EDUCATIONAL ARTICLE: STRUCTURAL ANALYSIS OF VIRAL FACTORIES: FROM CONVENTIONAL MICROSCOPY TO ELECTRON TOMOGRAPHY.

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Abstract

Viral factories are large structures built by cellular and viral components where viruses insert the macromolecular complexes needed for genome replication and morphogenesis of new viral particles. We are studying how factories are built and work with the assistance of a variety of electron microscopy (EM) methods. Our goal is to obtain three-dimensional (3D) maps of these very complex structures to study how replication complexes are formed, how replicated genomes are transferred to assembly sites and how new, infectious viral particles assemble and mature inside the factories. In the present work we revise EM techniques that provide 3D information of cells to describe how we can apply them to characterize the complex organization of virus factories. Reconstructions from serial sections and metal replicas after freeze-fracture provide 3D maps of medium resolution that we plan to use as complementary tools to assist in the interpretation of the very complex maps of cells to be obtained by electron tomography (ET). Within this context viruses are providing us with models to understand cellular trafficking pathways and cell architecture.

Resumen

Las factorías virales son grandes estructuras construidas por componentes celulares y virales, donde los virus insertan los complejos macromoleculares necesarios para la replicación del genoma y la morfogénesis de nuevas partículas virales. Estamos estudiando cómo las factorías se construyen aplicando varios métodos de microscopía electrónica (ME). Nuestro objetivo es obtener mapas tridimensionales (3D) de estas estructuras complejas para estudiar cómo se forman los complejos replicativos, cómo se transfiere el genoma replicado a los sitios de ensamblaje y cómo nuevas partículas infecciosas se ensamblan y maduran en las factorías virales. En este trabajo revisamos las técnicas de ME que aportan información 3D de las células y cómo podemos aplicarlas para caracterizar la compleja organización de las factorías virales. Las reconstrucciones generadas a partir de cortes seriados y las réplicas metálicas de células procesadas mediante criofractura proporcionan mapas 3D de media resolución que planteamos usar como herramientas complementarias para ayudar a la interpretación de los mapas celulares de gran complejidad que se obtendrán por tomografía electrónica. En este contexto los virus nos están aportando modelos para comprender la arquitectura y las rutas de tráfico celular.

Keywords: 3DEM, oriented serial sectioning, metal replication, viral factories.

Introduction

The structure of infected cells is radically modified by many viruses. The formation of viral factories, a massive recruitment of organelles at the replication and assembly sites induced by viral factors [1], is perhaps the clearest example. Many different non-related viruses build factories in host cells. In our laboratory, *Bunyamwera* virus (BUNV) is one of the experimental models we use for studying viral factories. *Bunyamwera* virus, an arthropod borne virus (arbovirus) belonging to the *Bunyaviridae* family (*Orthobunyavirus* Genus), is transmitted by mosquitoes (usually from the *Aedes* Genus) as do other arboviruses. Some of those arboviruses cause serious diseases in humans, such as meningitis, encephalitis or hemorrhagic fever [2], like for example *La-Crosse* virus in the U.S.A

[3] or *Oropouche* virus in Brazil [4]. These viruses are gaining more sanitary importance and have been catalogued as emergent or re-emergent disease agents [5]. Human activities and climate change have been described as major causes of the spreading of these viruses [6]. *Bunyamwera* virus factory is visualized as a recruitment of mitochondria and endoplasmic reticulum elements around the Golgi apparatus [1] where virus assembly occurs [7]. In this cell-viral hybrid compartment, many interactions are necessary for the construction of macromolecular structures of viral origin, for example viral tubes [7], and for viral assembly and maturation along the Golgi stack [8]. Our long term aim is to obtain 3D reconstructions of these viral factories with enough resolution to build their molecular maps. This ambitious project will have to be accompanied by technical developments such as the implementation of ET for large regions of eukaryotic cells [9]. In this work we describe how to generate useful three-dimensional information to assist in the interpretation of the very complex maps of cells to be obtained by ET. This is possible thanks to the study of oriented sections, 3D reconstruction of serial sections and analysis of metal replicas of freeze fractured cells followed by transmission electron microscopy (TEM). With these techniques we are studying viral factory dynamics and how cells maintain and modify their architecture.

Oriented sectioning of cellular monolayers.

