Journal of Structural Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Subtomogram analysis using the Volta phase plate

Maryam Khoshouei [a,1], Stefan Pfeffer [a,1], Wolfgang Baumeister [a], Friedrich Förster [a,b,*], Radostin Danev [a,*]

[a] Max-Planck Institute of Biochemistry, Department of Molecular Structural Biology, Am Klopferspitz 18, 82152 Martinsried, Germany
[b] Cryo-Electron Microscopy, Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form 6 May 2016
Accepted 24 May 2016
Available online xxxx

Keywords:
Cryo-electron tomography
Subtomogram analysis
Volta phase plate
Ribosome

ABSTRACT

Cryo-electron tomography (CET) and subtomogram analysis allow studying the structures of macromolecular complexes in their natural context. The radiation sensitivity of vitrified biological specimens and the resulting low signal-to-noise ratio (SNR) in CET limit the amount of structural information that can be mined from tomographic data. The Volta phase plate (VPP) has emerged as an effective means to increase the SNR and hence contrast compared to 'conventional' defocus-based phase contrast transmission electron microscopy (CTEM). Here, we assess the performance of the VPP compared to CTEM in subtomogram analysis, using the mammalian 80S ribosome as a test case. Accurate focusing is the major factor for achieving high resolution with the VPP, as highlighted by a comparison of slightly different focusing strategies. From only 1400 subtomograms, the VPP yields a subtomogram average of the mammalian 80S ribosome at 9.6 Å resolution without laborious contrast transfer function (CTF) correction. The subtomogram averages obtained using CTEM approaches are comparable, but suffer from lower signal transfer in certain frequency bands due to the oscillations of the CTF. Our study demonstrates that the VPP is a valuable tool for subtomogram analysis, because it enables improved performance and efficiency in terms of structure localization and number of subtomograms required for a given resolution.

1. Introduction

Cryo-electron tomography (CET) can provide three-dimensional (3D) views of structures and molecular organizations in a wide range of cryo-preserved specimens under near-to-native conditions (Lucic et al., 2013). It is applicable to a variety of cell-free systems, isolated cellular organelles and even intact cells. In combination with advanced image processing methods ('subtomogram analysis'), the location, orientation and, in particular, the structure of larger macromolecular complexes can be extracted from tomographic data (Briggs, 2013; Forster and Hegerl, 2007). CET is an excellent method for studying the molecular machine responsible for cellular protein synthesis, the ribosome, in a native context (Brandt et al., 2009; Myasnikov et al., 2014; Pfeffer et al., 2015; Pfeffer et al., 2012; Pfeffer et al., 2014). The ribosome is a universally conserved RNA-protein complex of 3–4 MDa size consisting of two subunits, which in concert mediate decoding of messenger RNA and formation of peptide bonds in the growing polypeptide chain (Steitz, 2008). Due to its fundamental importance, the ribosome has been extensively studied using single particle cryo-electron microscopy (cryo-EM) (Anger et al., 2013; Behrmann et al., 2015; Voorhees et al., 2014) and X-ray crystallography (Ben-Shem et al., 2010; Ben-Shem et al., 2011; Jenner et al., 2012; Schuwirth et al., 2005; Selmer et al., 2006; Yusupov et al., 2001), yielding structural insights into protein synthesis with atomic detail.

The low signal-to-noise ratio (SNR) of CET data limits their molecular interpretability in two ways: (*i*) CET is mostly used to image native or close-to-native samples, which contain numerous different types of macromolecular complexes. In-depth analysis of a macromolecule of interest requires its specific and sensitive detection, for example by template matching (Forster et al., 2010; Frangakis et al., 2002). The SNR is a key determinant for the specificity and sensitivity of the localization of macromolecules. (*ii*) Subtomogram averaging allows *in silico* enhancement of the signal and hence increases the resolution compared to that of raw tomograms. The attainable resolution depends not only on the number of averaged subtomograms, but also on their SNR, which determines how accurately they can be aligned to a common reference.

