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Cryo X-ray nano-tomography of vaccinia virus infected cells

Francisco Javier Chichón [a,1], Maria Josefa Rodríguez [a,1], Eva Pereiro [b], Michele Chiappi [a], Beatriz Perdiguero [a], Peter Guttmann [c], Stephan Werner [c], Stefan Rehbein [c], Gerd Schneider [c], Mariano Esteban [a], José L. Carrascosa [a,d,*]

[a] Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CNB-CSIC), Campus Cantoblanco, 28049 Madrid, Spain
[b] ALBA Synchrotron Light Source, MISTRAL Beamline – Experiments Division, 08290 Cerdanyola del Vallès, Barcelona, Spain
[c] Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Institute Soft Matters and Functional Materials, Electron Storage Ring BESSY II, Albert-Einstein-Str. 15, 12489 Berlin, Germany
[d] Instituto Madrileño de Estudios Avanzados en Nanociencia (IMDEA Nanociencia) Cantoblanco, Madrid, Spain

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ABSTRACT

We have performed full-field cryo X-ray microscopy in the water window photon energy range on vaccinia virus (VACV) infected cells to produce tomographic reconstructions. PtK2 cells were infected with a GFP-expressing VACV strain and frozen by plunge fast freezing. The infected cells were selected by light fluorescence microscopy of the GFP marker and subsequently imaged in the X-ray microscope under cryogenic conditions. Tomographic tilt series of X-ray images were used to yield three-dimensional reconstructions showing different cell organelles (nuclei, mitochondria, filaments), together with other structures derived from the virus infection. Among them, it was possible to detect viral factories and two types of viral particles related to different maturation steps of VACV (immature and mature particles), which were compared to images obtained by standard electron microscopy of the same type of cells. In addition, the effect of radiation damage during X-ray tomographic acquisition was analyzed. Thin sections studied by electron microscopy revealed that the morphological features of the cells do not present noticeable changes after irradiation. Our findings show that cryo X-ray nano-tomography is a powerful tool for collecting three-dimensional structural information from frozen, unfixed, unstained whole cells with sufficient resolution to detect different virus particles exhibiting distinct maturation levels.

1. Introduction

The process of virus infection of eukaryotic cells is a classical example on how the coupling in the host cell of the assembly of viral structural components to their spatial and temporal organization leads to efficient production of progeny. Viral components are produced under a temporal control, and they are subsequently transported to the proper assembly sites using cellular pathways. These areas define assembly factories where the concentration and access of viral components are optimized. During these steps there are extensive interactions between viral and cellular components that have been studied for decades as a main target to design antiviral strategies, as well as an attractive model system to understand the molecular basis for cell control and macromolecular interactions. In particular, the role of cell membranes in the viral assembly has been extensively documented for many viral systems, including the involvement of endoplasmic reticulum (ER) for rotavirus, the role of ER–Golgi intermediate compartment for coronavirus and poxvirus assembly and maturation, and the plasma membrane in the production of retrovirus, orthomyxovirus and paramyxovirus, among others (reviewed in Hunter, 2007).

The study of viral life cycles involves the correlation of biochemical and structural approaches to yield the proper detailed description of the different steps of the cycle: specific recognition of the host, incorporation of the viral genome into the cell, transcription and replication of the genome, assembly of the infective virus and the final exit from the host cell (reviewed in Flint et al., 2009). While improved visible light microscopy has been widely used for whole cell structural analyses of viral infection, the increasing demand of higher resolution to get deeper insights into these processes have led to the incremental use of powerful methods, as electron cryo-tomography (Lucic et al., 2005), revealing molecular details albeit with the cost of a reduction of the complexity or scale of the sample, while increasing the sample preparation difficulty. The inherent limitation of the analysis at the cellular level (sample thicknesses about 300–500 nm for electron

* Corresponding author at: Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CNB-CSIC), Campus Cantoblanco, 28049 Madrid, Spain. Fax: +34 915854506.
E-mail address: jlcarras@cnb.csic.es (J.L. Carrascosa).
[1] Both authors contributed equally to this work.

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microscopes), together with the consequent lack of quantification due to the sample limitations imposed by high resolution methods, have become critical problems for the correlation of these qualitative structural data with the quantitative data derived from whole populations using biochemical, genetic and proteome analyses (Baumeister and Steven, 2000; Martone et al., 2003).

There are different alternatives to overcome the resolution limits imposed either by visible light microscopy based analyses, or by the penetration power of transmission electron microscopy (TEM) to deal with thick, whole cell samples. Different super-resolution visible light fluorescence microscopy methods (Galbraith and Galbraith, 2011; Jones et al., 2011), and the use of improved sectioning methods in electron tomography (Pierson et al., 2010; Rigort et al., 2010) are opening new possibilities for the comprehensive study of the cellular cartography. The use of X-ray microscopy is another powerful alternative, based on the high penetration power of soft X-rays in the water window photon energy range (just below the O absorption K edge at 543 eV) in hydrated biological material (sample thicknesses $\leqslant$10 microns). The use of diffractive optics in full-field X-ray microscopes (Chao et al., 2005; Rehbein et al., 2009; Schneider et al., 1995), together with the incorporation of cryogenic procedures for sample preparation and data acquisition (Schneider, 1998), have led to the recent success of X-ray microscopy for 3-D imaging of cells yielding tomographic reconstructions at resolutions in the order of 20–30 nm (Hanssen et al., 2010; Larabell and Le Gros, 2003; Larabell and Nugent, 2010; Schneider et al., 2002, 2003, 2010; Weiss et al., 2000).

