Video Article

In situ TEM of Biological Assemblies in Liquid

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URL: https://www.jove.com/video/50936
DOI: doi:10.3791/50936

Keywords: Bioengineering, Issue 82, Microfluidics, Transmission Electron Microscopy (TEM), *In situ* imaging, Rotavirus, simian rotavirus double-layered particles (DLPs), 3D structure determination

Date Published: 12/30/2013

Citation: Dukes, M.J., Gilmore, B.L., Tanner, J.R., McDonald, S.M., Kelly, D.F. *In situ* TEM of Biological Assemblies in Liquid. *J. Vis. Exp.* (82), e50936, doi:10.3791/50936 (2013).

Abstract

Researchers regularly use Transmission Electron Microscopes (TEMs) to examine biological entities and to assess new materials. Here, we describe an additional application for these instruments- viewing viral assemblies in a liquid environment. This exciting and novel method of visualizing biological structures utilizes a recently developed microfluidic-based specimen holder. Our video article demonstrates how to assemble and use a microfluidic holder to image liquid specimens within a TEM. In particular, we use simian rotavirus double-layered particles (DLPs) as our model system. We also describe steps to coat the surface of the liquid chamber with affinity biofilms that tether DLPs to the viewing window. This permits us to image assemblies in a manner that is suitable for 3D structure determination. Thus, we present a first glimpse of subviral particles in a native liquid environment.

Video Link

The video component of this article can be found at https://www.jove.com/video/50936/

Introduction

A common goal of biologists and engineers is to understand the inner-workings of molecular machines. Transmission Electron Microscopes (TEMs) are ideal instruments to visualize these intricate details at near-atomic resolution[1-2]. In order to sustain the high vacuum system of a TEM, biological samples are typically embedded in thin films of vitreous ice[3], sugars[4], heavy metal salts[5], or some combination thereof[6]. As a result, images of embedded specimens may reveal only limited snapshots of dynamic processes.

Early attempts to maintain biological specimens hydrated in environmental liquid chambers were undertaken by Parsons and colleagues using differential pumping stages. Electron diffraction patterns of unstained catalase crystals were successfully recorded to a resolution of 3 Å in a hydrated state[7-8]. In addition, phase-separated lipid domains could be examined in hydrated membranes of human erythrocytes[9-10]. However, motion caused by diffusing liquid and its interference with the electron beam, resulted in severe resolution loss and further experiments using biological specimens were not attempted until recently.

Newly developed microfluidic specimen holders have been introduced that utilize semiconductor microchips to form a micro-scaled environmental chamber. These devices can maintain samples in liquid while they are positioned in a TEM column[11-12]. This technical breakthrough in TEM imaging has allowed researchers to view, for the first time, progressive events at the molecular level[13]. We refer to this new modality as "*in situ* molecular microscopy" as experiments can now be performed "inside" the EM column[14-15]. The overall goal of this method is to image biological assemblies in liquid in order to observe their dynamic behaviors at nanometer resolution. The rationale behind the developed technique is to record real-time observations and examine new properties of biological machinery in solution. This methodology expands the use of TEMs for broader purposes in cellular and molecular biology[12-16].

In the current video article, we present a comprehensive protocol to assemble and use a commercially available microfluidic specimen holder. These specialized holders utilize silicon nitride microchips produced with integrated spacers to form a liquid chamber that encloses minute volumes of solution. Thin, transparent windows are etched into the microchips for imaging purposes[12]. We demonstrate the proper use of a microfluidic holder to examine simian rotavirus double-layered particles (DLPs) in liquid using a TEM. To ensure that biological assemblies, such as DLPs, do not rapidly diffuse over great distances while imaging, we employ the Affinity Capture approach to tether them to the surface of the microfluidic chamber[16]. This molecular capture step has a *major advantage over alternative techniques* for imaging biological specimens in liquid because it allows for the acquisition of images that will be used for downstream processing routines. This capture step used in conjunction with microfluidic imaging is unique to our procedures[17]. Readers employing structural biology applications using TEM or microfluidic imaging chambers may consider the use of affinity capture techniques when dynamic observations at the molecular level are the end goal.