Cultured cells grown in monolayers represent the basic model system for the characterization of many viruses. The standard procedure for thin-sectioning of these samples starts with a fixation *in situ* followed by removal of the cells to prepare a pellet. After embedding in a resin, cells inside the pellet are randomly oriented in all directions [10]. In thin-sections from these samples, a global understanding of cell content is difficult, and detection of scarce or non-randomly distributed elements could be rather difficult. Moreover, viral factories and their internal elements are not randomly distributed inside infected cells. To avoid these limitations, we have implemented the oriented sectioning technique. The principle is simple: if we cut a group of cells with the same orientation with respect to their physical support, we will be able to cover all planes of all cells in the monolayer in an oriented manner. The protocol we use is as follows: Monolayers of cultured cells are grown on Thermanox© plastic support (Nunc) (Figure 1A and B). Cells infected with BUNV are fixed at different times post-infection with 1% glutaraldehyde and 0.5% tannic acid, during 1 h at room temperature (RT), and washed two times in HEPES buffer. The cellular monolayer on the plastic support is very delicate and Thermanox© sheets have to be picked up and manipulated quite carefully. Therefore, in all the subsequent steps, cells are washed or incubated by transferring the monolayers on Thermanox© between eppendorfs containing the next solution (instead of emptying and refilling the same eppendorf).

