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Pushing the resolution limits in cryo electron tomography of biological structures

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Summary

Cryo electron tomography is a three-dimensional imaging technique that is suitable for imaging snapshots of the structural arrangements of biomolecular complexes and macromolecules, both *in vitro* and in the context of the cell. In terms of attainable resolution, cryo electron tomographic reconstructions now show resolvable details in the 5–10 nm range, connecting optical microscopy with molecular imaging techniques. In view of the current developments in super-resolution light microscopy and correlative light and electron microscopy, cryo electron tomography will be increasingly important in structural biology as a tool to bridge light microscopy with molecular imaging techniques like NMR, X-ray diffraction and single particle electron microscopy. In cell biology, one goal, often referred to as visual proteomics, is the molecular mapping of whole cells. To achieve this goal and link cryo electron tomography to these high-resolution techniques, increasing the attainable resolution to 2–5 nm is vital. Here, we provide an overview of technical factors that limit the resolution in cryo electron tomography and discuss how during data acquisition and image processing these can be optimized to attain the highest possible resolution. Also, existing resolution measurement approaches and current technological developments that potentially increase the resolution in cryo electron tomography are discussed.

Introduction

Cryo electron tomography (cryo ET) is a three-dimensional imaging technique that is suitable for imaging biological structures with a resolution in the order of nanometres. With cryo electron tomography, the three-dimensional shape of single and unique structures, such as macromolecular complexes, pleomorphic viruses, small bacteria and slices or thin areas of cells can be visualized (Medalia *et al.*, 2002; Koning & Koster, 2009). Cryo electron tomography bridges the resolution gap between light microscopy and molecular imaging techniques, e.g. X-ray crystallography and single particle cryo electron microscopy (Figure 1). In fluorescence light microscopy, fluorescently labelled molecular structures can be localized with high resolution, though the information about underlying structural arrangements and the cellular context is limited.

A powerful emerging application of cryo electron tomography in the field of structural cell biology is the localization and direct visualization of vitrified macromolecular structures in a snapshot of their cellular context. In principle, this approach would enable the three-dimensional spatial mapping of molecules in the cell's interior. This application is sometimes referred to as visual proteomics (Nickell *et al.*, 2006). The basic idea is that available molecular models, which are determined by other techniques such as single particle electron microscopy, X-ray crystallography, NMR, are placed into the cryo electron tomogram of the cell. The correct spatial location and orientation of these molecular models in the cellular context is determined by computational procedures, some of which are referred to as template matching or docking. From both experimental and theoretical work it is concluded that the reliability and robustness of docking approaches becomes significantly higher when the resolution of the cryo electron tomograms is sufficient to provide unambiguous structural clues. Some experimental data have shown that a resolution of 0.8 nm is sufficient to resolve the presence of α-helices in cryo electron reconstructions providing sufficient information for

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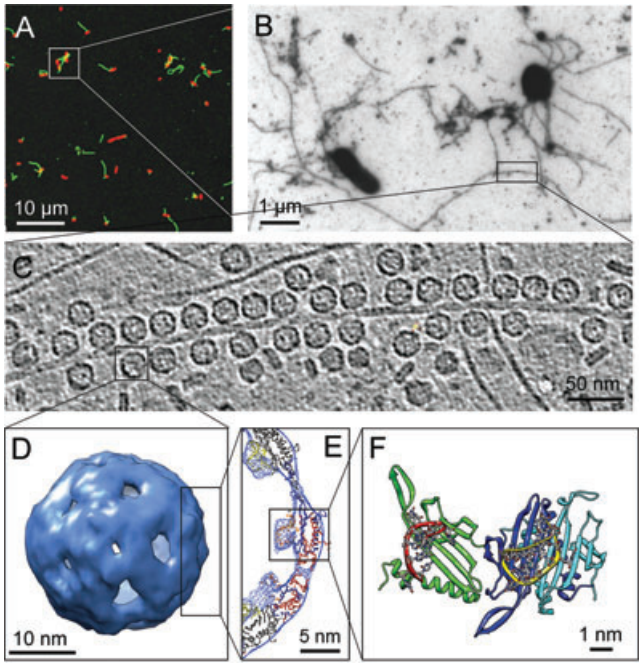