Protocol

1. Prepare Affinity Capture Devices[16]

1. Clean the silicon nitride E-chips by incubating them in 15 ml of acetone for 2 min followed by 15 ml of methanol for 2 min (**Figure 1A**). Allow chips to dry under laminar air-flow.
2. Incubate the dried chips on a heated stir plate (without stirring) for 1.5 hr at 150 °C, then allow them to cool to room temperature before use.
3. Use Hamilton syringes to compose lipid mixtures in small glass tubes to contain 25% chloroform, 55% DLPC (1,2-dilauroyl-phosphocholine) in chloroform (1 mg/ml) and 20% Ni-NTA lipid (1,2-dioleoyl-iminodiacetic acid-succinyl-nickel salt) in chloroform (1 mg/ml) for a total volume of 40 µl.
4. Apply a 1 µl aliquot of the mixture over each 15 µl drop of Milli-Q water on a piece of Parafilm in a humid Petri dish (**Figure 1B**). Incubate samples on ice for at least 60 min.

2. Capture Macromolecules[17]

1. Place an E-chip with a 150 nm integrated spacer on top of a monolayer sample and incubate for 1 min (**Figure 1C**).
2. Gently lift the chip off of the sample and add a 3 µl aliquot of His-tagged Protein A. Incubate for 1 min at room temperature (**Figure 1D**).
3. Blot away the excess drop using Whatman #1 filter paper and immediately add a 3 µl aliquot of antibody solution. Incubate for 1 min at room temperature.
4. Remove the excess solution using a Hamilton syringe and immediately add a 1 µl aliquot of rotavirus DLPs (0.1 mg/ml) in buffer solution containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$ and 10 mM $CaCl_2$. Incubate for at least 2 min at room temperature. The preparation of DLPs has been described previously[18].

3. Assemble the Microfluidic Chamber and Load the *In situ* Specimen Holder

1. Load the wet E-chip containing the viral sample into the tip of the microfluidic specimen holder. Glow-discharge a second flat E-chip for 1 min then loaded on top of the spacer chip (**Figure 2,** panels 1-5).
2. Alternatively, to increase the contrast of biological macromolecules, heavy metal stain (*e.g.* 0.2% uranyl formate) can be added to wet specimens prior to placing the second E-chip on the wet specimen. However, the E-chip containing the specimen will need to be washed with Milli-Q water prior to adding the contrast reagent.
3. Sandwich the entire assembly together to form a sealed enclosure, held in place mechanically within the holder by 3 brass screws (**Figure 2**, panels 6-8).
4. Following assembly, the tip of the holder is pumped to 10^{-6} Torr using a turbo-pumped dry pumping station before placing the holder inside the TEM.

4. Imaging in Liquid Using a Transmission Electron Microscope

1. Load the *in situ* specimen holder into a Transmission Electron Microscope (FEI Company) equipped with a LaB_6 filament and operating at 120 kV.
2. Turn on the TEM filament and adjust the eucentric height of the microscope stage with respect to the specimen by using the wobbler function to tilt the sample from -15° to +15° back and forth in the column. This procedure adjusts the stage in the z-direction to help account for the proper thickness of the liquid chamber. This step also ensures an accurate magnification is used when recording images.
3. Record images along the edges and in the corner regions of the microfluidic chamber first. These areas typically contain the thinnest solution. Record images of specimens under low-dose conditions (1-3 electrons/$Å^2$) using a CCD camera. Use nominal magnifications of 6,000X - 30,000X.
4. Determine the proper defocus value by focusing at the edge of fluidic chamber. Use a value of -1.5 µm to record images at 30,000X magnification. If thick solution is encountered or if contrast agent is not used in the specimen preparation, use higher defocus values in the range of -2 to -4 µm.
5. To ensure solution is contained in the microfluidic chamber throughout the experiments, focus the electron beam until the bubbles are formed in the liquid within the device.

