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ScienceDirect
Journal of Structural Biology 160 (2007) 305–312
Journal of Structural Biology
www.elsevier.com/locate/yjsbi

On the freezing and identification of lipid monolayer 2-D arrays for cryoelectron microscopy

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Received 18 June 2005; received in revised form 2 April 2007; accepted 4 April 2007
Available online 6 May 2007

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; Automated microscopy

1. 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 laboratory 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, 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.

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

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doi:10.1016/j.jsb.2007.04.011

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 shown 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, 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.

2. Methods

2.1. 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.

2.2. CryoEM specimen recovery and 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 s 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 s 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.