3D in SEM, (S)TEM, Ion Imaging, incl. FIB-SEM and SBF-SEM

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Focussed ion beam/scanning electron microscopy (FIB/SEM) has the potential of a powerful tool in virus research

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Introduction: Microscopic examinations of virus infected cells including localization of viral and cellular proteins are crucial tools in virus research. Viral tegument protein accumulations can be found at the cell base in cells infected with the human cytomegalovirus (HCMV) in fluorescent microscopy. However, light microscopy is not powerful enough for characterization of these accumulations in more detail and especially to answer the two questions (I) are these proteins part of infectious (DNA containing) virus particles and (II) are these accumulations intra- or extracellular. We have recently shown that the resolution of FIB/SEM is comparable to transmission electron microscopy (TEM) [1]. Hence, we applied FIB/SEM tomography to HCMV infected cells to investigate these accumulations of viral protein at the electron microscopic level and to access them from a different direction than usually given by ultrathin sectioning and TEM.

Experimental procedure: Fibroblasts were seeded on sapphire discs with (*protocol A*) or without (*protocol B*) coordinate system (Wohlwend GmbH, Switzerland), respectively, and infected the next day with either GFP-labeled (*protocol A,* Figure 1) HCMV or non-labeled HCMV (*protocol B*, Figure 2). The z-stack in Figure 1A was acquired with the fluorescence microscope Axio-Observer.Z1 (Zeiss, Germany) from HCMV infected fibroblasts stained for a viral protein as described in [2]. The cells infected with GFP-labeled HCMV were imaged immediately before high pressure freezing (Figure 1B). For the FIB/SEM samples, high pressure freezing, freeze substitution and embedding in epoxy resin was conducted as described in [3]. The height of the resin block was reduced to 1 mm with a jigsaw after removal of the sapphire disc and the resulting resin disc was mounted on an SEM specimen stub and coated with 5 nm of platinum by electron beam evaporation [1]. In the FIB/SEM Helios Nanolab 600 (FEI, Eindhoven, The Netherlands) we located the area of interest at a relatively high acceleration voltage of 10 kV (Figure 1C and 1D). For FIB milling of the samples shown in Figure 2C and 2D an additional layer of platinum was deposited using ion-beam induced deposition (IBID) [4], starting with a deposition of a thin layer at 48 pA, followed by a thicker layer at 0.28 nA. For all images in Figure 2 a regular cross-section was milled, followed by a cleaning cross-section at 53°. The sample was then tilted back to 52° and the block-face was imaged with the through the lens mode with an extraction voltage of 70 V at an acceleration voltage of 5 kV.

Conclusion: We could show that the FIB/SEM approach can be successfully applied for virological research since we were able to visualize virus particles, their membranes and the different states of HCMV capsid formation inside the nucleus in high resolution. Hence, we were able to clarify that the accumulations of viral protein detected in the fluorescence microscope obviously originate from infectious virus particles underneath the cell monolayer. This result supplements the model of HCMV egress and has not been shown so far. In order to facilitate the localization of virus infected cells in the FIB/SEM we used GFP-labeled virus particles together with sapphire discs with a coordinate system. By performing FIB/SEM in combination with this correlative approach, we established a tool for further experiments aiming at visualization of virus infected cells in three dimensions in order to answer urgent questions concerning the mechanisms of virus entry and morphogenesis.

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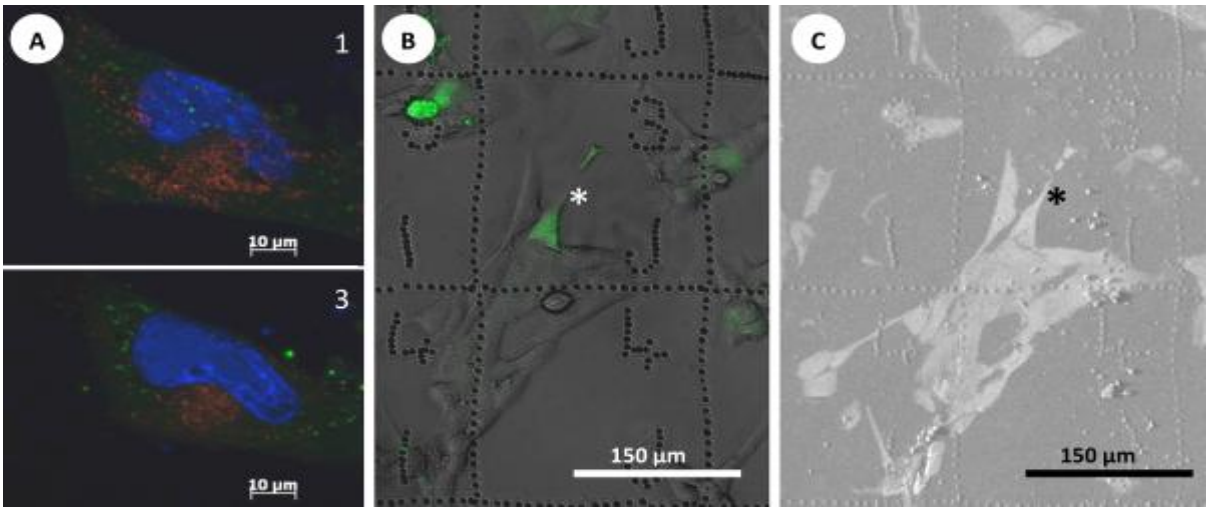