The estimation of the effective resolution attainable by cryo X-ray nano-tomography (cryo-X-nano-tomo) of whole cell samples is a critical parameter to evaluate its possibilities for the detailed analysis of the cartography of cellular components. A quantitative estimation for such a resolution was experimentally obtained by comparison of the tomographic reconstructions of a large and complex virus (VACV) using both X-ray and electron microscopies (Carrascosa et al., 2009). The resolution from X-ray tomograms using a zone plate of 25 nm was found to be only 3,7 times lower than that obtained from electron microscopy (*i.e.* 25.7 nm vs 6.7 nm), thus opening the possibility to resolve structural features of small cellular complexes and aggregates in whole cells at resolutions between light and electron microscopies. As those resolution estimations were done on purified virus preparations in buffer, a critical question is whether such features could be distinguished within the complex and crowded cellular media. In this work we show the possibility to detect VACV individual particles in the cytoplasm of an infected whole cell without chemical fixation, sectioning or labeling by cryo X-ray tomography with an estimated 3D-resolution up to 55 nm using a zone plate of 40 nm, which allowed fulfilling better depth of focus requirements.

Vaccinia virus (VAVC) is the best known member of the Poxviridae family. The virus particle is relatively large ($360 \times 270 \times 250\ nm^3$), enclosing a core with a double stranded DNA containing more than 200 open reading frames coding for viral specific proteins (Cyrklaff et al., 2005; Moss, 2007). The assembly of VACV is very complex, starting with the modification of cellular membranes at the ER by incorporation of viral proteins, giving rise to crescents. These modified membranes evolve to rounded immature virus particles (IV) which contain a complex viroplasm. The IVs incorporate viral DNA and they enter in a complex maturation process involving the reorganization of viral envelopes, the formation of a distinct core, and a severe change in the overall structure, yielding the characteristic brick-shaped mature virus (MV). These particles, although infective, are not the final form of the viral maturation: they are able to interact with the trans-Golgi network to produce a wrapped form of the virus (WV), which is transported towards the outer plasma membrane of the cell using a kinesin-based microtubular mechanism. Later in the infection cycle, the virus particles associate to the cell membrane and are further ejected from the cells using actin polymerization to efficiently spread the virus along large microvilli (reviewed in Flint et al., 2009; Moss, 2007).

The maturation process of VACV is carried out entirely in the cell cytoplasm, and it involves multiple sets of temporal interactions between cellular membranes and other organelles with viral components. Due to the complexity of these interactions, their study has been difficult and several controversies related to the total number of viral membranes and their cellular origin are still open (reviewed in Rodriguez et al., 2006). Part of these problems are derived from the fact that most of the studies were based on the very limited and partial information that is retrieved from ultra-thin sections of 50–100 nm from a total cell thickness of 10–20 μm or more. Furthermore, the electron microscope images from those sections overlap their structural features in two-dimensional projections, thus leading to conflicting interpretations. The use of electron tomography was a main step towards the understanding of the virus structure (Cyrklaff et al., 2005), assembly (Chichon et al., 2009) and disassembly (Cyrklaff et al., 2007). Tomographic reconstructions reveal genuine three-dimensional information, thus avoiding the superposition of information found in projected images. Nevertheless, even in these cases the information is retrieved from a small percentage of the volume of the whole cell, thus preventing the quantitative analysis of the viral life cycle at the cellular level. In an attempt to circumvent these problems, we have explored the combination of fast freezing of VACV infected cells with X-ray microscopy to get insights into the viral infection process at the whole cell level taking profit of the penetration power of X-rays. The VACV structural knowledge obtained by the analysis of the same cell type by standard electron microscopy approaches allowed us to identify the viral related structures within the X-ray tomograms. Our results demonstrate that it is possible to detect different types of viral assembly intermediates within the cytoplasm of whole cells. In addition, we studied the effect of radiation damage by TEM analysis of plastic embedded thin sections of X-ray irradiated cells. The results revealed no noticeable changes in the cellular ultrastructure during the tomographic data acquisition by X-ray microscopy.

2. Materials and methods

2.1. Cells and viruses

The established chick embryo cell line DF-1 and PtK2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were maintained in a humidified air 5% CO_2 atmosphere at 37 °C. Virus infections were performed with 2% FCS for both cell types. The VACV strains used in this work include Western Reserve (WR), modified vaccinia virus Ankara (MVA) obtained from the Ankara strain after 586 serial passages in primary chick embryo fibroblast cells (CEF) (derived from clone F6 at passage 585, kindly provided by G. Sutter, Germany) and MVA-C-ΔF1L expressing from the TK locus gp120 and Gag-Pol-Nef proteins of HIV-1 (subtype C) under the transcriptional control of the synthetic early/late promoter and with the anti-apoptotic virus F1L gene replaced by rsGFP gene (Perdiguero et al., in preparation).

2.2. Analysis of virus growth

To determine virus growth profiles, monolayers of PtK2 cells grown in 12-well tissue culture plates were infected at 0.01 PFU/cell with WR, MVA or recombinant MVA-C-ΔF1L(GFP). Following virus adsorption for 60 min at 37 °C, the inoculum was removed,