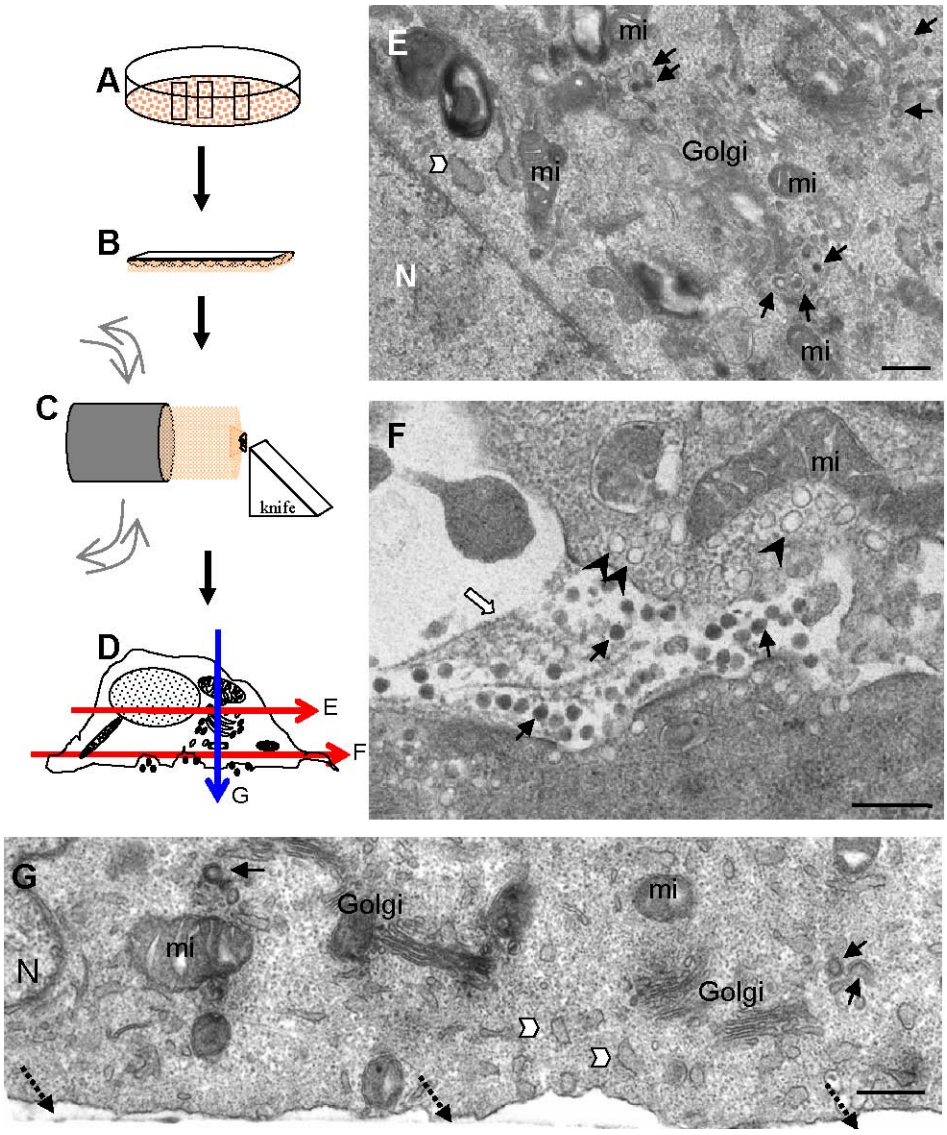

Figure 1: Oriented sectioning of cell monolayers. A-D: Scheme showing the procedure to obtain oriented sections. A: Confluent cultured cells growing on pieces of Thermanox©. B: Cells on a plastic piece after embedding in an epoxy resin. C: Cell monolayer is glued on a resin capsule and mounted on the ultramicrotome unit where a trapezoid is trimmed. D: Starting at base-level and moving towards the apical zone of the cells, the serial sections are collected on grids, stained with uranyl acetate and lead citrate, and analysed by TEM. E-F: Micrographs of sections oriented in parallel to the base of the cell. E: Micrograph of an area of an oriented section in the "middle" of the cell (3µm). A BUNV factory containing numerous viral assemblies (arrows) can be seen in Golgi membranes near the nucleus (N). Mitochondria (mi) and rough endoplasmic reticulum (white arrowhead) surround the factory. F: Micrograph of a section cut very close to the base of the cells, where released virions (black arrows), caveolae (arrowheads) and elements of the cortical cytoskeleton (white arrow) can be seen. G: Elements of the factory as visualized in a section perpendicular to the base of the cell. Discontinuous arrows point to the plastic base. Bars: 300 nm.

Post-fixation is done by incubating the monolayers for 1h at 4ºC in 1% osmium tetroxide and 0.8% potassium ferricyanide to fix and stain cell lipids and then washed two times in HEPES (pH 7.4). Cells are then treated 40 min at 4ºC with 2% uranyl acetate, which protects membranes from extraction during dehydration. After that we apply short dehydration steps in increasing concentrations of acetone (50, 70, 90 and 100%, 10min each, at 4°C) to minimize the extraction of soluble components. Finally, the monolayer is processed by embedding in the epoxy resin EML-812 (Taab Laboratories, Adermaston, Berkshire, UK) as previously described [11] but using a flat embedding mold (Ted Pella, Inc.) instead of regular capsules. Once embedded, the monolayer has to be physically adapted to the ultramicrotome unit. This can be done by mounting the monolayer on an epoxy resin capsule with standard glue for plastic material (Figure 1A to D). Basically, the monolayer can be oriented in two main ways: to obtain sections in planes parallel to the base of the cells (Figure 1E and 1F) or perpendicular to the cell base (Figure 1G). Oriented sections are showing new aspects on BUNV factory organization inside cells. Different viral assemblies concentrate in distinct planes of the cell: viral tubes and immature viruses are mainly located at 3-6 µm from the cell base (Figure 1E) while infectious virions are massively released from the cell basal zone (Figure 1F). Another important advantage of this oriented sectioning is that images of particular structures visualized by EM can be correlated adequately with the images obtained by confocal microscopy.

Serial Sections.

As a first approach for 3D analysis of cells at the EM level, we have incorporated a method for serial section reconstruction: a series of consecutive sections of cells embedded in an epoxy resin are obtained, imaged and processed to model a low resolution 3D map of the specimen of interest. The voxel size achieved by this technique can have an anisotropic resolution of about 4 nm in XY axis and 50 nm in Z axis [Fontana J., López-Montero N., Elliott R.M., Fernández J.J. and Risco C., unpublished results]. This resolution is enough for learning about contacts between different elements like synapses between neurons [12], or for getting specific information about spatial distribution of small elements [13]. In our laboratory we have optimized the protocol of serial section reconstruction for studying contacts between different organelles in viral factories. To this end, the sample is chemically fixed with a mixture of 1% glutaraldehyde and 0.5% tannic acid in HEPES buffer (pH 7.4) for 30 min at room temperature and embedded in an epoxy resin as previously described [14]. Ultrathin consecutive sections about 50 nm thick are collected on Formvar-coated parallel-bar copper grids with a perfect-loop (Diatome) and stained 30 min with saturated aqueous uranyl acetate and 2 min with lead citrate. To obtain long ribbons it is important to improve the adhesion between two consecutive sections. In addition, this will facilitate collection of the sections [15]. To this purpose, the quality of trimming is critical. Small trapezoids with parallel upper and lower sides and large bases represent the most adequate shape for the pyramid top. It is important to pick-up the sections in parallel to the bars of the grid, allowing the same regions to be seen all over the series (Figure 2A). Detailed protocols about how to solve problems while serial sectioning can be found in reference [15]. Slot grids can help when very long series are required, although they require a careful manipulation [16]. Samples are then studied by TEM. It is important to start studying a central section of the series, because when an interesting region is found, it can be easily followed up and down the ribbon. After selecting and photographing the region of interest in all sections, the photographs are digitalized (Figure 2B) and normalized using the Bsoft software [17]. This normalization allows to compare all the micrographs of the series by giving the same mean and standard

