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Quantifying resolution limiting factors in subtomogram averaged cryo-electron tomography using simulations

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ABSTRACT

Cryo-electron tomography (CET) is the only available technique capable of characterizing the structure of biological macromolecules in conditions close to the native state. With the advent of subtomogram averaging, as a post-processing step to CET, resolutions in the (sub-) nanometer range have become within reach. In addition to advances in instrumentation and experiments, the reconstruction scheme has improved by inclusion of more accurate contrast transfer function (CTF) correction methods, better defocus estimation, and better alignments of the tilt-series and subtomograms. To quantify the importance of each contribution, we have split the full process from data collection to reconstruction into different steps. For the purpose of evaluation we have acquired tilt-series of ribosomes in such a way that we could precisely determine the defocus of each macromolecule. Then, we simulated tilt-series using the InSilicoTEM package and applied tomogram reconstruction and subtomogram averaging. Through large scale simulations under different conditions and parameter settings we find that tilt-series alignment is the resolution limiting factor for our experimental data. Using simulations, we find that when this alignment inaccuracy is alleviated, tilted CTF correction improves the final resolution, or equivalently, the same resolution can be achieved using less particles. Furthermore, we predict from which resolution onwards better CTF correction and defocus estimation methods are required. We obtain a final average using 3198 ribosomes with a resolution of 2.2 nm on the experimental data. Our simulations suggest that with the same number of particles a resolution of 1.2 nm could be achieved by improving the tilt-series alignment.

1. Introduction

Cryo-electron tomography (CET) is an essential technique to study the structure of macromolecules *in situ*, i.e. embedded in their native environment. A typical CET acquisition consists of a thin specimen that is tilted in order to acquire projections of the specimen at different angles. These projections are then used to reconstruct a three-dimensional (3D) volume. A major problem of this technique is radiation damage imposed by the electrons onto the specimen. This limits the amount of electrons that can be used for imaging which results in very noisy images. Nevertheless, this restriction can be overcome using subtomogram averaging. If a specimen contains many copies of an identical structure, the reconstructed subvolumes of these structures can be aligned and averaged to increase the signal-to-noise ratio (SNR) of the final reconstruction. Prerequisites for subtomogram averaging are that these copies can be identified in the reconstructed volume and aligned.

Projections in CET are intentionally recorded with underfocus. This defocusing allows contrast-generating interference of the undiffracted beam with the beam that is phase-shifted by the specimen. The contrast transfer function (CTF) describes the contrast transfer due to aberrations such as defocusing, astigmatism and spherical aberration. In effect, the CTF is an oscillating function of spatial frequency and depends on the defocus. These oscillations result in contrast inversions at certain spatial frequencies. Therefore, it is necessary to correct for the oscillations in order to interpret structures at a resolution beyond the first zero-crossing.

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Recently, different studies tackled a number of problems associated with CET. Due to the extremely low SNR per projection, defocus estimation requires either a special averaging technique (Fernández et al., 2006; Xiong et al., 2009), using magnification correction (Zanetti et al., 2009) or a new acquisition procedure (Eibauer et al., 2012). Due to the tilted geometry, required for tomography, CTF correction needs to account for the defocus gradient perpendicular to the tilt-axis (Philippsen et al., 2007; Voortman et al., 2011; Voortman et al., 2012; Winkler and Taylor, 2003; Fernández et al., 2006; Xiong et al., 2009; Zanetti et al., 2009). Furthermore, some researchers studied CTF correction methods that consider the defocus gradient within the specimen along the optical axis (Kazantsev et al., 2010; Jensen and Kornberg, 2000; Voortman et al., 2012). Using these methods together with subtomogram averaging, Eibauer et al. (2012) reported a resolution of 1.68 nm on mycobacterial membrane protein MspA.

Similar to single-particle analysis (SPA) (Rosenthal and Henderson, 2003), the resolution after subtomogram averaging depends on the number of particles but also on the accuracy of defocus estimation, CTF correction method as well as tilt-series and subtomogram alignment. In order to get a better understanding of what is currently limiting the resolution, it is needed to quantify the influence of the different processing steps.

In this study we investigate the influence of defocus estimation, CTF correction, tilt-series alignment and subtomogram alignment, primarily using simulations. We use an extended acquisition scheme to determine the defocus and defocus gradient for each projection in a tilt-series, providing us with orientation and planarity of the sample and thus the defocus at the positions of each macromolecule. We acquired experimental data using this scheme and simulate tilt-series which match the experimental conditions. After subtomogram averaging, we show that the experimental data is in good agreement with the simulations. Using these simulations we quantify the influence of defocus estimation and CTF correction, but also tilt-series alignment and subtomogram alignment on the resolution.

2. Experimental methods

2.1. Protein purification and sample incubation

Escherichia coli MRE600 were cultured up to an OD600 of 1.0. Then the cell membranes were disrupted in a French Press and the ribosomes were purified following the protocol described in Fechter et al. (2009).

