput of automated data collection. In a recent experiment, more than 280,000 particles of GroEL were acquired during a single experiment in which data were continuously acquired for 25 h (Stagg et al., 2006). Examples of the distribution of the GroEL across the holes are shown in Figure 5.

Discussion

The new grids are now in regular and routine use at the National Resource for Automated Molecular Microscopy. They provide a clean, controlled, and very thin holey carbon substrate. Use of these grids has improved the overall success rates in obtaining large areas of ice with suitable thickness and dramatically improved the distribution of single particles across the holes compared to Quantifoil grids with similar geometry, particularly when imaging small macromolecules. The templates that are used to form the holes can be designed using a wide range of geometries, but the 2-μm-diameter holes with the 4-μm pitch spacing were found to provide a suitable substrate for almost all specimens tested.

The thickness of the perforated carbon foil is ~10–20 nm. Although the thickness of the carbon film can be easily increased, use of an ~10-nm carbon film layer results

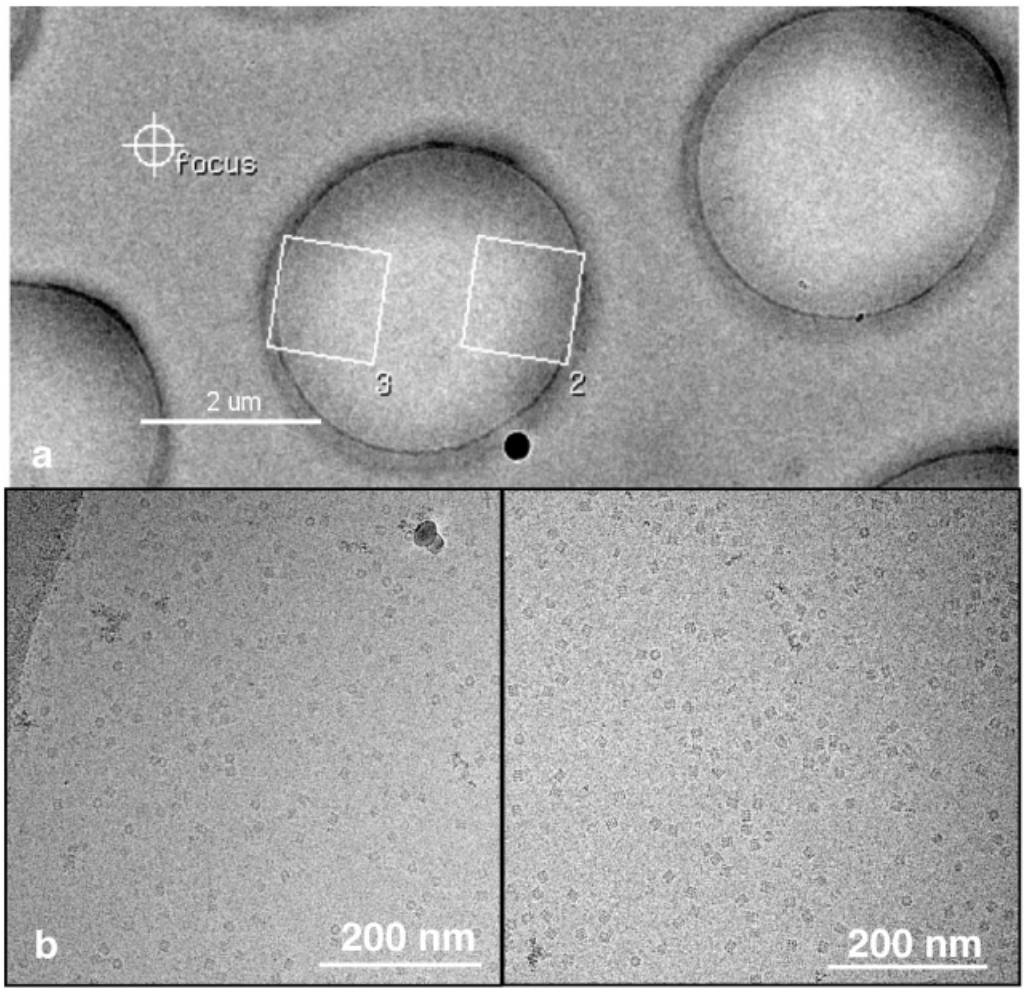

Figure 5. GroEL suspended in vitreous ice over the carbon grid. **a:** Intermediate magnification image of a hole indicating two areas from which high magnification images were acquired. **b:** High magnification images of targets 2 and 3. The distribution of particles is more even and consistent than observed on Quantifoil grids and has allowed us to acquire more than 250,000 single particles from a single grid during a 24-h session at the microscope.

in very thin layers of ice across the holes (on the order of 150 Å), which is desirable for imaging very small particles. The carbon is also of very high quality and provides the potential for examining the specimen over the carbon itself rather than just over the holes. This may be an advantage for samples in short supply because, even if the sample does not distribute over the holes, it can still be imaged over the carbon film.

Two issues that often impact the cryoEM specimen preparation process are (1) agglomeration of particles near hole edges in holey carbon films and (2) variations in vitreous ice thickness. Using an improved robust pattern transfer technique to fabricate holey carbon grids, both of these issues were greatly reduced, which led to a threefold increase in the number of images collected in a single session. This will potentially allow users to improve data throughput in a single experiment.

Samples of these grids are available on request from the National Resource for Automated Molecular Microscopy (http://nramm.scripps.edu) and are available for sale from Protochips, Inc. (http://www.protochips.com). John Damiano, Stephen E. Mick, David P. Nackashi, and Joel Quispe have commercial interests in Protochips, Inc.

Acknowledgments

This research was conducted at the National Resource for Automated Molecular Microscopy, which is supported by the National Institutes of Health though the National Center for Research Resources' P41 program (RR17573). We thank Arthur Horwich and George Farr for the GroEL, Ruben Diaz for the TMV and Mark Yeager and Kelly Dryden for the rotavirus samples.

References

Downing, K.H. (2003). Support films with uniform hole size. *Microsc Today* **11**, 54.

Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowall, A.W. & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. *Q Rev Biophys* **21**, 129–228.

Ermantraut, E., Wohlfart, K. & Tichelaar, W. (1998). Perforated support foils with re-defined hole size, shape and arrangement. *Ultramicroscopy* **74**, 75–81.

Henderson, R. (1995). The potential and limitations of neutrons, electrons, and X-rays for atomic resolution microscopy of unstained biological macromolecules. *Q Rev Biophys* **28**, 171–193.

Murray, J. (1987). Preparation of holey carbon films suitable for cryo-electron microscopy. *J Electron Microsc Tech* **5**, 285–290.

Quispe, J., Banez, R., Carragher, B. & Potter, C.S. (2004). Improving automation for cryo-EM specimen preparation. *Microsc Microanal* **10**(Suppl. S02), 1508–1509.

Stagg, S.M., Lander, G., Pulokas, J., Fellmann, D., Cheng, A., Quispe, J.D., Mallick, S.P., Avila, R.M., Carragher, B. & Potter, C.S. (2006). Automated cryoEM data acquisition and analysis of 284,742 particles of GroEL. *J Struct Biol* **155**, 470–481.

Suloway, C., Pulokas, J., Fellmann, D., Cheng, A., Guerra, F., Quispe, J., Stagg, S., Potter, C.S. & Carragher, B. (2005). Automated molecular microscopy: The new Leginon system. *J Struct Biol* **151**, 41–60.

Taylor, K.A. & Glaeser, R.M. (1974). Electron diffraction of frozen, hydrated protein crystals. *Science* **186**, 1036–1037.