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Method Article

Suspending samples over carbon holey films increases heterogeneity of molecular orientations in negative stain electron microscopy

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A B S T R A C T

Negative stain electron microscopy (NSEM) is a simple and cost effective method to analyze a wide variety of specimens, especially proteins. In traditional NSEM, the protein sample is applied to and supported by a continuous carbon film. Unfortunately, many proteins stick to the carbon film with a limited number of orientations. Because the restricted orientation limits the available views of the molecule, information about the three-dimensional structure of the molecule is likewise limited. The method presented here overcomes this limitation by using a carbon holey film combined with 1-octadecanol as a spreading agent. We demonstrate this method with solubilized envelope (Env) proteins from HIV, which typically show a restricted orientation on continuous carbon film, and show the following:

- 1-octadecanol added to negative stain aids the formation of a continuous sample-stain layer spanning the holes of a holey carbon film.
- Samples negatively stained over holes show less restricted orientation, resulting in better single particle reconstructions.

A R T I C L E I N F O

Method name: Negative stain over holes

Keywords: 1-Octadecanol, Uranyl formate, Carbon film, Preferred orientation, Single particle reconstructions

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More specific subject area:	*Negative stain electron microscopy*
Method name:	*Negative stain over holes*
Name and reference of original method:	*N/A*
Resource availability:	*N/A*

Method details

Background

Traditional NSEM over a continuous carbon film often gives a limited number of molecular views, due to a limited number of orientations on the film [1]. Harris and Scheffler demonstrated that it was possible to reduce or eliminate the restricted orientation seen on carbon films by imaging molecules suspended over the holes of a carbon holey film using ammonium molybdate with trehalose for the negative stain [2,3]. However, when using uranyl acetate for the stain, they reported considerable instability of the sample in the electron beam [2]. Our own attempts with uranyl formate stain yielded a high fraction of empty holes. We therefore adopted the strategy of adding 1-octadecanol, which was originally proposed as a spreading agent for traditional NSEM over continuous carbon films [4]. As shown here, when 1-octadecanol is used with uranyl formate over carbon holey films, we are able to form a continuous sample-stain layer that fills the majority of the holes in the carbon film.

Equipment needed

- Forceps, #5
- Electron microscope
- Standard laboratory pipets, tubes and glassware

Materials

- Protein sample, $\gtrsim$ 0.1 mg/ml
- Compatible dilution buffer (*e.g.* 20 mM HEPES, 150 mM NaCl, 2% glycerol, pH 7.4)
- Deionized water
- Uranyl formate (Electron Microscopy Sciences #22450), 0.5−0.6% in deionized water.
- 0.015% 1-octadecanol (Sigma-Aldrich #74723) in hexane (Sigma-Aldrich #296090)
- Carbon holey grids (*e.g.* C-flat™ grids, Electron Microscopy Sciences #CF31)
- Whatman Grade 1 filter paper cut into small triangles

NSEM grid preparation

1 As needed, dilute ~1–3 μg of protein with dilution buffer to give a volume of ~25 μl at a final concentration of 40–100 μg/ml protein. This volume is sufficient to make five NSEM grids.
2 Do not glow discharge the carbon holey grids. Unlike traditional NSEM over continuous carbon films, you want the carbon film to remain hydrophobic in this technique, so that your sample preferentially stays in the holes rather than sticking to the film.
3 Load ~5 μL of sample onto carbon side of the grid and incubate for 5–10 s.
4 Use a filter paper triangle to wick off excess sample solution leaving a thin film on the grid
5 Immediately rinse the sample by adding 5 μL of deionized water and incubating for 5 s.
6 Wick off and immediately add 5 μL of uranyl formate stain solution.
7 Immediately add 1 μL of 1-octadecanol solution to the dome of stain droplet and incubate for 1 m.

8 Wick off excess stain and leave a very thin film of stain.
9 Allow the sample to air dry.
10 Examine samples in electron microscope as needed.

