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Correlative VIS-fluorescence and soft X-ray cryo-microscopy/tomography of adherent cells

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ABSTRACT

Soft X-ray cryo-microscopy/tomography of vitreous samples is becoming a valuable tool in structural cell biology. Within the 'water-window' wavelength region (2.34–4.37 nm), it provides absorption contrast images with high signal to noise ratio and resolution of a few tens of nanometer. Soft X-rays with wavelengths close to the K-absorption edge of oxygen penetrate biological samples with thicknesses in the micrometer range. Here, we report on the application of a recently established extension of the transmission soft X-ray cryo-microscope (HZB TXM) at the beamline U41-XM of the BESSY II electron storage ring by an in-column epi-fluorescence and reflected light cryo-microscope. We demonstrate the new capability for correlative fluorescence and soft X-ray cryo-microscopy/tomography of this instrument along a typical life science experimental approach – the correlation of a fluorophore-tagged protein (pUL34-GFP of pseudorabies virus, PrV, the nuclear membrane-anchored component of the nuclear egress complex of the *Herpesviridae* which interacts with viral pUL31) in PrV pUL34-GFP/pUL31 coexpressing mammalian cells, with virus-induced vesicular structures in the nucleus, expanding the nucleoplasmic reticulum. Taken together, our results demonstrate new possibilities to study the role of specific proteins in substructures of adherent cells, especially of the nucleus *in toto*, accessible to electron microscopy in thinned samples only.

1. Introduction

Coming from an electron cryo-microscopy background (Grünewald et al., 2003; Vanhecke et al., 2011), soft X-ray cryo-microscopy, experienced hands-on at the HZB TXM at beamline U41-XM of the BESSY II electron storage ring in Berlin/Germany, offers several unique characteristics making it a complementary tool in structural cell biology. (i) The limit in sample thickness is in the micrometer range, and many mammalian cell models can be imaged intact with all their organelles and the nucleus well resolved to count and chart specific features (Schneider et al., 2010; for yeast cells, see Uchida et al., 2011). While for electron cryo-microscopy the cellular sample has to be as thin as possible, for X-ray cryo-microscopy one has to revisit the thickness limit of plunge freezing for cryo-immobilization (Dubochet et al., 1988). Tomography is key to mine the wealth of information, as soft X-ray cryo-microscopic images are too 'crowded' to reveal much detail, and the reconstructed volumes are almost cubes instead of thin slabs (Larabell and Nugent, 2010; Weiss et al., 2000). (ii) Radiation damage seems less of an issue (Schneider, 1998), at least at lower resolution. Thus, a 'low dose approach' as in electron cryo-microscopy is not necessary, and the entire dynamic range of the detector is used yielding high signal/low noise data. (iii) Imaging in the wavelength region between the innershell absorption edges of oxygen and carbon (2.34–4.37 nm), known as 'water window' (Thieme et al., 1993; Wolter, 1952), ice contamination is accordingly hard to see, and is mostly not interfering with data acquisition. (iv) One key part of the instrument, the zone plate focusing element, does not look like an objective, but is a high-precision in-house development of the HZB Nano-Lab (Rehbein et al., 2009; Werner et al., 2010). (v) Fascinatingly, the object is illuminated with soft

Abbreviations: DIC, differential interference contrast; GFP, green fluorescent protein; NEC, nuclear egress complex; PrV, pseudorabies virus.

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X-ray radiation by an elliptically shaped capillary condenser mirror of a defined length (Guttmann et al., 2009). (vi) Even though it seems conceptionally to be a 'light' microscope, one can see nuclear pores with it (Schneider et al., 2010). Taken together, it would be nice to have such an instrument in our own lab. Unfortunately, it (still) needs a 'huge lamp' (for development of a laboratory soft X-ray microscope, see Bertilson et al., 2011b).

But even good things can be improved. As a cell biologist, reading about the plan to establish correlative fluorescence and X-ray cryo-microscopy was stimulating (Le Gros et al., 2009; McDermott et al., 2009; Schneider et al., 2006), getting hands on it is thrilling. Since beginning of 2011 we have worked with the newest extension of the HZB TXM in Berlin – an in-column epi-fluorescence and reflected light microscope (for technical description see Schneider et al., this issue). The goal is to correlate results of cell imaging using a rainbow of fluorescent tags to elucidate the role of specific proteins in the cell (Wiedenmann et al., 2009; Wombacher and Cornish, 2011) with nanometer-resolved structures from soft X-ray microscopy (Falcone et al., 2011), in vitreous samples, i.e. with the best possible preservation of ultrastructure (Dubochet, 2007). In electron cryo-microscopy/tomography, already several correlative approaches and solutions exist (for a recent review, see Briegel et al., 2010).

Here, we have chosen an application of one of our recent projects – characterization of the herpesvirus 'life' cycle with cryo-microscopy (Ibiricu et al., 2011; Maurer et al., 2008) – to demonstrate as a proof of principle the new capability of the HZB TXM instrument. Herpesviruses are complex viruses with a distinct replication cycle involving cytoplasmic and nuclear compartments of infected cells. Viral capsids assemble in the nucleus and gain access to the cytosol by vesicular transport through the nuclear envelope, mediated by the viral nuclear egress complex (NEC) consisting of pUL34, a type II membrane anchored protein, interacting with pUL31 (for review see Mettenleiter et al., 2009). Heterologous coexpression of just these two proteins in mammalian cells results in the budding of vesicles from the inner nuclear envelope into the perinuclear space (Klupp et al., 2007).

We share our experience in sample preparation for soft X-ray cryo-microscopy, and discuss further improvements.