Fig. 1. How cryo electron tomography bridges the resolution gap between light microscopy and molecular imaging. A: Light microscopy image of fluorescently labelled *E.coli* cells (red) and pili covered with phages (green). From Daehnel *et al.*, 2005. B: Low-magnification TEM image of negatively stained *E.coli.* C: Virtual section through a cryo electron tomogram showing the F-pilus decorated with MS2 bacteriophages. D: Isosurface of the MS2 capsid after averaging of sub tomograms in C. E: Docking of MS2 capsid X-ray structure (PDB 1ZDI) into an icosahedral single particle reconstruction. F: Closer view of the crystal structure showing genomic RNA (red and yellow) connected to capsid protein.

high-resolution docking of macromolecules. Although that resolution cannot be obtained in cryo ET for most specimens, it is clear that technological developments are geared toward the highest possible resolution.

To obtain the highest possible resolution in electron tomography, it is essential to optimize the image acquisition parameters and image processing tools. In data acquisition, the most important factors are radiation damage, total electron dose, sample thickness and the tilt scheme that is used. Of the required tomographic image processing steps, the alignment of the tilt series is crucial, whereas CTF correction and noise filtering are powerful tools to increase the contrast in noisy tomographic reconstructions. High-resolution models of *in situ* macromolecular complexes can be obtained by sub-tomogram averaging of identical structures, implicitly increasing the signal-to-noise ratio by averaging. Current technological approaches that are undertaken that have a positive effect of the attainable resolution are dual-axis tilt cryo electron tomography, the use of direct electron detectors and the adaptation of the image formation process by incorporating phase plates. In order to assess the quantitative effects these methodological and instrumentation developments have on resolving structural features, it has become increasingly important to define a quantitative measure for the resolution of cryo electron tomograms and define a numerical indicator for their quality.

Here we give an overview of the parameters that affect resolution during data collection, point to some key topics in image processing and describe current approaches and methodologies to measure the resolution of cryo electron tomograms.

Parameters that influence the resolution during data acquisition

Radiation damage, total electron dose and magnification

The main resolution limiting factor in cryo electron tomography is the total electron dose that is used to acquire a tilt series. Increasing exposure of vitrified biological samples to the electron beam causes the breakage of covalent bonds that progressively leads to structural degradation. Therefore, the total electron dose that can be used for imaging is limited. Realizing that for high-resolution electron tomography the number of images composing the tilt series needs to be large to cover a wide angular range as finely as possible, the limited dose will have the inevitable consequence that the individual images in the tilt series are very noisy. For an optimal resolution, the total electron dose that is available to acquire a tilt series is a trade-off between an increase of the signal-to-noise ratio in the individual images and an increase of physical deterioration of the specimen.

The optimal magnification at which cryo electron tomograms are recorded is determined by the desired resolution. At low magnification the field of view is larger, and when the electron dose to which the specimen is exposed is kept constant more electrons hit the detector, resulting in a higher signal-to-noise ratio per pixel. The minimal magnification is determined by the required image resolution. The magnification should be chosen such that the pixel size on the specimen level is small enough to properly sample the desired structural detail that needs to be resolved. Lower magnifications will lead to resolution loss due to undersampling, whereas oversampling will result in increased beam damage without any increase in resolvable detail. It should also be taken into account that the modulation transfer function (MTF) of detectors falls off at high spatial frequencies and that it can be more effective to acquire images with the individual pixels of the camera software or hardware binned into larger pixels, e.g. that four pixels contribute to one binned pixel that result in a more favourable signal-to-noise ratio. In practice, the pixel size at the level of the specimen should be chosen at least 4× smaller than the desired resolution.