* Corresponding authors at: Max-Planck Institute of Biochemistry, Department of Molecular Structural Biology, Am Klopferspitz 18, 82152 Martinsried, Germany.
E-mail addresses: foerster@biochem.mpg.de (F. Förster), danev@biochem.mpg.de (R. Danev).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.jsb.2016.05.009
1047-8477/

Both the detection fidelity and the accuracy of subtomogram alignment depend primarily on the low-frequency components of the signal, which are by far the strongest for any macromolecular complex (Scheres and Chen, 2012). Defocus-based phase contrast severely dampens the low-frequency components and has contrast transfer function (CTF)-induced contrast oscillations at higher frequencies, which necessitate elaborate correction methods for faithful interpretation of the data. These contrast oscillations also result in the absence of signal for spatial frequencies located close to the CTF zeros. In comparison, a phase plate (PP) introduces a relative phase shift between the scattered and unscattered electrons, which allows imaging in or close-to focus with strong and continuous contrast transfer from low to high frequencies. The increase of low frequency contrast, in particular, promises to be highly beneficial for CET because particles can be localized and aligned more accurately.

The recently introduced Volta PP (VPP) is the first implementation of a PP for electron microscopy that is sufficiently durable and easy to use for routine work (Danev et al., 2014; Fukuda et al., 2015). The VPP consists of a thin amorphous carbon film mounted on a supporting aperture positioned at the back-focal plane of the objective lens of the microscope. The Volta potential created by the electron beam on the surface of the heated amorphous carbon film induces a phase shift for the unscattered electron beam, which leads to a change in the contrast transfer function and improved contrast (Danev et al., 2014). After initial conditioning of the VPP, the phase shift remains approximately constant and changes only very gradually. Tomographic data acquisition with the VPP requires accurate focusing in order to provide uniform, oscillation-free information transfer throughout the frequency spectrum. The position of the sample changes during sequential tilting of the specimen, requiring accurate measurement and adjustment of the microscope defocus for each tilt image. Perpendicular to the tilt-axis tilt images exhibit a defocus gradient, which complicates data acquisition further. The VPP has already proven its value for CET (Asano et al., 2015; Fukuda et al., 2015; Mahamid et al., 2016) and cryo-EM single particle analysis (Danev and Baumeister, 2016; Khoshouei et al., 2016) but its performance in subtomogram analysis has not yet been quantitatively evaluated.

Here, we assess the performance of the VPP for subtomogram analysis and compare it to CTEM. In particular, we investigate whether subtomogram averages of subnanometer resolution can be obtained using the VPP and what is the best strategy for data acquisition. We chose the mammalian 80S ribosome as a test sample because it is highly abundant, structurally well characterized and, due to its size, suitable for subtomogram analysis.

