Published in final edited form as:

J Struct Biol. 2007 December ; 160(3): 305–312.

ON THE FREEZING AND IDENTIFICATION OF LIPID MONOLAYER 2-D ARRAYS FOR CRYOELECTRON MICROSCOPY

Dianne W. Taylor[&], Deborah F. Kelly^{&,1}, Anchi Cheng[†], and Kenneth A. Taylor^{&§}

[&]*Institute of Molecular Biophysics Florida State University, Tallahassee, FL 32306-4380*

[†]*Center for Automated Molecular Imaging, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037*

Abstract

Lipid monolayers provide a convenient vehicle for the crystallization of biological macromolecules for 3-D electron microscopy. Although numerous examples of 3-D images from 2-D protein arrays have been described from negatively stained specimens, only six structures have been done from frozen hydrated specimens. We describe here a method that makes high quality frozen-hydrated specimens of lipid monolayer arrays for cryoelectron microscopy. The method uses holey carbon films with patterned holes for monolayer recovery, blotting and plunge freezing to produce thin aqueous films which cover >90% of the available grid area. With this method, even specimens with relatively infrequent crystals can be screened using automated data collection techniques. Though developed for microscopic examination of 2-D arrays, the method may have wider application to the preparation of single particle specimens for 3-D image reconstruction.

Keywords

Electron Crystallography

INTRODUCTION

Since the technique of lipid monolayer crystallization was first demonstrated (Uzgiris and Kornberg, 1983), it has been applied to a large number of specimens from which a number of 3-D reconstructions have been published (for the most recent reviews see (Chiu et al., 1997; Ellis and Hebert, 2001)). However, most 3-D imaging has been accomplished with specimens preserved for electron microscopy using negative stains, primarily uranyl acetate. Our lab has published the only 3-D reconstructions of lipid monolayer crystals preserved frozen hydrated for cryoelectron microscopy (cryoEM) (Tang et al., 2001; Wendt et al., 2001; Liu et al., 2003; Liu et al., 2004; Kelly et al., 2006; Liu et al., 2006) showing that 3-D reconstructions can be obtained from unstained 2-D arrays formed by this method. Frozen hydrated specimens are generally preferred over negatively stained specimens because contrast is due to the difference in scattering between the atoms of the structure and aqueous solvent, the possibility of specimen flattening during drying is prevented as is differential staining between top and bottom surfaces.

[§]Corresponding Author, Voice Phone (850) 644-3357, Fax (850) 644-7244, E-mail: taylor@bio.fsu.edu.

¹Current address: Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

One of the major problems in working with lipid monolayer specimens is the preparation of quality specimens for freezing and cryoEM. There are several issues that need to be resolved to obtain a 3-D reconstruction from lipid monolayer 2-D arrays. These include efficient recovery of samples from the monolayer surface without disruption of their long range order, blotting that leaves only a thin water film with vitrification after freezing over a large proportion of the grid, and finally identification of the crystalline arrays.

Several methods have been utilized for the recovery of lipid monolayer 2-D arrays. The simplest of these is the application of a hydrophobic carbon film to the free surface of the monolayer. With this method, the specimen grid with a carbon film is applied to the air-water interface; the grid is lifted after a short time interval and negatively stained, usually with uranyl acetate. Topologically, this places the lipid between the crystalline array and the carbon film and often leads to disruption of the array (Darst et al., 1991).

Two approaches have been developed to place the protein array in contact with the carbon film with the goal of reducing the disruption that occurs when the lipid is placed in contact with the carbon. One of these “lowers” the monolayer onto a hydrophilic carbon film. This approach has sometimes yielded larger, less broken arrays (Darst et al., 1991). Although some projections have been published from frozen hydrated lipid monolayer arrays recovered on continuous carbon films, no 3-D reconstructions have been produced to our knowledge. The second method utilizes a wire loop for recovery of the arrays (Asturias and Kornberg, 1995). This method has been used for preparing specimens for cryoEM but no 3-D reconstructions of the ice embedded specimens have been produced (Asturias et al., 1997).