The sensitivity of biological specimens to the electron beam depends on several other factors. Lower accelerating voltages increase electron-specimen interactions, leading to

faster sample damage and simultaneously increase image contrast. To a minor extend, lower electron radiation dose rates, short exposure times and lower sample temperatures were reported to be advantageous for imaging. Also, biological structures with different composition and chemical bonds (e.g. proteins and RNA) can have different sensitivity to the electron beam, which can be observed by differences in radiation damage as a function of electron dose. In most cases, the total electron dose that is chosen depends on a combination of the biological structure and the desired resolvable details. To image structures that maintain their molecular integrity, the electron dose should not exceed 10^3 e/nm^{-2}. An electron dose in the order of 10^4 e/nm^{-2} can be used for imaging cellular structures, whereas 10^5 e/nm^{-2} can be used for membranous structures (Grimm *et al.*, 1998).

Sample thickness

The sample thickness is an important resolution determining factor in cryo electron tomography. Upon increasing sample thickness, the relative amount of inelastically scattered electrons–which do not contribute to the high-resolution information in the image of the sample but add to the noise level in the image–increases, leading to lower signal-to-noise ratios (Koster *et al.*, 1997). Therefore, the maximal achievable resolution in thick specimens is worse than in thin samples.

It is useful to express the sample thickness in terms of the mean free path of an electron transiting through the sample since it depends on the used acceleration voltage of the electron microscope and the material of the sample. The mean free path is the average distance between two consecutive scattering events of one electron. The mean free path of amorphous ice is about ∼200 nm for 120 kV, ∼270 nm for 200 kV and ∼350 nm for 300 kV. Note that upon tilting an electron microscopy sample, typically having a slab geometry, the effective sample thickness increases with 1/cos(tilt angle) and the thickness increases up to 3× at 70°. In practical terms, cryo electron tomography can be used for samples with a thickness up to 500 nm.

An electron energy filter can remove inelastically scattered electrons, allowing only elastically scattered electrons to pass through to the detector (zero-loss imaging). This effectively will increase the signal-to-noise level in the images. Zero-loss imaging is very useful in cryo electron tomography, especially when thick samples are imaged. Although in theory resolutions can be achieved ranging from 2 nm in samples that are not thicker than ∼100 and 10 nm in ∼500 nm thick samples, in practice the resolution appears to be a factor of 2–5 worse.

Data collection tilting geometry and number of images

The data collection tilting geometry is an important factor that influences the resolution. It has been determined that a minimal number of projection images is needed to fully sample an object in Fourier space to achieve a certain resolution (Crowther *et al.*, 1970). For noise-free images of a spherical sample the maximal resolution (r) that can be attained is determined by a combination of the sample thickness (D) and the angular tilt increment (α_0, in radians) and is given by $r = D\,\alpha_0$. In practice, for electron tomography the resolution will be worse since images of vitrified samples are noisy, samples have slab geometry and the effective thickness of the sample increases with tilt angle. To overcome this latter effect, tilt geometries with decreasing angular increments at higher tilts were developed (Saxton & Baumeister, 1984).

Since vitrified samples that have slab geometry are supported by a metal grid and mounted inside a cryo holder, they cannot be imaged over the full angular range, but instead are imaged over a smaller range, typically ranging from +60° to −60°. This implies that the object is not completely sampled in Fourier space and a missing data wedge exists. The combination of incomplete sampling and rotation along one axis results in an anisotropic resolution within the tomographic reconstruction, being worst in the direction of the electron beam and best along the tilt axis.

To reduce this anisotropy, a second tomographic tilt series can be recorded of the feature of interest along an axis perpendicular to the first tilt series. The combination of the two series reduces the missing data wedge in Fourier space into a missing data pyramid and improves the resolution in the direction orthogonal to the primary tilt axis. The application of dual tilt cryo tomography will require that the total electron dose per tilt series is halved. In addition, the quality of the combined tomogram will rely on how well the two tomograms can be combined (e.g. aligned to each other).