Methods validation

To assess the tendency to show a restricted orientation, we examined a soluble HIV Env construct (CH505TFchim.6R.SOSIP.664.v4.1/293 F, graciously supplied to us by Dr. Kevin Saunders) by negative stain prepared over holes as described above. We considered collecting data from the carbon film areas of the same sample grid to use as an internal control; however, we deemed the carbon film in C-flat holey grids to be too thick and grainy to make this a fair comparison. Instead we prepared our own grids with a thin, continuous carbon film to use for controls. When preparing the sample grid, we concurrently prepared a separate control grid on a continuous carbon film, which was otherwise treated identically, including the addition of 1-octadecaol. Typical results are shown in Fig. 1. The sample prepared over continuous carbon film shows Env molecules that are mostly triangular, or shaped like a 3-bladed propeller (Fig. 1A). These shapes indicate that the most common view of the molecule is looking down the molecule's 3-fold symmetry axis. That is, the proteins sit on the carbon film with a restricted orientation. In contrast, the Env shapes in the sample prepared over holes show a more varied appearance (Fig. 1D), indicating less restricted orientation. Both samples showed similar stability in the electron beam and sensitivity to radiation damage. They were stable at low illumination levels or during 1-second exposures at higher illumination. Exposing either sample to a focused electron beam induced radiation damage, seen as an excessive graininess in the exposed region.

To further test the restricted orientation, we performed a 3D single particle analysis on the two samples. Both samples were imaged on a Philips EM420 electron microscope with a CCD camera (4pi Analysis) at a magnification of 82,000x, corresponding to sampling size of 4 Å/pixel, to give about 20 micrographs each. Electron micrographs were imported into the EMAN2 image processing software suite [5]. Images of individual molecules were computationally cut out with a square window 64 pixels wide and placed into a stack of ~11,000 images for each sample. Image stacks were subjected to ten rounds of iterative 2D class averaging. Class averages were used to generate *ab initio* 3D models, assuming C3 symmetry. The initial models were low-pass filtered to 30-Å resolution and used as

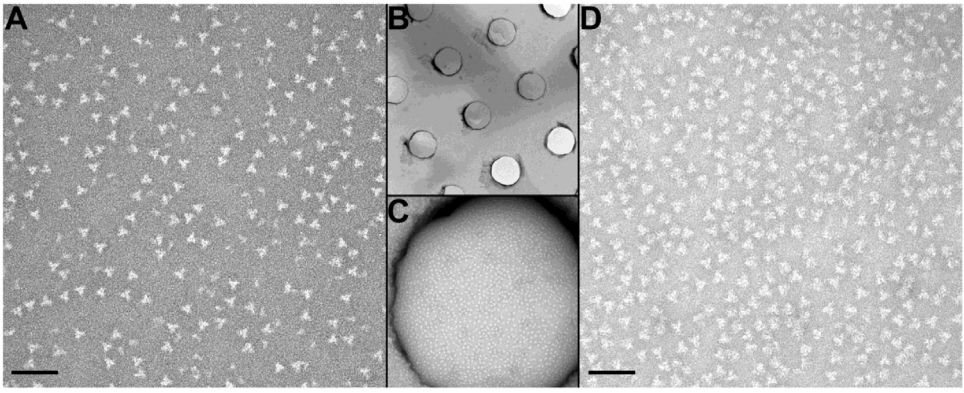

Fig. 1. Negatively stained HIV Env proteins appear as white structures on a dark background. A. When negatively stained over continuous carbon, most molecules appear as a roughly triangular shape. B. Low magnification view of a sample negatively stained over a C-flat holey grid shows a few empty holes; but most of the 1-μm diameter holes are spanned by stain. C. An intermediate magnification view of a single 1-μm hole shows a uniform sample-stain layer. D. This image is an enlargement of the central region of C, and shows that the molecules within the holes have a varied appearance. Scale bars =500 Å.

starting models for six rounds of iterative 3D refinement with EMAN, assuming C3 symmetry, to generate final 3D reconstructions.

Fig. 2 compares the 3D reconstructions from samples prepared over continuous carbon films (left) or over holes (right). For both reconstructions, the overall shape is similar (Fig. 2A). However, for the sample imaged over continuous carbon (left), the data shows a markedly restricted orientation. The restricted orientation is noted in two ways. First, many views are missing in the class averages from the sample imaged over continuous carbon (Fig. 2B, left). Second, most views are clustered near the "top view," which looks down the three-fold symmetry axis of the molecule (Fig. 2C, left). In the sample imaged over continuous carbon, the missing views correspond to side views of the molecule. These

