Abstract

We report on the use of EM tomography to visualize individual proteins in negative stained samples. A negative stain for tomography must not degrade under the electron dose used to collect a set of dual axis tomographic images at the necessary magnification. This precondition was verified experimentally for a tungsten-based stain for pixel sizes as small as 0.3 nm. Results show individual molecules on the surfaces of influenza virus and in post synaptic densities. Corresponding surface renderings of proteins obtained from virtual sections by thresholding demonstrate a good fit to known structures. Negative stain tomography thus provides a way to visualizing both the conformational richness and clustering properties of some complex proteins.

Negative staining has been a pivotal technique for visualizing in exquisite detail the structure of protein molecules and oligomers. The most abundant glycoprotein of the influenza virus, hemagglutinin, was observed for the first time after extraction from the virions [1]. Later, the structure of the holoenzyme, calcium calmodulin kinase II (CaMKII), a major component of synapses, was determined by imaging negative stained extracts with electron microscopy [2]. More recently there has been new evidence that this holoenzyme assumes a disk-shape in solution [3].

Current methods allow tomograms to be calculated by back projection from series of tilt images collected at high resolution by electron microscopy (EM). Gold particles are used as fiduciary markers to align sets of tilt images at pixel accuracy [4], thereby yielding high signal to noise ratios. In calculated virtual sections, one pixel thick, details commensurate to the resolution can now be sorted out by eye, and then selected, segmented by thresholding, and rendered. The tungsten-based negative stain tolerates the doses needed to image at high magnifications (Fig 1A-C). Virtual sections from tomography reveal fine details in images obtained from averaging ~140 tilt images acquired at 300 keV at a magnification giving a pixel size of 0.29-0.37 nm, using a dose of ~10⁶ e⁻/nm².

Glycoproteins were observed on influenza virus adhered to a substrate, while CaMKII molecules were observed on rat hippocampal post synaptic densities (PSD) obtained by cell fractionation [5]. Results show macromolecules of shapes and dimensions very similar to those obtained after studying crystals (Fig. 1). In particular, the shapes of the hemagglutinin and the CaMKII (Fig. 2) molecules as well as their dimensions are reproduced accurately in the negative stained samples. Thus, negative stain tomography provides an alternative method to view fine details of molecular structure in protein complexes. It can be performed on very small sample, and has potential for imaging molecules in larger protein complexes. Since it requires exposure of proteins to heavy metals, each application must be evaluated separately by comparison with the known structure of the protein to determine that damage from the stain in absent. The present study demonstrates examples where the negative stain tomography does provide useful structural data.
Methods: Influenza virus A/Japan/305/57, H2N2 (Charles River); PSDs extracted from rat brain by cell fractionation [5]. Samples negative stained on glow-discharged Formvar-carbon coated grids with Nano-W (Nanoprobes Inc.). Fiduciary markers: 5 or 10 nm gold spheres coated with protein (Ted Pella): minimum five per tomogram. Tecnai EM operated at 300 keV to minimize stain degradation, observed otherwise. Tomography experiment: section tilted every 2° around two arbitrary perpendicular axes; total ~140 images. Tomograms calculated using IMOD (Boulder Laboratory, CO). Virtual sections of thickness equal to the initial pixel size extensively analyzed first by eye. Images modified only with spatial filtering, binning and contrast adjustment. Rendering by thresholding with AMIRA (Visage Imaging, Inc).

References
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