The most efficient method for monolayer recovery utilizes holey carbon films (Kubalek et al., 1991). That the method has been used in a large number of studies utilizing both negatively stained and frozen hydrated specimens is testament to its efficiency at recovering these monolayer crystals. An extensive study was performed comparing perturbations of the specimen on lifting with either a solid carbon substrate or a reticulated carbon film (Brisson et al., 1999). They observed that transfer onto the solid carbon resulted in extensive damage to the monolayer with huge variability in the extent and quality of transferred material. On the other hand, several studies have showed that lipid monolayer arrays recovered with reticulated carbon films can diffract electrons to ~3 Å resolution (Kubalek et al., 1991; Avila-Sakar and Chiu, 1996; Celia et al., 1999).

Our laboratory has published six 3-D image reconstructions of proteins crystallized on lipid monolayers (Tang et al., 2001; Wendt et al., 2001; Liu et al., 2003; Liu et al., 2004; Kelly et al., 2006; Liu et al., 2006) and preserved frozen hydrated for image analysis. These studies have been facilitated by an adaptation of the Kubalek method for monolayer specimen recovery (Kubalek et al., 1991). During these six studies, we developed an efficient technique for freezing the specimens within thin vitreous films for cryoEM. The method has been briefly described in these papers but a detailed description has not been produced that fully documents the extremely high quality of the specimens that are routinely obtained. In addition, we report here on initial efforts to identify 2-D arrays within the holes of Quantifoil grids that will facilitate automated data acquisition in future studies.

METHODS

Specimen film preparation

Four of the six 3-D reconstructions published by our group, recovered the lipid monolayer arrays using reticulated films made in house by the method of Fukami and Adachi (1965). This method utilizes surfactants of various kinds to vary the hydrophobicity of a clean glass slide in order to vary the hole size within the reticulated film. The films have a high content of

relatively round holes and with sufficient surrounding matrix for focus and astigmatism correction. However, the holes are irregularly spaced. The two most recent studies (Kelly et al., 2006; Liu et al., 2006) utilized Quantifoil grids (Quantifoil Micro Tools, GmbH, Germany). These grids are characterized by a regular array of holes of uniform size and spacing that are ideally adapted for automated image acquisition such as provided by the Leginon software (Suloway et al., 2005).

We have successfully used Quantifoil grids (Ermantraut et al., 1998) with hole sizes up to 3.5 μm either as precarbon coated films or uncoated films that we carbon coated ourselves. For uncoated grids, the cleaning procedures are the same after the initial carbon coating. Residual plastic and any organic contaminants must be removed, otherwise monolayer recovery is inefficient. Thus, the grids are cleaned by placing them, carbon side up, on filter paper dampened with ethyl acetate. This puts the “grid bar” side facing the filter paper and the carbon film safely removed from the filter paper. The removal of the film and any surfactant must be completely done or the residue can contaminate the surface of the carbon. This would disrupt the lipid monolayer when it comes into contact with the grid resulting in poor recovery efficiency. After cleaning, another layer of carbon is deposited on the side of the film which will be applied to the monolayer surface. We apply two light coats of carbon to this side at 90° to each other. We prefer 300 mesh grids for surveying negatively stained specimens and 200 mesh grids for frozen hydrated specimens.

CryoEM Specimen Recovery & Freezing

To recover the monolayers for cryoEM in the frozen hydrated state, the grid is placed on the lipid monolayer, with the *grid bar* side facing the monolayer (Fig. 1A). The grid is left in contact with the monolayer film for ~30 seconds to allow the grid bars time to sink through the monolayer so that the carbon film can make contact with the lipid surface. The preference for 200 mesh grids for this purpose is due to the necessity of a large enough opening in the grid square for this contact to occur. The grid is then lifted from the air-water interface (Fig. 1B), blotted on the grid bar side with filter paper (Whatman 542), and plunge frozen in liquid ethane (Fig. 1C) (Dubochet et al., 1988). The exact time that the filter paper remains in contact with the grid prior to freezing is variable and depends on the size of the drop of mother liquor. As a guide, we wait for 2-3 seconds after the bulk of the water has been removed. This time point is judged according to the moment that the grid is no longer clearly visible through the filter paper. We generally do our freezing in a 4 °C cold room with minimal air circulation.

