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Correlative light electron microscopy of giant viruses with the SECOM system

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Abstract

Bright-field transmission electron microscopy (TEM), TEM-negative staining technique, resin-embedding and ultramicrotomy, scanning TEM, scanning electron microscopy, atomic force microscopy and cryoelectron microscopy are imaging techniques used for describing giant viruses, their cycle and ultrastructure. Here we used the SECOM system, an integrated correlative light and electron microscopy using light and electronic imaging without sample transfer, to study cells infected with giant viruses, as shown by *Tupanvirus*, the ultrastructure of which was successfully observed. An improvement of the SECOM system with an eye to its use in fundamental and clinical research could be considered in the field of microbiology.

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Introduction

The discovery of giant viruses (GVs) has upended the definition of the virus by exceeding the barrier of particle size and genome [1,2]. *Tupanvirus* is a GV with a ~450 nm capsid containing fibrils and large cylindrical tail (~550 × ~450 nm including fibrils) attached to the base of the capsid that is the longest described in the virosphere. The average length of a complete virion is ~1.2 μm, which makes them one of the longest viral particles [3].

Bright-field transmission electron microscopy (TEM), TEM-negative staining technique and resin embedding and ultramicrotomy have been used, respectively, to obtain nanometer-scale information about GV morphology [1,2,4–7], to picture whole-GV morphology [2,3,7–9], to study the ultrastructure of GV and

to describe infection cycles over time [1–5,8–10]. Scanning TEM [8], scanning electron microscopy (SEM) [11] and atomic force microscopy have been used to study sections of GV-infected cells [12] and to characterize GV morphology. Cryoelectron microscopy has been a popular technique because it enables the preservation of GV ultrastructure [9,10,12–15].

Because the diameter of GVs is larger than the optical resolution limit, GVs are readily visible in bright-field transmitted or fluorescence light microscopy (LM) [2,3,10]. But despite this unique property, LM is not as often used as electron microscopy (EM) for characterizing GV morphology or infection cycle because of limitations in resolution. Correlative light and electron microscopy (CLEM) helps to bridge this gap between LM and EM of GV-infected cells with the combination of the specificity of fluorescent labeling and the high-resolution structural information of EM, making it the perfect tool to study the complex relationship between form and function in biology. The SECOM system is a system for integrated CLEM wherein light and electron imaging are performed in one system without the need for sample transfer [16,17].

Here we report our study of cells infected with GVs using integrated CLEM with the goal of determining whether GVs such as *Tupanvirus* [3] could be detected using the SECOM system so we could study their unique ultrastructure.

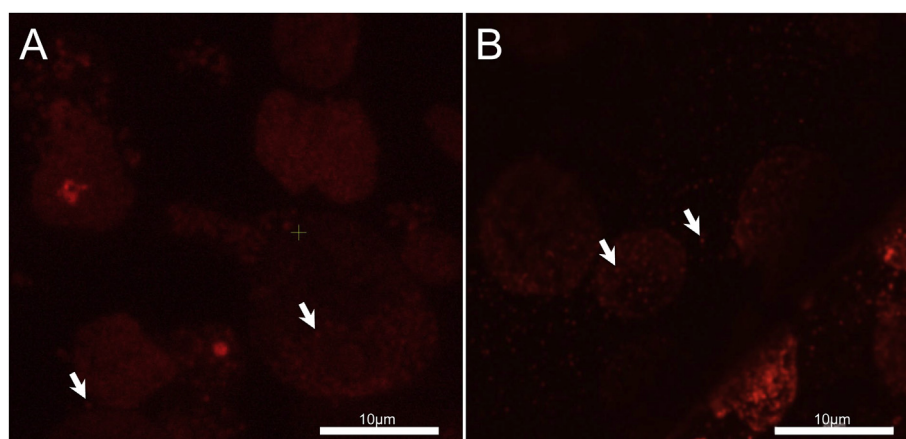


FIG. 1. (A) SECOM system fluorescence image of 100 nm thick ultrathin section on indium tin oxide slide using 60× water immersion objective with NA of 1.2 (B) Confocal laser scanning microscope reference image of 100 nm thick ultrathin section on grid using 63× objective lens (Z maximal projection). White arrows point to intracellular or extracellular single *Tupanvirus* particles in (A) and (B).