2. Materials and methods

2.1. Sample preparation and CET

Ribosomes were pelleted (TLA 100, 30 min, 150,000 g, 4 °C) from rabbit reticulocyte lysate (Promega, USA) and resuspended in ribosome buffer (20 mM Hepes, pH 7.6; 50 mM KCl; 2 mM $MgCl_2$). After applying 3 µl of ribosomes to plasma-cleaned (20 s) Quantifoil R 2/1 (Quantifoil, Germany) holey carbon copper grids, 3 µl of 10-nm colloidal gold in ribosome buffer were added and the sample was vitrified in liquid ethane using a Vitrobot Mark IV (FEI, USA). The Vitrobot was operated at ambient temperature (22 °C) and humidity (60%) using a blotting time of 3 s and a blot force of 0. Tilt series were acquired on an FEI Titan Krios transmission electron microscope equipped with a K2 Summit direct electron detector with a Quantum energy filter (Gatan, USA), operated in movie mode with 4–7 frames per projection image. Exposure time varied from 0.8 to 1.4 s and 2.0 to 2.8 s for CTEM and VPP data acquisition, respectively, due to variations in the FEG output current. Cumulative electron dose was kept constant in all datasets. The TEM was operated at an acceleration voltage of 300 kV, an object pixel size of 2.62 Å, and nominal defocus of 2.4–3.7 µm (defocus dataset) or 0–200 nm (VPP dataset). The standard autofocusing procedure implemented in SerialEM was used for focusing. For retention of high-resolution signal in VPP data, it was important to use no defocus offset for focus measurement and to set the target defocus to 0 (Fig. 3). Single-axis tilt series were recorded over an angular range of −60° to +60° in two halves (first half: 20° to −60°; second half; 22° to 60°) with an angular increment of 2° and a cumulative electron dose of 90–100 $e^-/Å^2$ using the SerialEM acquisition software (Mastronarde, 2005). Acquisition of a tomogram took approximately 45 min. Conditioning of the VPP was achieved by pre-irradiation for 60 s after setting the on-plane condition and aligning the beam shift pivot points. The conditioning applied a total dose of 12 nC (0.2 nA beam current * 60 s) to the phase plate which was estimated to generate an initial phase shift of 0.35 π (from Fig. 3 in (Danev and Baumeister, 2016)). Tilt series added a dose of ~30 nC (61 images * 2.5 s exposure * 0.2 nA beam current) which means that at the end of acquiring a tomogram the total dose on the phase plate was ~42 nC corresponding to a phase shift of ~0.55 π. The irradiated area on the VPP was changed after each tomogram.

2.2. Image processing

Frames from the K2 direct electron detector were aligned using in-house developed motion correction software based on the algorithm described in (Li et al., 2013). For the defocus dataset, tilt-dependent, local correction of phase reversals due to the contrast transfer function was performed using MATLAB scripts and PyTom (Hrabe et al., 2012) on single projections, as described in (Eibauer et al., 2012). For the PP dataset, no CTF correction was performed. Tomogram reconstruction (object pixel size: 2.1 nm) and template matching against a single particle cryo-EM reconstruction of the human 80S ribosome (EMD-5592) (Anger et al., 2013) filtered to 5 nm resolution were accomplished using weighted backprojection in PyTom (Hrabe et al., 2012) as described in (Pfeffer et al., 2012). Candidate particles in each tomogram were selected according to Gaussian distributed constrained correlation coefficients obtained from template matching. For the retained particles, 1 × down sampled subtomograms (80^3 voxels, object pixel size: 0.524 nm) were reconstructed individually from the weighted aligned projections using the full tilt range (−60° to +60°). After iterative subtomogram alignment and averaging with PyTom (Hrabe et al., 2012), unbinned subtomograms (160^3 voxels, object pixel: 0.262 nm) were reconstructed using a reduced tilt range (−20° to +20°) in order to restrict the cumulative electron dose to 30 $e^-/Å^2$. Subtomograms were iteratively aligned with PyTom until convergence, using a single particle reconstruction of the human ribosome (EMD-5592) (Anger et al., 2013) filtered to 2 nm resolution as a reference for the first iteration. For the subsequent iterations, the average from the previous iteration served as a reference. Angular sampling and bandpass filter for alignment were automatically set in PyTom according to the determined resolution from the previous iteration. For analysis of the relationship between particle number and resolution, differently sized subsets of random subtomograms were either averaged using alignment shifts and rotations determined during alignment of the complete set of 1400 subtomograms, or aligned separately only among the reduced number of particles. The resolution of all subtomogram averages was assessed by Fourier shell correlation (FSC = 0.33) against EMD-5592 (Fourier shell cross resolution, FSCR). For visualization, the resolution-limited maps were sharpened using an empirically

Please cite this article in press as: Khoshouei, M., et al. Subtomogram analysis using the Volta phase plate. J. Struct. Biol. (2016), http://dx.doi.org/10.1016/j.jsb.2016.05.009