RESULTS

For cryoEM specimen preparation, the excess solution on the grid is removed by touching the face of the grid with a piece of filter paper so that solution is removed uniformly and rapidly across the grid surface. Our early attempts at blotting from the front side of the grid were not generally successful. By recovering monolayer samples with the “back” or grid bar side, as opposed to the “front” or carbon film side, the grid bars act to prevent contact between the filter paper and monolayer thereby producing low array disruption. Blotting from the back side also creates a small humidity chamber with the lipid retarding evaporation from the free side of the grid, the wet filter paper retarding evaporation from the other side and the grid bars providing the spacer. The monolayer itself makes the entire surface of the grid hydrophilic so that the crystallization medium can be easily blotted away from grids that were initially hydrophobic. With lipid monolayer specimens, the volume of aqueous phase recovered along with the monolayer crystals is also rather constant, facilitating blotting in a consistent manner.

We have invariably used copper grids for specimen recovery. These grids have the disadvantage that they contract at low temperatures more than the carbon film thereby causing a phenomenon known as “cryocrinkling” (Booy and Pawley, 1993). To overcome this

temperature induced wrinkling, we have tried this method with molybdenum grids, which are the grids of choice for high resolution crystallography, coated with reticulated films but the much thicker grid bars required working with 100 mesh grids. Unfortunately, at the moment, we don't know for sure, but doubt whether the carbon films that surround the holes and the water surface tension are capable of creating a flat specimen within the hole for high resolution work even if used in conjunction with molybdenum grids.

Reproducibility is usually high using this method. Once crystallization attempts are predictably producing crystals in high quantity across much of the monolayer, this method seems to recover them for cryoEM just as easily as for negative stain EM. The quality of the specimen is illustrated by the high percentage of grid squares that are intact and electron transparent (Fig. 2A). Most holes within the intact squares, shown here at 170x magnification, contain thin ice (Fig. 2B) estimated to be 100-200 Å thick. At a magnification of 3,500x individual holes can be screened for the presence of crystals (Fig. 2C) by the appearance of a ring of thicker ice film around the edges of the holes. This thicker ice is due to a lip of carbon film around the hole edge (see Figure 4). At higher magnification (29,000x) the presence of crystalline arrays within the holes can be demonstrated by computing the Fourier transform (Fig. 2D).

Utility for Automated Crystal Screening and Data Collection

When the monolayer specimen consists of contiguous crystals, as is the case with chicken smooth muscle α -actinin (Taylor and Taylor, 1993), we are unable to determine visually the presence of arrays within the holes. The array thickness for α -actinin is $\sim 65\text{\AA}$ and without a distinct boundary between array and surrounding ice, there is nothing to distinguish a crystal from simply thicker ice or uncrystallized protein. In our experience, protein arrays almost never continue past the edge of the holes onto the surrounding matrix, a quality that might make it easier to identify their presence. This is not true for all specimens. Actin rafts, $\sim 100\text{\AA}$ thick, in various forms usually can be identified visually because they do not cover the hole uniformly and sometimes extend over the edge into the surrounding matrix. Thicker, well ordered rafts have very straight edges which have enough contrast to be detected in ice. In most cases, identification of crystals within holes requires recording a low exposure image on a CCD camera and computing its Fourier transform. However, for automated data collection, some criterion for selection of holes with a high probability of containing arrays is desirable for efficiency.

In our experience with various forms of 2-D arrays of chicken smooth muscle α -actinin, the presence of a slight ring around the edge of holes is diagnostic of thin ice across the hole. Using this "vitrification" ring as a classification criteria, four categories of holes can be defined: (1) clear/flat (Fig. 3A), (2) clear with a ring (Fig. 3B), (3) thick with a ring (Fig. 3C) and (4) too thick (Fig. 3D). In the experiment illustrated in Figures 2 and 3, from high magnification exposures of 44 intact holes of various types, 10% were clear/flat and these did not show a reciprocal lattice in their Fourier transforms. These are probably "dry" holes. Of the 40 holes with a vitrification ring around the edge, 18 (45%) showed a clear reciprocal lattice in their Fourier transforms. Another 8 images with obviously thick ice showed a reciprocal lattice in 3 cases (38%) but these would probably be less desirable for data collection. Therefore conservatively, 40% of holes contain potential data for a 3-D reconstruction.