Representative Results

Representative images of DLPs in liquid using E-chips that were glow-discharged (**Figure 3A**) show fewer DLPs in a given viewing area, presumably due to diffusion, in comparison to DLPs that are enriched on Affinity Capture devices (**Figure 3B**). The addition of uranyl formate in the imaging chamber enhances the contrast of the specimen and hence the visibility of individual DLPs in solution (**Figure 3C**, top panel). Better contrast allows for downstream image processing such as particle averaging (**Figure 3C**, bottom panel) and 3D reconstruction routines (**Figure 3C**, inset) as previously described[17]. Briefly, for the 3D reconstruction we selected 600 particles of DLPs using the SPIDER software package[19]. Selected particles were refined against an initial model of the rotavirus DLP[20] that was filtered to 80 Å resolution. We used the RELION software package[21] to compute a 3D reconstruction at a resolution of approximately 25 Å. Our 3D map in in good agreement with the initial model and with an independent cryo-EM reconstruction that was calculated using the same parameters and DLP sample[17].

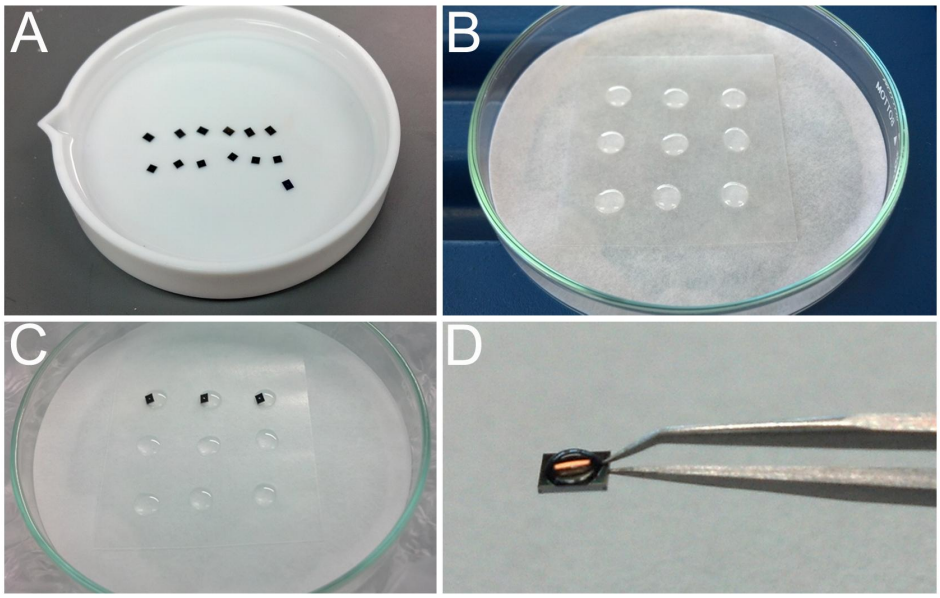

Figure 1. Preparing silicon nitride microchips for liquid imaging. A) Silicon nitride microchips are cleaned by submerging for 2 min in acetone followed by 2 min in methanol to remove residual photoresist used in the manufacture process. **B**) Lipid monolayer samples are prepared on drops of Milli-Q water added to Parafilm in a humid Petri dish[17]. **C**) Cleaned microchips are placed on top of the monolayers and removed after a 1 min incubation step. **D**) Molecular adaptors (*i.e.* protein A and antibodies in solution) are added directly to the monolayer coated chips and the excess solution is removed prior to adding DLPs. The wet specimen chip is then loaded into the microfluidic holder.