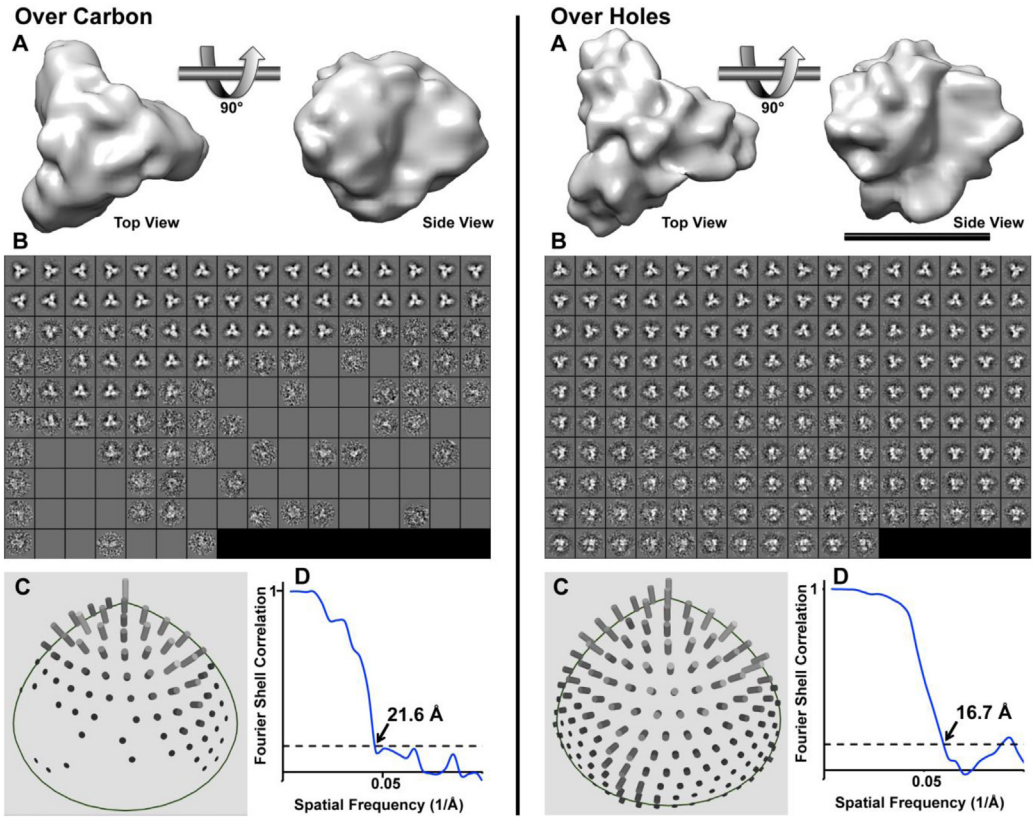

Fig. 2. Left and right sides show the results of single particle 3-dimensional reconstructions from samples negatively stained over continuous carbon film or over holes, respectively. A. The final 3D maps are seen as surface representations in top and side views. Scale bar =100 Å. B. The data used to generate the final maps are shown here as two-dimensional class averages. Each image in B corresponds to a different view of the molecule. The view looking down the three-fold symmetry axis of the molecule is at the top-left in B. This view is the same orientation as the top view in A, but seen as a 2D projection in B rather than the surface representation shown in A. On the left, many images in B are missing. The missing images indicate that views of the molecule in those orientations were not observed in the specimen over continuous carbon. C. This 3D graph represents the spread of the raw data over different views of the molecule in three dimensions. The graph samples 1/3 of the hemisphere that encompasses the unique views of the 3-fold symmetric HIV Env molecule. Each class average in B is represented here as a cylinder. The location of the cylinder represents direction of view in three dimensions. For example, the top view, looking down the 3-fold symmetry axis, corresponds to the cylinder at the top center in C. Side views are represented by cylinders at the bottom, along the equator of the 1/3 hemisphere. The height of each cylinder corresponds to the number of images found in that view. An ideal specimen would have an even distribution of views; that is, the 1/3 hemisphere would be evenly covered with cylinders of equal height. D. A graph of Fourier Shell Correlation indicates the final resolution of each reconstruction (*6*), given by the reciprocal of the spatial frequency where the curve falls to a value of 0.143 (dashed line) and labeled on the graph.

side views are critical for obtaining optimal resolution. In the sample imaged over continuous carbon (Fig. 2D, left), the final resolution was 21.6 Å, as judged by the Fourier Shell Coefficient plot [6]. In contrast, the sample imaged over holes displayed much less restricted orientation. There were no missing views (Fig. 2B, right); and the views were more evenly distributed (Fig. 2C, right). The final resolution was slightly better, 16.7 Å (Fig. 2D, right).

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