Taking the data in Figure 3, we plotted the radial density across the hole and onto the carbon matrix to provide a quantitative criterion by which routines implemented in Leginon could distinguish holes with high probability. These four examples of holes were all taken from the same micrograph so there are no issues as to beam fluctuations. The figures, show the raw data curve (Fig. 4A) and a curve normalized to the carbon matrix (Fig. 4B). It is clear from the graphs that the large lip at the edge of the holes, apparently a result of carbon evaporation followed by removal of the matrix, creates thick ice around the perimeter but the ice thickness

in the center of the holes is relatively constant suggesting good water spreading. Thus, hole distinction must be done mainly through the profile of the ice thickness at the edge of the holes rather than at the center. The distinction between clear-flat and clear-ring holes is quite small, yet clear-flat holes never yielded sampled diffraction, whereas clear-ring holes usually did. Thick ring holes also generally yielded sampled diffraction yet even these holes had similar ice thickness in the central part when the profiles were normalized to the carbon matrix density and differed only by an obviously wider ice ring around the edge. Thus, the water seems to have spread uniformly across the hole, beading up only near the thick lip. We take this to mean that as long as there was a ring of water around the perimeter of the hole, that water was present across the monolayer film in sufficient quantity to preserve the arrays at the moment of freezing. Protein would be present in high concentration even in dry holes that never contained arrays, which may account for the small differences in density towards the center across all hole-types.

Using the grid illustrated in Figure 2A, 52% of the grid squares have ice of potentially useful thickness while the remaining holes either have thick ice or have broken support films. This 200 mesh Quantifoil grid shows 220 visible grid squares and 114 that might be useful. Each useable grid square has 675 holes 2 μm in diameter. Of the roughly 600 holes that contain the vitrification ring, combined with a success rate of $\sim 40\%$, about 240 holes / grid square contain 2-D arrays. Thus, over this entire grid, there could be $\sim 28,000$ holes containing arrays of varying size and quality but which display diffraction when the entire image of the hole is transformed. This enormous number of potential arrays provides ample opportunity to choose only the ones with the best diffraction to include in the data sets.

DISCUSSION

Lipid monolayer crystallization has enormous potential for the production of ordered arrays of soluble proteins, or extrinsic membrane bound proteins for structural work. The large number of papers reporting lipid monolayer 2-D arrays of a variety of specimens attests to the wide applicability of the method (for recent reports see (Ganser et al., 2003; Mayo et al., 2003; Makhov et al., 2004)). While several efforts at preserving these arrays in the frozen hydrated state have shown promise, with the exception of six 3-D reconstructions from ice embedded specimens, all 3-D reconstruction work has been done on negatively stained specimens. Frozen hydrated specimens have several advantages for structural work: (1) the contrast is the native contrast between protein and solution, (2) refinements of atomic models using standard crystallographic refinement programs can be done without attempting to define the relationship between specimen and stain, (3) specimen distortion on drying, such as flattening and non-uniform stain distribution between top and bottom of the specimen are avoided (see detailed discussions in (Frank, 1996)).

For a specimen preparation method to be viable for cryoEM it must produce a large number of useable grid squares with thin ice over the holes. If the number of useable grid squares is small and the number of grid squares containing arrays is small, then cryoEM becomes very tedious because the product of two small probabilities is very small. The use of holey carbon films has resulted in very high efficiency of specimen recovery from the monolayer surface compared with continuous carbon films. Needed was a high efficiency method for producing thin, vitrified films of these samples which the present technique provides.

Brisson et al. (1999) have systematically compared lipid monolayer protein arrays recovered on holey carbon and continuous carbon films and found that in addition to differences in efficiency of transfer, that the structure of the protein at the monolayer is correctly maintained only when the monolayer is transferred to a grid with a holey film. Indeed, their work documents a clear change in structure on transfer to a continuous carbon grid. Thus, where the

relationship of the protein with the lipid is important, holey carbon films are preferable over continuous carbon grids.

In our work with different protein samples, we found that when the monolayer specimens were recovered with holey films by placing the grid bar side on the monolayer followed by recovery and blotting, that a large proportion of the grid squares had thin ice and that a large number of the holes within the grid squares had thin ice. This made the chances of finding arrays within holes containing thin ice high enough that data could be obtained with a reasonable effort. Recovering monolayer specimens using Quantifoil grids then permitted grid screening with Leginon. Combined with further screening according to the radial density criteria provides a rapid means of identifying crystals making automated data collection highly feasible.

