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Short communication

Preparation of viral samples within biocontainment for ultrastructural analysis: Utilization of an innovative processing capsule for negative staining

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A B S T R A C T

Transmission electron microscopy can be used to observe the ultrastructure of viruses and other microbial pathogens with nanometer resolution. In a transmission electron microscope (TEM), the image is created by passing an electron beam through a specimen with contrast generated by electron scattering from dense elements in the specimen. Viruses do not normally contain dense elements, so a negative stain that places dense heavy metal salts around the sample is added to create a dark border. To prepare a virus sample for a negative stain transmission electron microscopy, a virus suspension is applied to a TEM grid specimen support, which is a 3 mm diameter fragile specimen screen coated with a few nanometers of plastic film. Then, deionized (dI) water rinses and a negative stain solution are applied to the grid. All infectious viruses must be handled in a biosafety cabinet (BSC) and many require a biocontainment laboratory environment. Staining viruses in biosafety levels (BSL) 3 and 4 is especially challenging because the support grids are small, fragile, and easily moved by air currents. In this study we evaluated a new device for negative staining viruses called mPrep/g capsule. It is a capsule that holds up to two TEM grids during all processing steps and for storage after staining is complete. This study reports that the mPrep/g capsule method is valid and effective to negative stain virus specimens, especially in high containment laboratory environments.

1. Introduction

Transmission electron microscopy is an effective tool for viewing the morphology and ultrastructure of small biological specimens that are too small to be seen with a traditional light microscope, such as viruses (Gentile and Gelderblom, 2014; Kruger et al., 2000; Curry et al., 2006; Goldsmith and Miller, 2009). TEMs shoot electrons through a very thin specimen. The electrons interact with the specimen and are focused by an objective lens to form a magnified image on the viewing screen or camera screen. Regions of the sample that bend or block electrons appear dark, while regions that are electron lucent appear white. Due to the fact that a TEM uses electrons, the resolution is much greater than that of a light microscope, because electrons have a much shorter wavelength than visible light.

Lack of electron dense matter makes viruses difficult to view under a TEM because the electrons are unable to interact with viral material and pass right through the sample. Negative staining is the most common method used to create contrast and view viruses under a TEM. The first negative staining procedure was proposed by Brenner and Horne in 1959, based on an experiment where Hall (1955) and Huxley (1957) observed the appearance of biological structures in reverse contrast when immersed in an electron-dense substance (Kiselev et al., 1990). The process of negative staining has been virtually unchanged over the past half century. Negative staining involves briefly applying a heavy metal salt solution to

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a coated TEM grid, with the virus sample already attached, in an attempt to surround the virus without infiltrating it (Brenner and Horne, 1959). This creates a dark border and maps out the particle's shape. Negative staining can be pictured as the immersion of an object within an electron-dense matrix (Kiselev et al., 1990). The two reagents used as negative stains in our experiments are uranyl acetate (UA) and potassium phosphotungstic acid (PTA). Both of these stains are commonly used to negatively stain small biological samples, such as viruses, protein complexes, and small nanoparticles (Harris, 2007; Bradley, 1967; Suzuki et al., 1987).

The conventional negative staining technique is referred to as the single-droplet negative staining technique (Harris, 2007). It is a manual method that requires precise handling of small and fragile TEM grids with forceps while applying small amounts of virus sample and stain. The typical preparation protocol involves applying a droplet of sample suspension onto the surface of a film-coated TEM grid (Fig. 1A). After attachment of the sample to the film surface, the grid is rinsed to remove non-adherent viruses, and then stained quickly with either UA or PTA for a few seconds to a minute, depending on the sample. Excess liquid is always wicked away from the edge of the grid with filter paper strips and the grid is stored in a grid box until TEM imaging.

The manual method requires the handling of multiple reagents with precise timing to ensure experimental accuracy and reproducibility. It is also time consuming because each TEM grid must be prepared individually. If not handled with care, TEM grids are easily punctured or bent by forceps or contaminated from being in the open air for too long. Processing numerous samples leads to difficulties in tracking the grids and ensuring similar processing for each sample. This sample preparation is more difficult when working in biosafety level (BSL) 3 and 4 biocontainment laboratories, because the required personal protective equipment is cumbersome. Our safety department requires personnel manipulating viruses in BSL-3 biocontainment laboratories to wear 1 to 2 pairs of gloves and work in a biosafety cabinet (BSC). Extra layering of gloves can reduce tactile sensitivity and restrict fine motor movement. The airflow of the BSC protects the user and helps prevent sample contamination; however, the airflow can also cause the samples and stains to dry faster, which can affect the results of a negative stain. The strong airflow in the BSC can also quickly suck up a grid if accidentally dropped. In BSL-4 biocontainment laboratories, personnel are required to wear a positive pressure suit which further restricts physical movement and the window of visibility. The technician wears at least 2 pairs of gloves, with the outer pair is a thick glove which reduces hand motion and tactile sensation, and is required to work in a BSC. Furthermore, the forceps used to handle TEM grids are small and sharp. It poses a risk to the technician due to their ability to puncture gloves. After the completion of staining procedures, the grids are inserted in microfuge tubes filled with Osmium Tetroxide vapor, and transferred out of the biocontainment laboratory. Prior to transfer from the biocontainment laboratory, all surfaces of the microfuge tubes are decontaminated with a disinfectant solution and sealed within double bags with disinfectant solution in the space between the bags. This procedure for removal of specimens from biocontainment can increase the damage to the negative stained EM grids.