deviation to all of them. The sections are aligned by selecting common spots (Figure 2C to E) between two consecutive sections using the free editor for serial section microscopy "Reconstruct" [18] (http://synapses.bu.edu/tools/index.htm), following the "tips for aligning sections" of the User´s manual. These include selecting traces all over the micrograph, ideally at least one in each corner of the micrograph and another one in the middle. To minimize distortions of the final volume it is important to avoid the selection of the same trace in consecutive pairs of sections. Reconstruct takes into account the potential deformations associated to the sectioning process or to irradiation in the electron microscope. The main difference between our studies and previously reported works on serial section reconstruction is in the segmentation procedure, that is, the assignment of identity to the different elements of the micrographs. In our studies we have employed the knowledge previously developed for electron tomography reconstruction instead of manual drawing of the elements to be reconstructed [19].

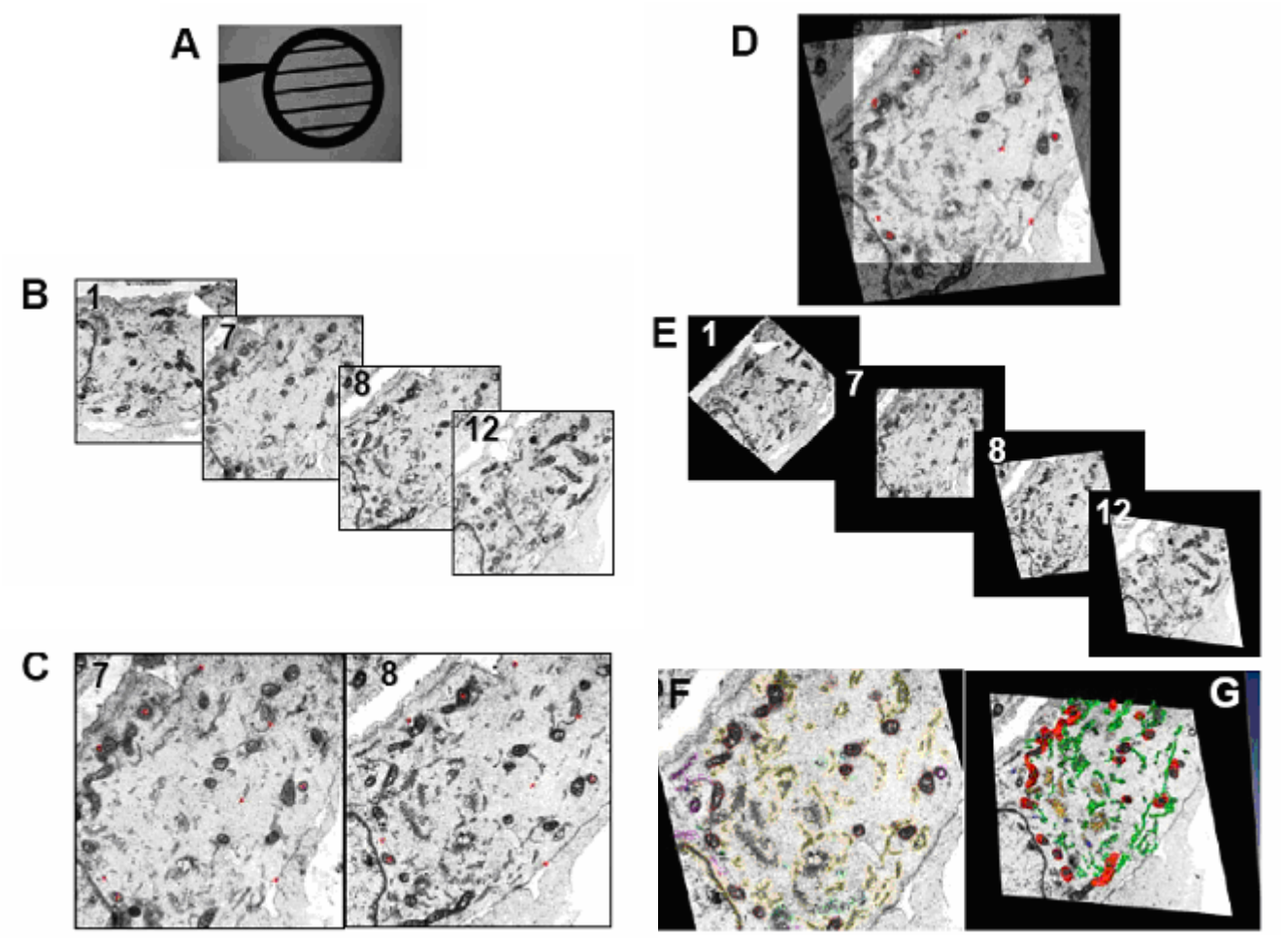

Figure 2. Serial Section Reconstruction. (A) Formvar coated parallel-bar copper grid with consecutive sections. (B) Four scanned micrographs of a selected zone out of a series of 15 sections. Note that the micrographs are not aligned. (C) Selection of common spots (red points) between two consecutive sections using Reconstruct software. (D) Two aligned consecutive sections. (E) The four micrographs shown in B after the alignment process. (F) Segmentation is done by selecting a mask that encloses the different elements of the factory using Amira software. (G) The quantity of material to be represented in the final 3D model is calculated for the different elements and color code for the final 3D map is established.