While recovery of lipid monolayers on holey carbon films is efficient, the specimens typically have the draw back for high resolution work of relatively imperfect flatness, compared to what can be obtained using high quality continuous carbon films. Flatness is further aggravated in the present work by the use of copper grids for specimen recovery. Although specimens appear well ordered in projection, order in images of tilted specimens is poor. Flatness may be only one of several factors contributing to this. We see no solution to the flatness problems in lipid monolayer specimens at the moment other than application of a “single particle” approach to analysis of these 2-D arrays.

Although we have used this method for the freezing of 2-D monolayer crystals, it may have wider application to the freezing of single particle specimens for cryoEM. Ice embedded specimens of molecules attached to positively charged lipid monolayers have been used for some single particle applications (Medalia et al., 2002; Azubel et al., 2004). In those cases the positive monolayer charge provided some purification and concentration of the specimen. Whether the monolayer recovery was done as described here is not known. We suggest that by anchoring a molecule or complex to the monolayer using charge, as above, or a long linker attached to a his6 tag in combination with a Ni chelating lipid (Kubalek et al., 1994), that a distribution of molecules in a thin ice film and with random orientations could be produced. The variability of defocus across the ice film would be minimal and the specimens produced could be readily applied to high throughput data collection methods currently under development (Suloway et al., 2005). The production of arrays could be avoided by using conditions that are not conducive to crystallization, such as short incubation times and no precipitants. Single particle specimens could potentially be produced within minutes of layering the monolayer over the protein containing buffer.

In summary, the steps used for preparation of frozen hydrated, lipid monolayer specimens include (1) carefully prepare clean, hydrophobic 200 mesh holey carbon films (in particular Quantifoil grids) free of detergents and other contaminants, (2) place the grid, bar side down, onto the monolayer surface, (3) wait ~30 seconds for the monolayer and reticulated film to come into contact, (4) blot excess fluid by placing a piece of filter paper (Whatman 542) against the grid bars, (5) wait 2-3 seconds after the grid disappears behind the filter paper and plunge immediately into liquid ethane. The frozen-hydrated sample may then be used for manual or automated screening and data collection procedures using low-dose conditions.

Acknowledgements

We thank Dr. Bridgett Carragher for her helpful comments on an earlier version of the manuscript. This research has been supported by NIH grants AR42872, AR47421, and GM64346 to the Cell Migration Consortium. The Center for Automated Molecular Imaging is supported by NIH grant RR17573.