Figure 1. Fibroblasts infected with GFP-labeled HCMV. (A) Two images from a z-stack (1 µm between images, numbers indicate the number of the optical section counted from the cell base to the top). In the first section patches of red fluorescence signal (viral tegument protein) are distributed throughout the entire plane, in the third section the accumulation is only located in the assembly complex (=circular structure enclosed by a kidney shaped nucleus in blue). (B) Combination of the transmitted light image with the fluorescence image of fibroblasts on the sapphire disc prior to high pressure freezing. Infected cells are indicated by green fluorescence. The cell of interest is marked with an asterisk. (C) It can then be easily found in the FIB/SEM after embedding.

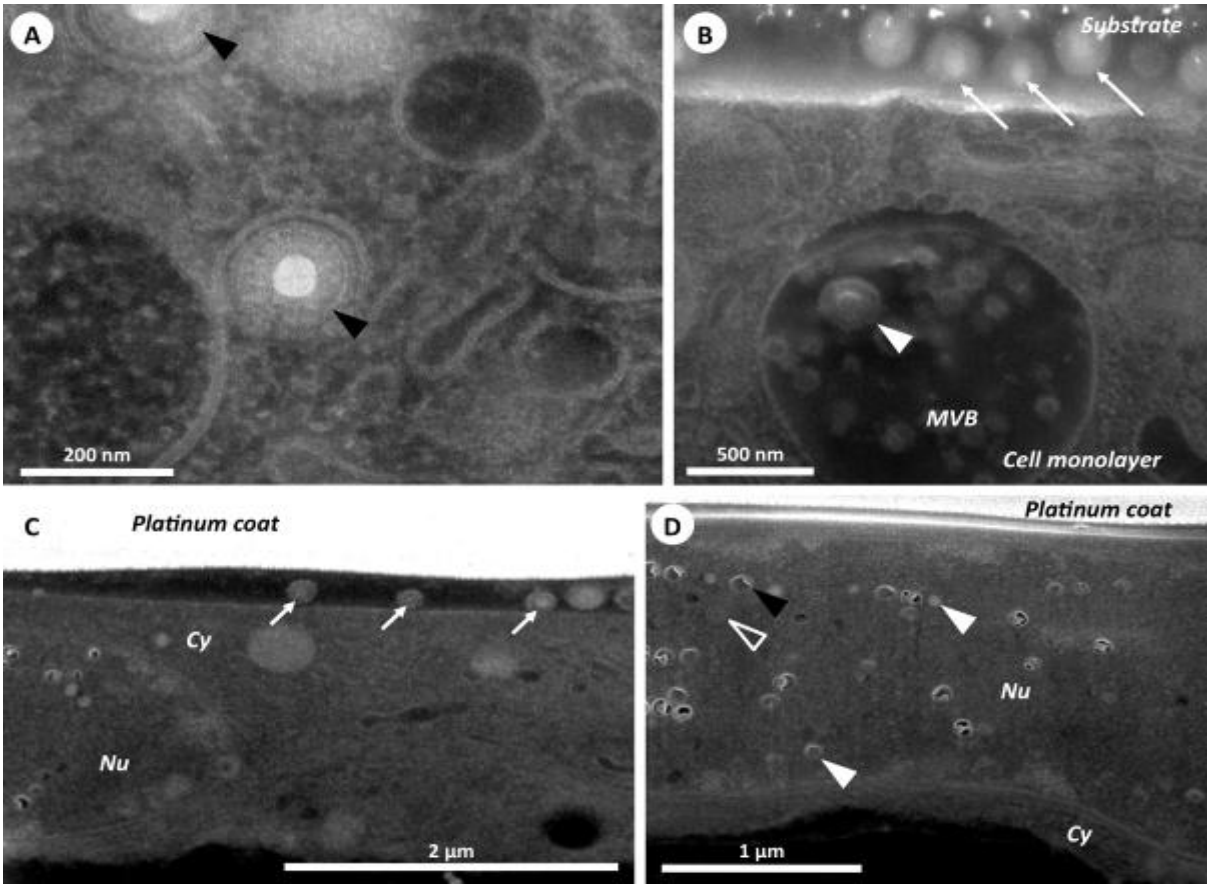

Figure 2. FIB/SEM images of other HCMV infected cells. (A) Virus particles (arrowheads) within a cell. Note the TEM like resolution of the membrane bilayers. (B) The interface between cell monolayer and the substrate is easily accessible with the FIB/SEM approach. Virus particles can be identified (white arrows). (C) and (D) Cross section after additional platinum coating to avoid beam damage (*Cy* cytoplasm, *Nu* nucleus). (C) Virus particles at the interface cell/substrate (white arrows). (D) Different states of virus capsid formation inside the nucleus. Virus capsids containing DNA (white arrowhead) can be distinguished from virus capsids with a scaffold ring (empty arrowhead) or empty capsids (black arrowhead).