We have drawn a mask around each different material using Amira (http://amira.zib.de) (Figure 2F). This mask is just a selection of the pixels to be included in the calculations that will render the final 3D model. When the mask is being drawn it is important to assure the selection of all the pixels of a particular material by including pixels of the surrounding area as well. Noise reduction is then performed by applying three iterations of a median filter [20]. The optimal threshold, that is the quantity of material previously selected with the mask to be included in the 3D model, is calculated for the different materials by using a previously implemented algorithm [21]. Thus, 3D reconstructions (Figure 2G and Figure 3) do not exclusively depend on the manual drawing of the limits of the different elements and higher resolution is obtained. However, the intrinsic problem of serial sections which is the poor resolution in the Z axis, is still present. A possibility for minimizing this limitation would be using thinner sections. In Figure 3 we can observe the details provided by these 3D maps. Contacts between the different structures included in the reconstructions are clearly seen. A careful study of these volumes will help us to understand how viral factories are built and work inside infected cells.

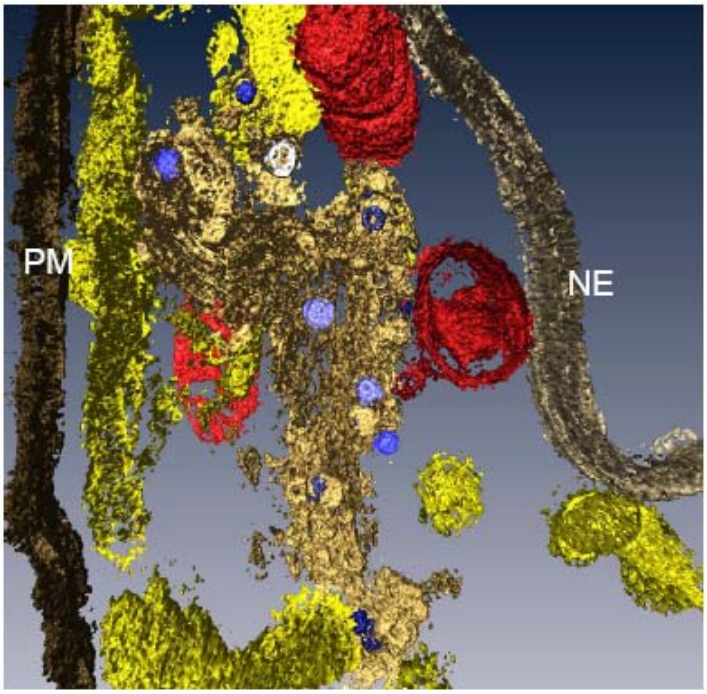

Figure 3. Serial section 3D reconstruction of a BUNV infected cell. New viral particles (purple) and viral tubes (white) are seen inside Golgi stacks (beige) surrounded by mitochondria (red) and RER elements (yellow). NE Nuclear Envelope. PM Plasma Membrane.