References

- Asturias FJ, Kornberg RD. A novel method for transfer of two-dimensional crystals from the air/water interface to specimen grids: EM sample preparation/lipid-layer crystallization. *J Struct Biol* 1995;114:60–66. [PubMed: 7772418]
- Asturias FJ, Meredith GD, Poglitsch CL, Kornberg RD. Two conformations of RNA polymerase II revealed by electron crystallography. *J Mol Biol* 1997;272:536–540. [PubMed: 9325110]
- Avila-Sakar AJ, Chiu W. Visualization of β -sheets and side-chain clusters in two-dimensional periodic arrays of streptavidin on phospholipid monolayers by electron crystallography. *Biophys J* 1996;70:57–68. [PubMed: 8770187]
- Azubel M, Wolf SG, Sperling J, Sperling R. Three-dimensional structure of the native spliceosome by cryo-electron microscopy. *Mol Cell* 2004;15:833–839. [PubMed: 15350226]
- Booy FP, Pawley JB. Cryo-crinkling: what happens to carbon films on copper grids at low temperature. *Ultramicroscopy* 1993;48:273–280. [PubMed: 8475597]
- Brisson A, Bergsma-Schutter W, Oling F, Lambert O, Reviakine I. Two-dimensional crystallization of proteins on lipid monolayers at the air-water interface and transfer to an electron microscopy grid. *J Cryst Growth* 1999;196:456–470.
- Celia H, Wilson-Kubalek E, Milligan RA, Teyton L. Structure and function of a membrane-bound murine MHC class I molecule. *Proc Natl Acad Sci USA* 1999;96:5634–5639. [PubMed: 10318936]
- Chiu W, Avila-Sakar AJ, Schmid MF. Electron crystallography of macromolecular periodic arrays on phospholipid monolayers. *Adv Biophys* 1997;34:161–172. [PubMed: 9204133]
- Darst SA, Ahlers M, Meller PH, Kubalek EW, Blankenburg R, Ribi HO, Ringsdorf H, Kornberg RD. Two-dimensional crystals of streptavidin on biotinylated lipid layers and their interactions with biotinylated macromolecules. *Biophys J* 1991;59:387–396. [PubMed: 1901232]
- Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, McDowell AW, Schultz P. Cryo-electron microscopy of vitrified specimens. *Q Rev Biophys* 1988;21:129–228. [PubMed: 3043536]
- Ellis MJ, Hebert H. Structure analysis of soluble proteins using electron crystallography. *Micron* 2001;32:541–550. [PubMed: 11163726]
- Ermanntraut E, Wohlfart K, Tichelaar W. Perforated support foils with pre-defined hole size, shape and arrangement. *Ultramicroscopy* 1998;74:75–81.
- Frank, J. Three-Dimensional Electron Microscopy of Macromolecular Assemblies. Academic Press; San Diego, CA: 1996.
- Fukami A, Adachi K. A new method of preparation of a self-perforated micro plastic grid and its application. *J Electron Microsc* 1965;14:112–118.
- Ganser BK, Cheng A, Sundquist WI, Yeager M. Three-dimensional structure of the M-MuLV CA protein on a lipid monolayer: a general model for retroviral capsid assembly. *EMBO J* 2003;22:2886–2892. [PubMed: 12805204]
- Kelly DF, Taylor DW, Bakolitsa C, Bobkov AA, Bankston L, Liddington RC, Taylor KA. Structure of the α -actinin-vinculin head domain complex determined by cryo-electron microscopy. *J Mol Biol* 2006;357:562–573. [PubMed: 16430917]
- Kelly DF, Taylor KA. Identification of the β 1-integrin binding site on α -actinin by cryo-electron microscopy. *J Struct Biol* 2005;149:290–302. [PubMed: 15721583]
- Kubalek EW, Kornberg RD, Darst SA. Improved transfer of two-dimensional crystals from the air/water interface to specimen support grids for high-resolution analysis by electron microscopy. *Ultramicroscopy* 1991;35:295–304. [PubMed: 1926634]
- Kubalek EW, Le Grice SF, Brown PO. Two-dimensional crystallization of histidine-tagged, HIV-1 reverse transcriptase promoted by a novel nickel-chelating lipid. *J Struct Biol* 1994;113:117–123. [PubMed: 7536435]
- Liu J, Taylor DW, Kremontsova EB, Trybus KM, Taylor KA. Three-dimensional structure of the myosin V inhibited state by cryoelectron tomography. *Nature* 2006;442:208–211. [PubMed: 16625208]
- Liu J, Taylor DW, Taylor KA. A 3-D reconstruction of smooth muscle α -actinin by CryoEm reveals two different conformations at the actin-binding region. *J Mol Biol* 2004;338:115–125. [PubMed: 15050827]

- Liu J, Wendt T, Taylor D, Taylor K. Refined model of the 10S conformation of smooth muscle myosin by cryo-electron microscopy 3D image reconstruction. *J Mol Biol* 2003;329:963–972. [PubMed: 12798686]
- Makhov AM, Taylor DW, Griffith JD. Two-dimensional crystallization of herpes simplex virus type 1 single-stranded DNA-binding protein, ICP8, on a lipid monolayer. *Biochim Biophys Acta* 2004;1701:101–108. [PubMed: 15450179]
- Mayo K, Huseby D, McDermott J, Arvidson B, Finlay L, Barklis E. Retrovirus capsid protein assembly arrangements. *J Mol Biol* 2003;325:225–237. [PubMed: 12473464]
- Medalia O, Typke D, Hegerl R, Angenitzki M, Sperling J, Sperling R. Cryoelectron microscopy and cryoelectron tomography of the nuclear pre-mRNA processing machine. *J Struct Biol* 2002;138:74–84. [PubMed: 12160703]
- Suloway C, Pulokas J, Fellmann D, Cheng A, Guerra F, Quispe J, Stagg S, Potter CS, Carragher B. Automated molecular microscopy: The new Leginon system. *J Struct Biol* 2005;151:41–60. [PubMed: 15890530]
- Tang J, Taylor DW, Taylor KA. The 3-D structure of α -actinin obtained by cryoelectron microscopy suggests a model for Ca^{2+} dependent actin binding. *J Mol Biol* 2001;310:845–858. [PubMed: 11453692]
- Taylor KA, Taylor DW. Projection image of smooth muscle α -actinin from two-dimensional crystals formed on positively charged lipid layers. *J Mol Biol* 1993;230:196–205. [PubMed: 8450536]
- Uzgiris EE, Kornberg RD. Two-dimensional crystallization technique for imaging macromolecules, with application to antigen-antibody-complement complexes. *Nature* 1983;301:125–129. [PubMed: 6823289]
- Wendt T, Taylor D, Trybus KM, Taylor K. Three-dimensional image reconstruction of dephosphorylated smooth muscle heavy meromyosin reveals asymmetry in the interaction between myosin heads and placement of subfragment 2. *Proc Natl Acad Sci USA* 2001;98:4361–4366. [PubMed: 11287639]