In this report we introduce a new method for negative staining grids in biocontainment laboratories that utilizes mPrep/g capsules, a capsule based device for grid handling and staining (Benmeradi et al., 2015; Goodman et al., 2015; Goodman and Kostrna, 2011). The mPrep/g capsule accommodates two TEM grids, minimizes direct handling, and thus reduces the potential for grid damage. The mPrep/g capsule attaches directly to a single or multichannel pipette similar to a pipette tip, allowing for the application of various liquids via pipet aspiration. This enables simultaneous preparation of multiple samples with duplicate grids (Fig. 1B). To negative stain with mPrep/g capsules the virus sample is first drawn into the capsule and held for 10 min to let the viruses adsorb onto the grid surfaces. The grids with adsorbed virus are subsequently washed with deionized (dI) water and stained with either UA or PTA for a few seconds to 1 min. This process uses the same protocol steps and reagents as the manual droplet method. The difference being that all work occurs inside the mPrep/g capsule with no physical handling of the grids. (Fig. 1C,D).

The purpose of this study was to evaluate mPrep/g capsules as a new method for negative staining of virus samples in biocontainment environments. The mPrep/g capsule method and the manual droplet method were compared, to determine its effectiveness of the mPrep/g negative staining system. This study also examined the EM image quality produced from two different virus inactivation procedures: 1) rapid inactivation, with 1% Osmium Tetroxide (OsO_4) vapor, and 2) a minimum 24 h inactivation with 2% glutaraldehyde, both were conducted using the mPrep/g capsules. Finally, we evaluated two of the most commonly used negative stains, UA and PTA, with regards to EM image quality.

2. Material and methods

2.1. Manual droplet method for negative staining in a biosafety level 2 (BSL-2) lab (Fig. 1A)

Inside the biocontainment laboratory BSC, the virus suspension was mixed well with the same volume of 4% glutaraldehyde to achieve a final concentration of 2% glutaraldehyde. Viruses were inactivated with 2% Glutaraldehyde inside a BSC for 24 h, according to industry standard practice (Moller et al., 2015; Rutala and Weber, 2008), prior to removal and transfer to the BSL-2 TEM facility. In the BSL-2 TEM facility, a drop (8 μl) of the glutaraldehyde treated virus suspension was placed onto a formvar/carbon coated TEM grid for 10 min in a moist chamber to reduce evaporation. It was important to make sure the grid did not dry. Using fine forceps to hold the grid, the liquid was wicked away from the grid surface from the side with filter paper. The grid was then washed three times by touching the grid to the surface of drops of dI water. Remaining water was wicked away by touching filter paper to the side of the grid. A small drop of stain (either 1% UA or 1% PTA) was applied to the grid and allowed to remain from 10 s to 1 min depending on the sample. The stain was wicked away by touching the edge of the grid to a piece of filter paper. The grid was air dried at room temperature and stored for subsequent TEM imaging.

2.2. MPrep/g capsule method for negative staining in biocontainment using aqueous glutaraldehyde and 1% osmium tetroxide vapor inactivation (Fig. 1C)

Step 1. Inside the biocontainment laboratory BSC, 40 μl of virus suspension was aspirated into the mPrep/g capsule attached to a pipette. The pipette remained attached to the mPrep/g capsule until the process was complete. Step 2. The pipette was placed on its side for 10 min with grids oriented horizontally to promote an even distribution of virus particles onto the grids. Step 3. The pipet was picked up and the plunger pressed to dispense the virus solution into a waste container. 40 μl of 2% glutaraldehyde fixative was aspirated into the capsules, covering the grids, and the pipette placed horizontally for 20 min. The fixative was subsequently expelled and 40 μl of dI water was aspirated into the capsules to wash away the fixative. The wash was repeated for a total of 3 rinse cycles. Step 4. 40 μl of stain (either 1% UA or 1% PTA) was aspirated into the capsules for 30 s (time may vary from 10 s to 1 min based on virus sample). Step 5. The mPrep/g capsule was removed from the pipettor and the grids blotted dry by touching filter paper to the edge of