Freeze-fracture and metal replication.

Metal replicas can also provide 3D information of cells at medium resolution [22]. This technique consists in making a metal replica of the cell surface or the intracellular structures for their posterior study by TEM. When combined with labelling procedures for specific molecules with antibodies tagged with colloidal gold, replicas of the cell surface can provide valuable information about the distribution of receptors [23]. It also allows the study of the interior of the cell if previously fractured (Figure 4). For the standard freeze-fracture procedure, a suspension of cells fixed with 0.1%

glutaraldehyde and cryoprotected with glycerol are fast-frozen in liquid ethane or propane and then fractured inside a freeze-fracture unit at -150ºC [24]. Freeze-fracture of infected cells offers a global view of the whole viral factory (Figure 4A). Changes in the general distribution of organelles during factory construction are appreciated: Golgi stacks and mitochondria form a large structure near the nucleus. This recruitment leaves peripheral areas of the cell mostly empty of organelles, aspect that is also observed in thin-sections (Figure 4B). Although the global view is similar both with thin-sections and freeze-fracture, the three-dimensionality offered by metal replicas of freezefractured cells provides additional details when studying small elements at higher magnification. An example of this is observed when looking for small tubular structures of viral origin that assemble in Golgi stacks [7]. Thin sections show these elements as cylindrical structures with a larger globular domain in one of the extremes (Figure 4C). Although thin-sections show these tubes in Golgi areas and apparently associated to Golgi membranes, freeze-fracture provides evidences of their spatial integration in Golgi stacks (Figures 4D and 4E). Globular heads are integrated in Golgi sacculi, frequently on the periphery of the stack and always surrounded by membranes of this organelle (Figure 4D). The cylindrical domain of tubes is also inserted in the Golgi structure, sometimes connecting two contiguous stacks (Figure 4E). Both surface replication and freeze-fracture can be combined with an etching step at -90ºC. Water molecules of the upper layer of the exposed structure are removed from the sample with the help of a cold trap, for example the knife used for fracturing. As the chamber is under high-vacuum, the water of the sample goes to the cold trap. Etching unmasks the molecules of the upper layer of the sample, exposing them for replication. If the replicas are done with metals of fine grain, such as tantalum, resolution increases considerably. This suggests that replicas can be used to assist in segmentation of complex volumes obtained by electron tomography [22]. Direct 3D views of cell structures can be obtained by tilting the replicas in the electron microscope and making stereo pairs [22].

Concluding remarks.

The study of cell organization with enough resolution to characterize macromolecular interactions is nowadays a big challenge for structural biologists. ET has become the method of reference to obtain 3D maps of large, complex structures such as organelles and cell regions [25-29]. Advances in sample processing by high-pressure freezing and cryosectioning are providing native material for the elaboration of 3D maps of cells with molecular resolution. These maps are going to be of a considerable complexity. The application of immunogold labelling methods and the elaboration of complementary 3D maps of the same cell structures by serial section reconstruction and metal replication will help us to perform a more accurate segmentation and interpretation of tomograms [30]. Within this context we are studying viral infection as a model because inside infected cells there is a massive production of a few classes of viral macromolecular complexes. This will facilitate the development of methods to follow small structures and to study macromolecular interactions within the very complex intracellular environment.