Materials and methods

Acanthamoeba castellanii samples in periodic acid–Schiff medium were infected with purified *Tupanvirus* with a multiplicity of infection of 10 at 30°C for 18 hours and stained with FM4-64FX (aldehyde fixable; F34653, Thermo Fisher) for 30 minutes at 30°C in the dark, then fixed overnight at 4°C with paraformaldehyde 4% in sodium cacodylate 0.1 M buffer. After rinsing two times for 15 minutes each with a cacodylate 0.1 M/saccharose 0.2 M in water solution, cells were dehydrated with ethanol 50%, 70% and 96%, for 15, 30 and 30 minutes, respectively. Cells were then placed for 1 hour in a mix of LR-White resin 100% (Polysciences, Ref. 17411 MUNC-500) and ethanol 96% in a 2:1 ratio. After 30 minutes in pure 100% LR-White resin, cells were placed in 100% LR-White resin overnight at room temperature. The day after, cells were placed for 1 hour in 100% resin at room temperature. A total of 1.5 mL of

Pure 100% LR-White resin was added on the cell pellet. Polymerization was achieved at 60°C for 3 days. Between all steps, the samples were ultracentrifuged at 5000 rpm, and the supernatant was discarded. Sections 70, 100 or 1000 nm thick were cut on a UC7 ultramicrotome (Leica). For TEM, 70 nm thick sections were deposited on 300 mesh copper/rhodium grids (Maxtaform HR25, TAAB). They were poststained with 5% uranyl acetate and lead citrate according to the Reynolds method [18]. Electron micrographs were obtained on a Morgani 268D (Philips); TEM was operated at 80 keV and was equipped with a 1024 × 1024 pixel MegaView3 camera. For fluorescence microscopy, 100 nm thick sections on grids were imaged with a confocal laser scanning AiryScan LS800 microscope (Zeiss). For the SECOM system, 100 nm thick uncontrasted sections were deposited on glass slides coated with a conducting layer of indium tin oxide, making it possible to image the sections with electrons while maintaining optical transparency for

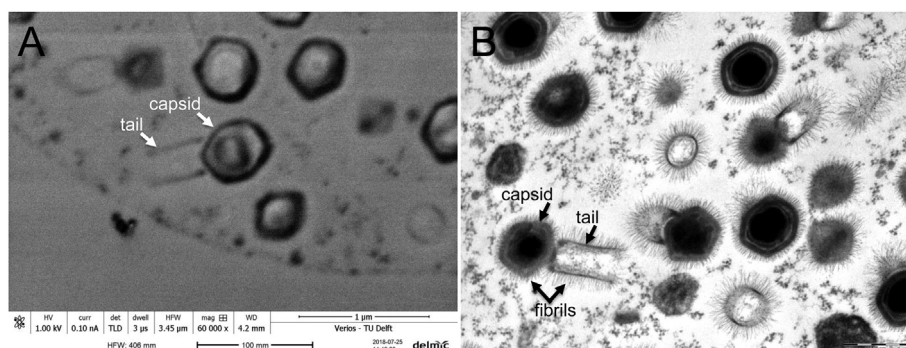


FIG. 2. (A) High-magnification scanning electron microscopy image of 100 nm thick ultrathin section with no EM stain acquired with FEI Verios 460 (FEI Company) and showing individual tupanviruses; virus particle shows typical capsid and attached tail. (B) High-magnification transmission electron microscopy image of 70 nm thick ultrathin section with EM stain acquired at 80 keV on Morgagni 268D (Philips) transmission electron microscope.

