In Situ Molecular Microscopy

Imaging Viruses in Liquid at the Nanoscale

We present a novel approach for visualizing viral assemblies in liquid using transmission electron microscopy (TEM). We utilize a microfluidic chamber that fits within a TEM specimen holder and while inserted in the column, is completely isolated from the vacuum. This configuration proves suitable for imaging and reconstructing viral complexes in solution at 2.5-nanometer resolution.

Background & Introduction

Understanding how molecular machines function is a primary goal of biologists and engineers. Transmission electron microscopes (TEM) are commonly used to peer into the world of macromolecules at the atomic level [1-2]. A current technical limitation is that samples must be fixed to sustain the vacuum system of a TEM. To accomplish this, specimens are typically frozen in a thin of vitreous ice [3]. Although ice maintains biological samples in a near-native environment, it also arrests functionality.

Recent advances in the development of graphene and silicon nitride provide new opportunities for real-time imaging at the nanoscale. When utilized as environmental chambers these materials permit researchers to perform experiments in situ or "inside" the TEM. In conjunction with new microfluidic based specimen holders, scientists have recently observed the growth of materials [4] and live cells engulfing nanoparticles [5]. One challenge with the technique is the movement that occurs when entities are freely diffusing in solution. Our recently developed Affinity Capture devices utilize especially coated silicon nitride microchips to tether protein complexes diffusing in liquid [6]. These devices can be produced using commercially available microchips and form a microfluidic chamber, suitable for TEM image acquisition. Two of these chips can be tightly sealed together with nanometer-thick spacers to accommodate samples in a self-contained "nanoscale biosphere" (fig. 1a). Transparent windows (~50 nm thick) etched into the chips allow the electron beam to penetrate the liquid chamber for imaging purposes. Our present work explains how this technology can be used to capture viral assemblies onto microchips decorated with antibodies against a capsid protein.
Rotavirus double-layered particles (DLPs) imaged using a microfluidic TEM platform yielded the first 3D view of biological assemblies in solution. We refer to this technique as in situ molecular microscopy in our recent publications [6-7].

**Experimental Design**

To engineer a system for capturing viral assemblies, we coated silicon nitride microchips with affinity biofilms doped with functionalized Ni-NTA (Nickel-nitrilotriacetic-acid) lipids as previously described [6]. His-tagged protein A was added to the Ni-NTA-coated microchips to serve as an adaptor molecule to bind to polyclonal antibodies against the viral protein, VP6. This system effectively mimics an antibody affinity column (fig. 1b). Rotavirus DLPs were purified using established protocols [8] and added to the microchips decorated with adaptors followed by the addition of contrast reagent. A second glow-discharged microchip was placed on top of the wet specimen chip to form the fluidics chamber that was positioned into the tip of a Poseidon TEM specimen holder (Protochip, Raleigh, NC). The holder containing the intact liquid chamber was inserted into a FEI Spirit BioTwin TEM equipped with a tungsten filament and operating at 120 kV. Specimens were examined under low-dose conditions and images of rotavirus particles suspended in liquid were recorded at 30,000x for processing routines with a final sampling of 10 Å/pixel.

**3D Structures of Viruses in Solution**

Individual particles were selected from images of DLPs in liquid using the Spider software package [9] (fig. 2a). To determine if dynamic features could be detected in the overall structure of DLPs, we calculated 3D reconstructions using the Relion software package [10]. Reconstruction and refinement procedures were iterated for 15 cycles outputting a single 3D reconstruction (fig. 2b) having a resolution of ~25 Å. The density map was in good agreement with the reference model derived from cryo-EM. We then used the Relion program to estimate the relative degree of
heterogeneity present in our sample. Following the first five rounds of refinement, statistical values indicated the presence of four variant structures. We refined each of the structures independently for an additional 10 iterations. The resulting 3D volumes were masked to 94 nm with a major structure containing 65% of the particles and another variant containing 23% (fig. 2b). Two additional reconstructions contained only 7% and 5% of the total particles present in the image stack (fig. 2b). The four individual reconstructions revealed a potentially more heterogeneous 3D view of DLPs in solution than what was reflected in the single density map calculated from the entire image stack.

To determine whether our sub-classes of reconstructions derived from DLPs in liquid would also be present in frozenhydrated samples, we collected images of ice-embedded DLPs prepared using the same experimental parameters (fig. 3). Images of frozen specimens revealed a similar degree of quality as the liquid specimens. The same refinement and reconstruction procedures were implemented in Relion to calculate a 3D volume. A single statistically significant species was present in image stack of ice-embedded DLPs, yielding a ~24-A reconstruction (fig. 3 inset). These findings suggest that frozen specimens may provide a less operationally dynamic view of 3D structures in comparison to the liquid specimens. We are currently working to further test this concept.

**Discussion**

Our techniques provide a new avenue for visualizing macromolecules using in situ molecular microscopy. In summary, we show the first single particle analysis of biological complexes contained within a liquid environment. We found that viral assemblies in solution exhibit greater variability in 3D than their frozen counterparts. Overall, we envision these new tools may be ideal for studying live macromolecular mechanisms, such as assembly pathways and viral entry into host cells. We anticipate this exciting new frontier may also permit cell biologists to study disease processes with a remarkable new level of resolution.

**References**


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