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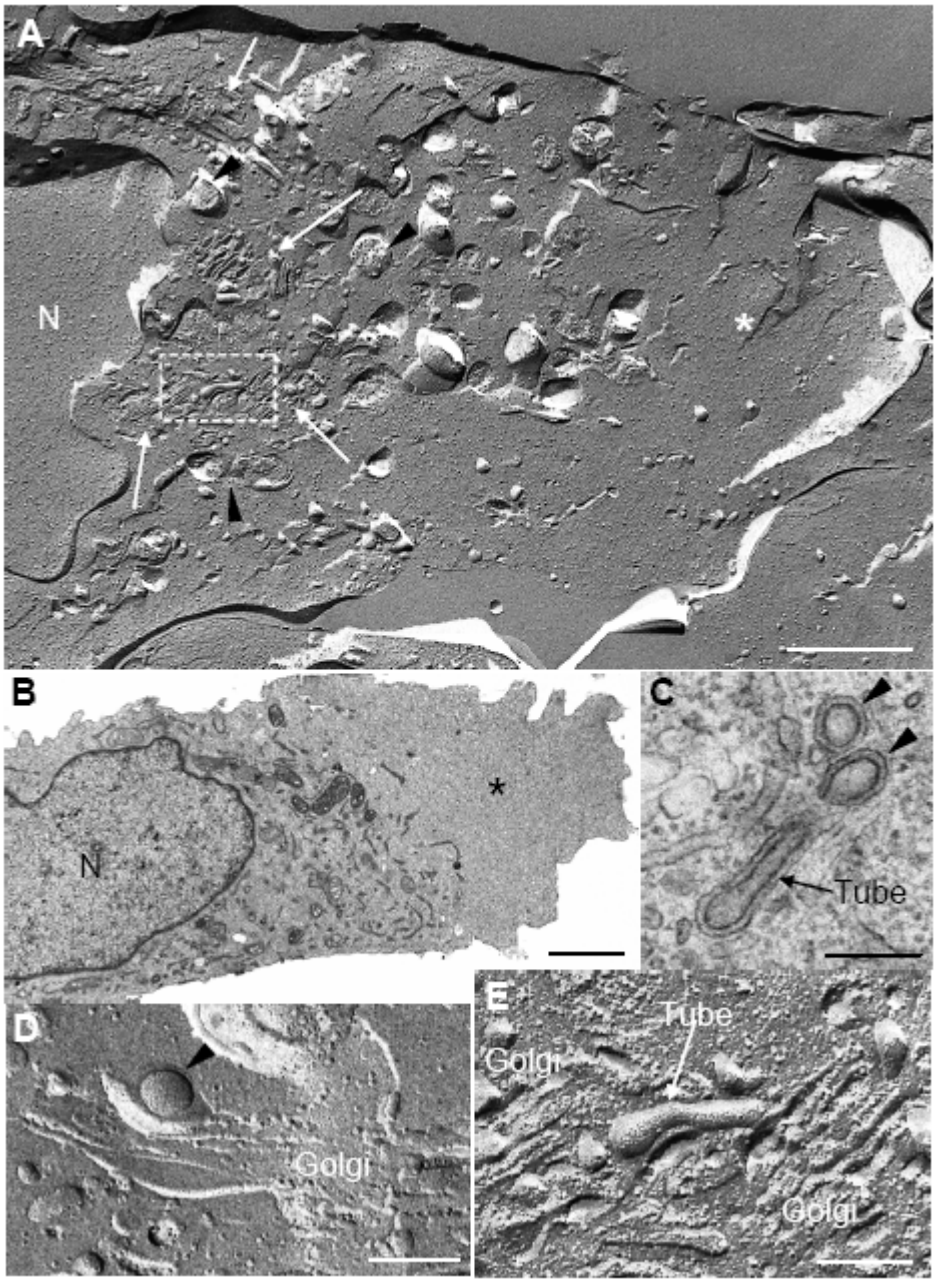

Figure 4. Freeze-fracture and metal replication to study virus factories. (A) Platinum/carbon replica of BHK-21 cells infected with Bunyamwera virus (BUNV) and processed by freeze-fracture in a BAF 060 unit (BAL-TEC, Liechtenstein). White arrows point to Golgi stacks while arrowheads point to recruited mitochondria. The white asterisk is on the peripheral region of the cell where organelles are missing. (B) Thin-section of a similar infected cell showing the recruitment of organelles to the perinuclear region and the empty periphery (black asterisk). (C) Thin section showing viral tubes and their globular domains (arrowheads) as seen when studying the Golgi stacks of the virus factory at higher magnification. (D) Freeze-fracture clearly shows that the globular domains (arrowhead) are always inside swollen Golgi sacculi. (E) Higher magnification of the factory area marked with a white rectangle in (A). A viral tube is seen as a "link" between two contiguous Golgi stacks, something difficult to appreciate in thin-sections. N: nucleus.

Bars: 1 μm in A and B; 200 nm in C-E.

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