Figure 1.

Schematic diagram illustrating monolayer recovery and freezing. The diagram is based on binding of chicken smooth muscle α -actinin to a peptide corresponding in sequence to the β 1-integrin cytoplasmic domain, which in turn is binding to a Ni chelating lipid (Kelly and Taylor, 2005). (A) To recover monolayer samples for cryoEM, we placed the grid bar side down onto the monolayer surface. After ~ 30 seconds, the grid sinks through the monolayer thereby facilitating contact between the lipid layer and the reticulated carbon film. The grid can then be lifted for freezing. A magnified representation of a single grid square is also indicated. (B) The orientation of the monolayer in the grid square of a Quantifoil grid allows blotting from the “back” side of the grid, as opposed to the “front” side on which the reticulated carbon film is located. (Recovery using the front side would normally be used for negative staining.) This highly magnified side view of the grid (with grid bars represented as copper colored surfaces) demonstrates how the grid bars help to shield the crystals from being disrupted by the filter paper during blotting. (C) After adequate blotting from the back side of the grid, the specimen is plunged into liquefied ethane (Dubochet et al., 1988) after which specimen manipulations are the same as for any other frozen specimen.

Figure 2.

(A) Low magnification survey view of the frozen-hydrated specimen recovered using a Quantifoil grid. Magnification of 170x. In this example, about 52% of the 220 grid squares would be potentially useful. The others have broken films or excessively thick ice. (B) Medium magnification (3500x) view of a grid square shown in (A). Here the reproducible character of the holes within a single grid square can be readily seen. Note that although the crystallization solution is topologically on the side of the grids with the grid bars, the ice is thinnest around the grid bars. This is counter to expectations, but is what occurs normally with this method. (C) High magnification view of one hole in the area shown in (B). The frozen hydrated holes are readily identified by the relatively thicker ring of ice near the edges of the holes. (D) High magnification (29,000x) of one hole with a crystal and its Fourier Transform in the lower right hand corner.

Figure 3.

Four examples of holes with variable ice thickness. In each image a white line to the left is used to indicate differences in the ice thickness at the edges. Qualitatively, the variations in the appearance of holes can be described as: **(A)** clear-flat hole – ordered diffraction is not obtained from images with this appearance; **(B)** clear with a ring – typically, these holes yield diffraction spots about 35% of the time; **(C)** thick with a ring – holes with this appearance show crystalline diffraction 45% of the time; **(D)** a hole with ice that is too thick for useful imaging although ordered arrays are commonly found in these holes about 38% of the time.

Figure 4.

Radial density plots obtained from the four hole-types shown in Figure 3. **(A)** Raw data. The large fluctuations near a radius of zero are due to the relatively few pixels in this region. Note that the clear-flat profile, which lacks the water ring around the lip of the hole, is noticeably different than the other three hole-types, which contain the ring. **(B)** The same data plotted but this time normalized to constant density for the carbon matrix. This would be correct only if the carbon matrix contained no ice film across it. Normalizing to the carbon matrix makes all four hole-types appear as if they had constant ice thickness, but this is probably not true since Fourier transforms of clear-flat holes never show crystalline diffraction whereas the other hole-types do.