The EF-G gene was inserted in *E. coli* following the procedure introduced by Dümmler et al. (2005). BL21 *E. coli* cells, transformed with the EF-G fuse gene, were cultured in LB medium at 37 °C up to an OD600 of 0.7. The expression of the EF-G was induced upon administration of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Four hours after induction the cells were harvested and the pellet dissolved in a buffer containing 25 mM Tris–HCl pH 7.1, 5% glycerol, 700 mM NaCl, 6 mM β-mercapto-ethanol, 0.1 mM PMSF and 0.1 mM benzamidine. The cells were then lysed by sonication and the debris and cell-membranes were removed by centrifugation (13,000 rpm, 45 min). The EF-G was separated by the contaminants present in the lysate loading the cell extract in a nickel–nitrilotriacetic acid (Ni–NTA) chromatographic column. The column was washed with 5 volumes of buffer containing 25 mM Tris–HCl pH 7.1, 300 mM NaCl, 5% glycerol, 20 mM Imidazole, 6 mM β-mercapto-ethanol, 0.1 mM PMSF and 0.1 mM benzamidine and then the EF-G was eluted with a buffer containing 25 mM Tris–HCl, pH 7.1, 300 mM NaCl, 5% glycerol, 300 mM imidazole, 6 mM β-mercapto-ethanol, 0.1 mM PMSF and 0.1 mM benzamidine.

The purified 50S, 30S and EF-G were dialysed in separate membrane against the same buffer: 2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl of pH 7.5, 0.1 mM EDTA, 1 mM DTT.

Equimolar amounts of 50S and 30S *E. coli* Ribosome were incubated for one hour with t-RNA fMet and m-RNA in a dialysis buffer. In another vial, with the same buffer composition, we incubated a 1.3 times excess of EF-G with a 10 times excess of fusidic acid. Then we mixed the solutions of the vials together in a 10 mM $MgCl_2$ buffer in order to obtain the 70S complex bound to t-RNA fMet and m-RNA and locked to EF-G by fusidic acid administration. 5 μL of 0.3 mg/mL of the complex were applied to glow-discharged quantifoil grids. The excess of liquid was blotted away in a vitrobot (FEI Company, 4 s blot time, Force 0, 100% humidity, 25 °C) and then the grid was flash-frozen in liquid ethane cooled down by liquid nitrogen.

2.2. Extended acquisition scheme

Improving the resolution beyond the first zero-crossing of the CTF requires a CTF correction step. In order to perform the CTF correction in such a way that it actually increases the resolution,

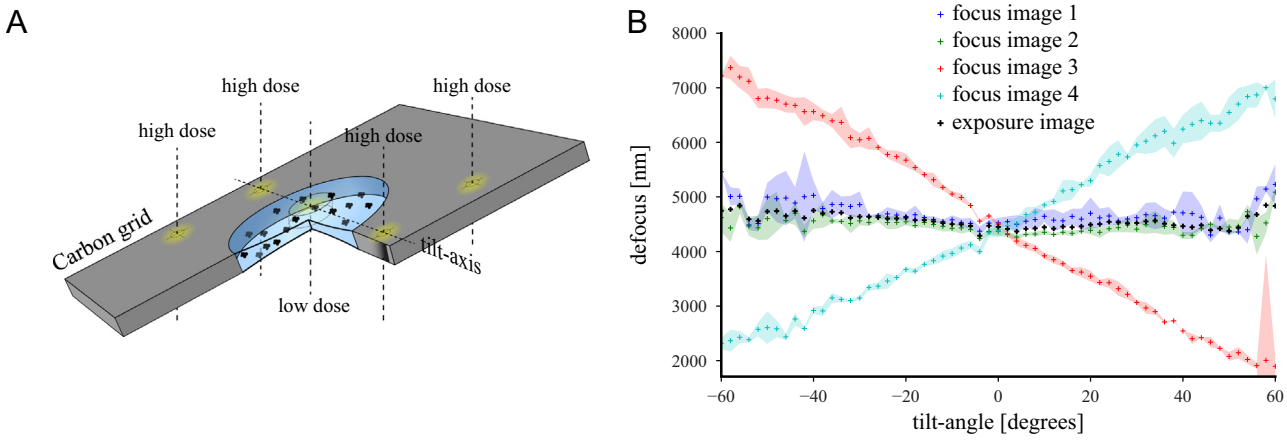

Fig.1. (A) Schematic overview of the extended acquisition scheme. The low-dose *exposure image* is located in a hole of the carbon-grid. Two high-dose *focus images* are located on the carbon, on the tilt-axis. The other two high-dose areas are located off-axis and positioned such that even at high tilt-angles the electron beam does not overlap with the *exposure image*. (B) Example of defocus estimation using the extended acquisition scheme. Four *focus images* are used to compute the defocus at the position of the *exposure image*. The color-shaded areas correspond to the uncertainty of the defocus estimate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)