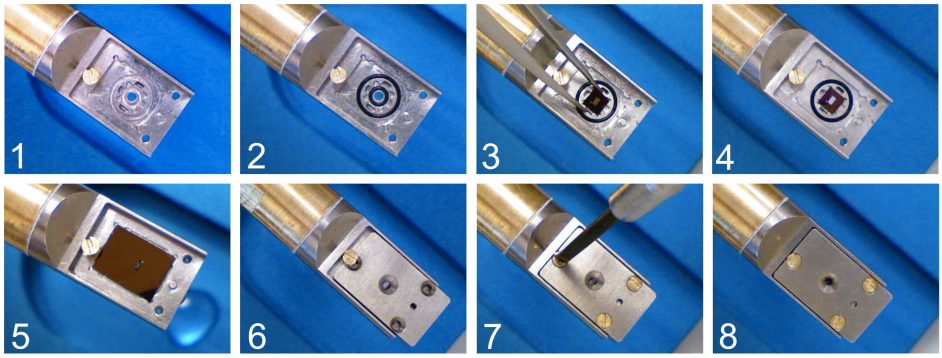

Figure 2. Assembling the liquid chamber of a microfluidic specimen holder. The tip of the specimen chamber is assembled by adding O-ring fittings to the empty tip (steps 1-2).The wet specimen chip containing integrated spacers is gently placed into the machined fittings within the tip of the holder (steps 3-4). A glow-discharged flat chip is placed over top of the specimen chip (step 5). The assembly is then covered with a metal face-plate (**step 6**) that is held in place by 3 brass screws (steps 7-8).