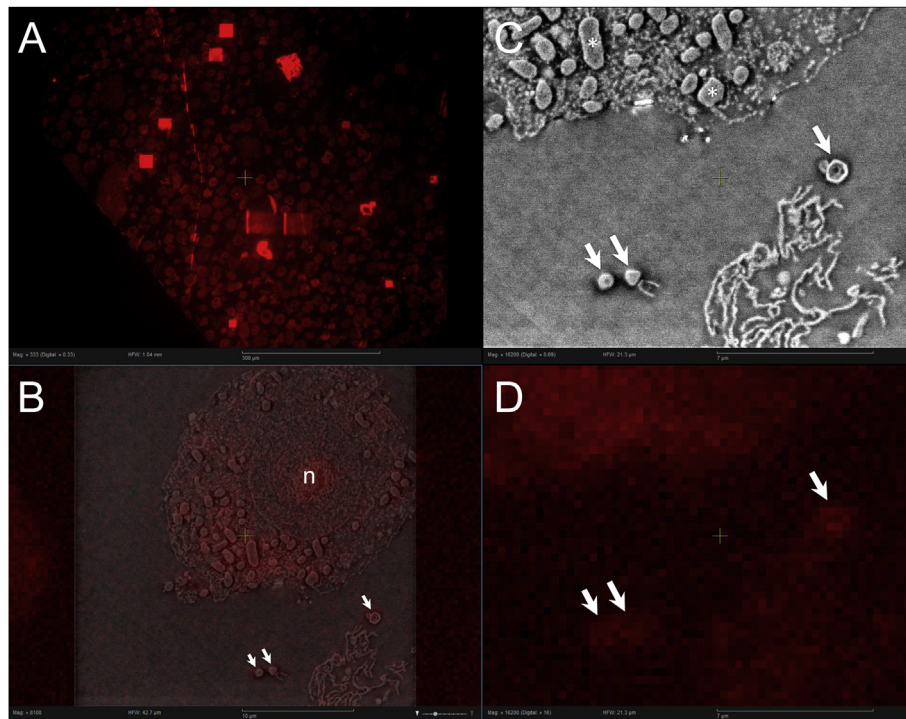


FIG. 3. Example of four-panel Odemis software interface of SECOM system with single and overlay channels. (A) Fluorescence light microscopy overview image of 100 nm thick ultrathin LR-white section containing *Tupanvirus*-infected cells. (B) Correlative light-electron microscopy overlay image of *Tupanvirus* particles (white arrows) located outside amoeba cell; n indicates amoeba nucleus. (C) Scanning electron microscopy of *Tupanvirus* particles depicted in (B). Depending on cutting plane of section, ultrastructural features such as capsid and tail can be seen. Mitochondria (asterisks) can be observed in amoeba cytoplasm. (D) Light microscopy of *Tupanvirus* particles (white arrows) depicted in (B) and detected by as fluorescent spots.

fluorescence imaging. The SECOM system was mounted on a Verios 460 (Thermo Fisher) SEM. The fluorescence images were obtained by excitation with a 467 nm light source.

Results and discussion

Thin sections containing whole infected cells were identified optically on the SECOM system using a low-magnification (40 \times) air objective lens, followed by imaging with a high NA (=1.2) 60 \times water immersion objective (Fig. 1(A)), where amoeba cells can clearly be identified.

EM images of the same (uncontrasted) sections were acquired on the Verios 460 SEM using the in-lens secondary electron detector in immersion mode. A 1 keV beam and 100 pA currents were used to obtain the high-resolution EM images which have sufficient contrast to enable the identification of virus particles as well as cell plasma membranes and intracellular organelles. Further, the high magnification SEM image of the virus (Fig. 2(A)) shows the possibility of locating and imaging an individual virus. A reference image of an EM-stained section acquired in a TEM is shown in Fig. 2(B).

The optical and electron beam imaging parameters were optimized at a location far away from the region of interest. Then the fluorescence image was acquired, followed by the electron image. Finally the overlay procedure was performed by electron beam exposure of a grid of points, from which the cathodoluminescence was detected by the camera and used to align the objective lens with the electron beam axis. The correlative imaging including the overlay is fully automated when giving a high-resolution image, with two mixing ratios of optical and electron contrast. The *Tupanvirus* was successfully identified and imaged on the SECOM system (Figs. 3 and 4). This also shows the optical LM, EM and LM/EM correlative image in the Odemis image acquisition interface.

Conclusion

The SECOM system can successfully detect GV particles by fluorescence LM while simultaneously providing information on the EM ultrastructure such as the *Tupanvirus* capsid and tail features. Although this ultrastructural information is not as high in resolution as that achieved in a conventional TEM (the fibrils

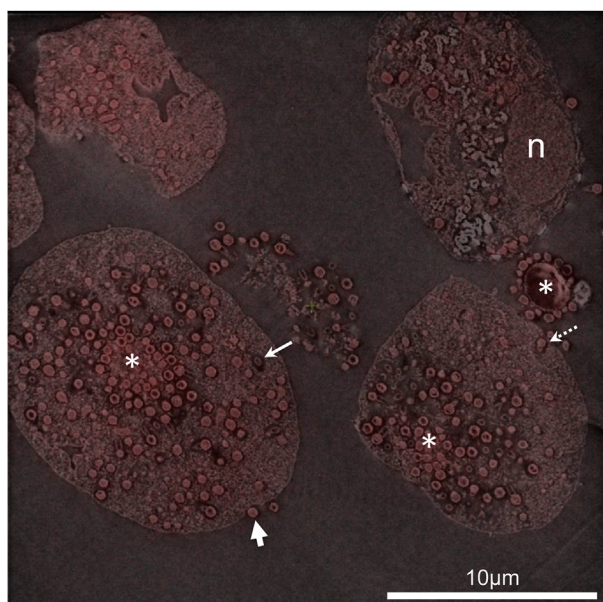


FIG. 4. Example of correlative light-electron microscopy overlay image of *Tupanvirus*-infected amoeba cells acquired with SECOM system. Nucleus (n) in cell as well as virus factories (asterisks) with multiplying viruses are shown. *Tupanvirus* particles can be found inside cells cytoplasm as well as inside cellular vacuoles or plasma membrane invaginations (solid and dashed thin arrows), probably on their way to externalization (thick arrow).

were not seen in SEM), it is sufficient for determining the gross morphology of GV particles and their locations regarding cellular compartments. In the field of virology the SECOM system has the potential to be an efficient tool for screening suspicious samples and virus-infected cells at low and higher resolution. More broadly, the SECOM system has the potential to be useful for fundamental and clinical research in the field of microbiology.

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Conflict of interest

None declared.

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