2. Materials and methods

2.1. Cells

Porcine epithelial-like embryonic EFN-R kidney cells stably coexpressing pseudorabies virus (PrV) pUL31 and pUL34, the latter fused to green fluorescent protein (GFP; pUL34-GFP; cell line designated as BK/EFN/UL31/34gfp, catalog No. RIE 1083 of the Collection of Cell Lines in Veterinary at the FLI, Greifswald-Insel Riems, Germany), were grown in Dulbecco's Modified Eagle Medium (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 10% (w/v) fetal calf serum and 1% (v/v) PSN Antibiotic Mixture (Gibco-Invitrogen). Generation and characterization of the cellular model are given in Klupp et al. (2007).

2.2. Sample preparation for X-ray cryo-microscopy

A detailed "*Protocol for partially coherent X-ray microscopy*" is provided in the Supplement of Schneider et al. (2010). Here, we highlight improvements or alternative procedures along this description, in parts depicted in Fig. 1.

For on-grid cell cultivation, custom-designed gold grids (new design with smaller slots, specified as HZB-2) were purchased from Gilder Grids, Grantham, UK. These grids were coated with a R2/2-perforated carbon foil by Quantifoil Micro Tools GmbH, Jena, Germany. This coat is a standard grid support in electron cryo-microscopy, and is compatible with adherent cell growth (Ermantraut et al., 1998; Maurer et al., 2008). Before cell incubation, grids on a glass slide with the carbon coat upwards were treated in a PDC-002 plasma cleaner (Harrick Plasma, Ithaca, NY, USA) to increase hydrophilicity. The coated side of the grid was marked with a permanent marker pen, and the mesh area was demarcated from the rest of the metal area of the grid by applying a thin line of PAP pen liquid blocker using the 45°-bended tip of tweezers (both items from Plano GmbH, Wetzlar, Germany). These grids, blocked from liquid spreading by the PAP pen line only on the carbon coat side, were placed on Whatman No. 50 hardened filter paper carbon-side up, and 2 µl of a 1:4 dilution of 200-nm FluoSpheres (carboxylate-modified polystyrene microspheres, red fluorescent proprietary dye, excitation max.: 580 nm, emission max.: 605 nm, 2% solids, catalog No. F-8810, Invitrogen, UK) were applied as alignment and correlation markers to the carbon-coated mesh area of the grids and thereby sucked through to the filter paper underneath. After 5 min of drying, the grids were submerged in 1 ml of fresh complete medium in plastic microscope slide growth chambers (µ-slide 2×9 well, Ibidi GmbH, Munich, Germany). At 90% confluency, cells from 25-ml cell-culture flasks were trypsinized, and were seeded onto the grids in the growth chamber slides at a cell density of approximately $3 \times 10^4\,cm^{-2}$. The slides were incubated on an aluminum rack at 37 °C, 5% CO_2 and

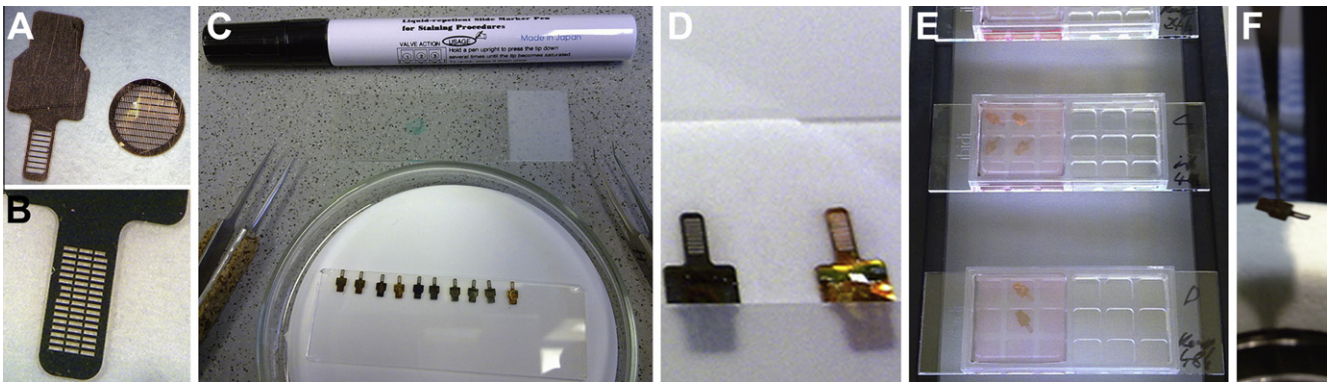

Fig.1. Sample grid preparation for soft X-ray cryo-microscopy/tomography at the BESSY II U41-XM beamline. (A) Due to the confined space for sample tilting in the microscope, a special grid design (IFR-1, left) is needed. For comparison, a standard 400 × 100 mesh grid for electron microscopy is shown (diameter: 3.05 mm, right). (B) Retaining the outer dimensions, an improved mesh design (HZB-2) with smaller slots helps to stabilize standard perforated carbon film (tested: Quantifoil R2/2). Furthermore, it alleviated targeting for correlative microscopy. (C) In order to prevent liquid to spread over the whole grid, liquid blocker (PAP pen) was applied as a thin line near the observation area of the grid. (D) Alignment marker bead suspension was blotted and dried on the carbon film of the glow-discharged grid. Note the green liquid blocker line on the carbon-coated side of the right grid. (E) On-grid cell incubation was performed in commercially available microscope slide growth chambers. (F) Grid handling with tweezers, and finally vitrification by plunge freezing of the sample, has to spare the clamping end of the grid for proper mounting in the Gatan model 630 specimen cryo-holder.