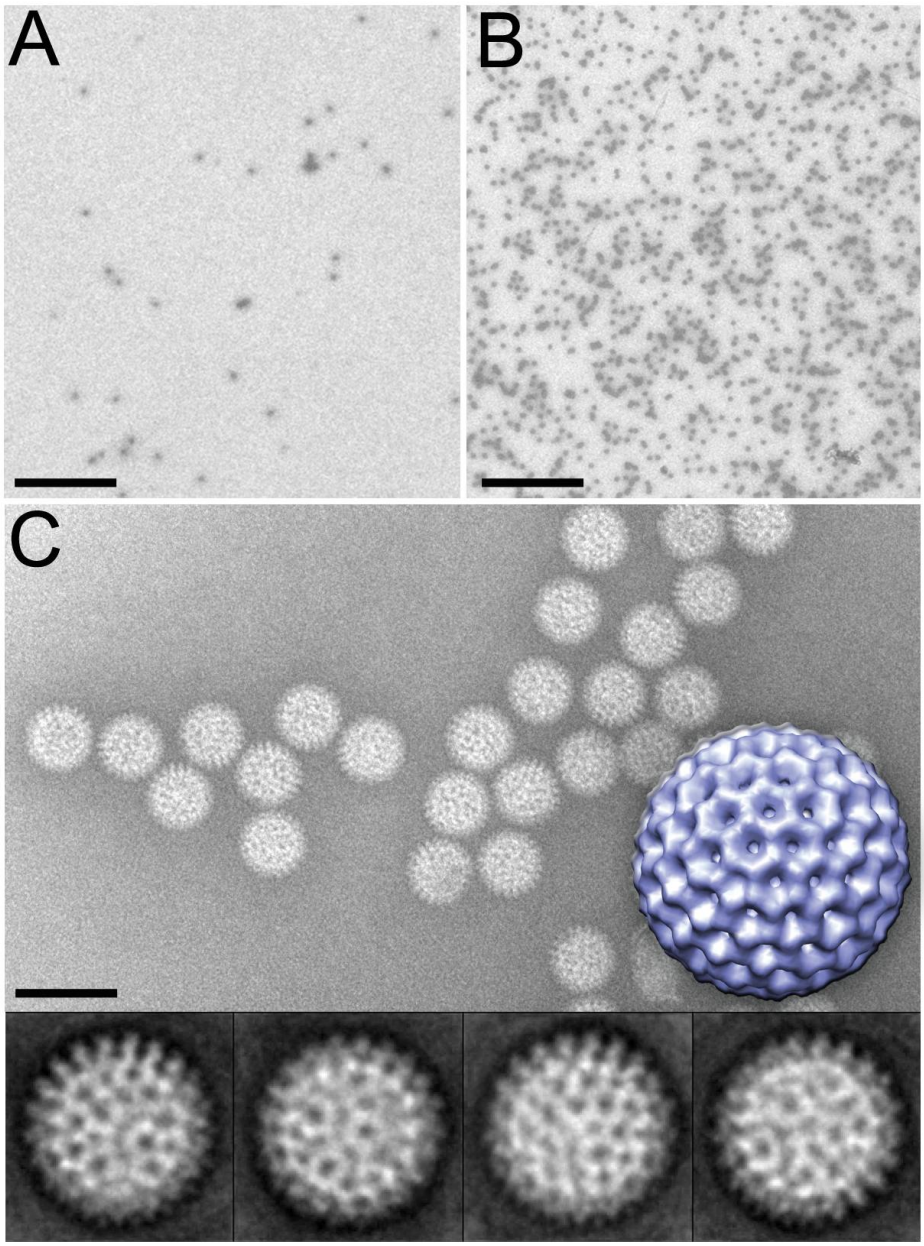

Figure 3. Representative results for DLPs in liquid. A) Glow-discharged microchips bind very few DLPs in solution in comparison to affinity-decorated microchips (**B**). Scale bars, 2 µm. **C**) Representative image (top panel) and 3D reconstruction (inset) of DLPs in liquid containing contrast reagent. Scale bar, 150 nm. The diameter of the reconstruction is 80 nm. Class averages of the DLPs in liquid (bottom panel) reveal nicely defined features along their outer surface. Individual panels are 110 nm.

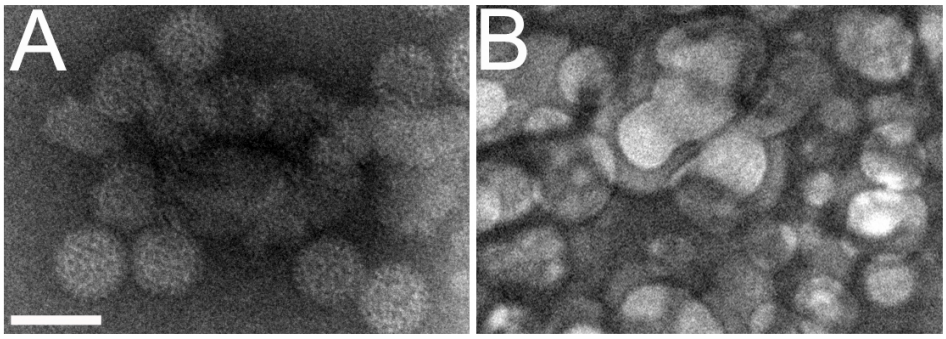

Figure 4. Representative results for bubble formation in liquid specimens in the TEM. Images of bubbles formed in liquid specimens upon the continuous exposure of the electron beam at 5 electrons/Å^2 for approximately 2 min (**A**) and 5 min (**B**). Scale bar is 100 nm.

Discussion

In our presented work, we employed the affinity capture approach to tether rotavirus DLPs to a microfluidic platform. This allowed for *in situ* imaging of macromolecular complexes in a liquid microenvironment. The *capture approach is significant with respect to other microfluidic imaging techniques* because it localizes biological specimens to the imaging window to negate large diffusive issues that arise while recording images in liquid. However, one of the *most critical steps in our protocol* involves the transfer of the lipid biofilm to the microchip surface. Should the lipid monolayer fail to spread evenly over the grid, this will result in a flawed experiment. An indication of a poor transfer step is that little to no viral assemblies will be bound to the fluidic chip. A limitation in the transfer step is thermal stability as relates to the fluidity of the lipid layer. If the transfer step is inadequate, as assessed by uneven spreading of the drop, the lipid layers should incubate on ice longer or in a vibration-free cold room. Also, the Petri dish must be adequately sealed with Parafilm to maintain the proper humidity.

An excess of viral complexes or adaptor proteins in the microchip samples can lead to excessive clustering of the particles. This is a limitation in the protocol that can easily be managed. To minimize the clustering effect, the protein factors can be incubated for a shorter time period or the virus sample should be further diluted. If an excess of nonspecific proteins are detected, the inclusion of freshly prepared imidazole (approximately 50 mM) in the buffer solution can decrease the background. The use of fresh imidazole is a *crucial step in the protocol*. Likewise, do not overload the chips with purified proteins as this will not ensure specific binding and will result in a flawed experiment. Images will contain an excess of background proteins and will not be suitable for downstream processing routines. An optimal concentration range for purified proteins is roughly 0.01-0.1 mg/ml. However, if this range does not provide the anticipated level of particles, the concentration of the sample should be increased until an optimal level has been achieved. An enrichment of viral particles should occur with respect to negative control samples that lack molecular adaptors, such as antibodies. Small amounts of nonspecific binding in your negative control may infrequently occur, however, the affinity-decorated microchips should always serve to enrich the viral particles. In the case of rotavirus, there is currently no reverse genetic system to introduce His tags into capsid proteins as a means of directly capturing viral complexes onto Ni-NTA decorated surfaces. His-tagged ribosomes, however, have been successfully recruited from bacterial lysates onto Ni-NTA decorated E-chips for liquid imaging experiments[16]. Thus providing evidence, albeit limited at this point, for native biological interactions to occur in liquid cells while in a TEM column.

The use of detergents, glycerol and high levels of sucrose must be avoided for *in situ* imaging. The use of these reagents will create artifacts in EM images that include excessive bubbling and muted features in particles, resulting in poor quality images. If these reagents are used in virus preparations, the sample can be dialyzed into buffer solution lacking these components. Affinity Capture devices can withstand some degree of detergents for a brief period of time, although this needs to be determined on a case-by-case basis. Please refer to the list of compatible reagents previously described[22].

To enable downstream image processing routines, a low concentration of contrast reagent can be added to the microfluidic chamber. This reagent does not fix protein samples, like traditional staining procedures[17]. To ensure liquid is present in the imaging chamber throughout the time-course of the experiments, focus the electron beam until bubble formation occurs (**Figure 4**). This is a simple measure to indicate the specimens remain hydrated. Liquid specimens encounter severe damage within 2 min (**Figure 4A**) of continuous expose of the electron beam at 5 electrons/Å^2. Prolonged continuous electron beam exposure for 5 min (**Figure 4B**) and beyond at this dosage will result in excessive bubble formation in the imaging area.

In situ molecular microscopy used in combination with Affinity Capture devices permits the examination of biological complexes in solution using transmission electron microscopy. These technical advancements are made possible by the use of newly developed microfluidic specimen holders. Such devices employ thin silicon nitride membranes to form a micro-scaled imaging chamber. Building upon the pioneering work of Parsons and colleagues[7-10] we provide a useful strategy to image individual viral assemblies in solution. The protocol described here explains methodologies that are compatible with single particle image processing routines and 3D reconstruction calculations. Future applications that will result from mastering these techniques may include live-EM imaging of macromolecules. We anticipate *in situ* imaging may reveal steps for viral assembly, transcription regulation or host cell invasion. In summary, the use of these protocols should permit researchers to investigate dynamic processes in solution in a completely new manner.

Disclosures

The author, Madeline J. Dukes, is an employee of Protochips, Inc.

Acknowledgements

The authors acknowledge Dr. Michael J. Friedlander, Director of the Virginia Tech Carilion Research Institute for encouraging our research endeavors. This project was supported by development funds to S.M.M and D.F.K. and in part by the Nano-Bio initiative of the Institute for Critical Technology and Applied Science at Virginia Tech.

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