Getting the best resolution from image processing

Image alignment

The alignment of the individual images in the tilt series is crucial for a high quality three-dimensional reconstruction. Inaccurate alignment will result in blurring of features and in lower signal-to-noise levels and lower resolution of the final tomogram. The image contrast, generated by the electron-beam interaction with frozen hydrated cellular structures that are composed of light elements, in the individual images of a tilt series will be very low. Therefore, in many cases, to facilitate the alignment of the tilt series strongly electron-scattering nano-sized gold particles are added to the sample prior to vitrification, that are used as fiducial markers. Fiducials are generally used in cryo electron tomography and result in good alignments. The alignment and distortion corrections are performed by three dimensional modelling of the fiducial positions. The use of fiducials also allows refinement and correction of a variety of image distortions, including image rotations, changes in magnification and tilt angle. Also

fiducial-less alignment procedures are used that are based on cross-correlation between the individual images of the tilt series. It should be stressed that the fiducials, and not the sample itself, are used for the alignment and that their proximity to the reconstructed object is of influence to the alignment accuracy.

CTF correction

Image formation in cryo electron microscopy on frozen-hydrated biological specimens can be mathematically described by the contrast transfer function (CTF), which includes a number of parameters such as defocus, astigmatism, beam coherence and energy spread. This is of importance as in tomographic tilt series the defocus value varies largely, both within one (tilted) image and between the different images of the tilt series. The defocus will vary more in areas further away from the tilt axes, whereas on the tilt axis it will remain constant. The shape of the CTF depends on the actual defocus. Identical structural features can exhibit a different appearance when imaged at a different defocus. In most cases, lower resolution features are not so much affected. To recover higher resolution structural information correctly from the acquired images, it is essential to correct for the defocus-dependent contrast changing effects of the CTF (Fernández *et al.*, 2006). Therefore, the CTF correction process is very important for high-resolution imaging, e.g. when sub-tomogram averaging methods are applied.

Denoising/Filtering

Noise reducing filtering is frequently used in a cryo electron tomogram to increase the signal-to-noise ratio for visualization and segmentation purposes. Low pass filtering, for a frequency range higher than the attained resolution, can be applied to reduce the noise level in the tomogram for improved visualization of structural features. Furthermore, non-linear anisotropic diffusion filters are used that incorporate the local shape of the structural element in the reconstruction into account. Local features that show high contrast will be enhanced and areas that exhibit low contrast will be blurred. The extend of filtering depends heavily on the sample, the biological question and on the resolution of the tomogram. When used properly, this might lead to a slight improvement of resolution due to the increase of contrast, though comparison with the unfiltered tomogram remains essential to avoid drawing conclusions from filtering artefacts.

Subtomogram averaging

Although cryo electron tomography is most suitable for imaging unique structures, it can also be applied to samples that contain multiple copies of (nearly) identical sub-structures, e.g. ribosomes in a cell. The resolvable details of these sub-structures can be improved by averaging, which improves the signal-to-noise ratio and isotropy of the data and therefore the resolution. The resulting resolution depends on the number of particles and on the particle alignment and classification quality prior to averaging. For cryo electron tomography applications where sub-tomogram averaging is not envisioned, a relatively large defocus is chosen ($\sim$ 8 um) to emphasize the spatial frequency range of 4 nm and less. For sub-tomogram averaging in most cases a higher resolution is aimed for and a lower defocus value has to be chosen ($\sim$2 um). Low defocus values result in higher resolutions in non CTF corrected sub-tomogram averaged reconstructions.

Measuring the resolution in cryo electron tomograms

Despite much progress in cryo ET, quantitative assessment of the attained resolution in tomograms has remained a challenging issue. In electron tomography the resolution is not limited by the resolving power of the imaging system (the transmission electron microscope lenses and the imaging detector) but more by the nature and characteristics of the specimen, the signal-to-noise level, the precision and robustness of the alignment and sub-tomogram averaging procedures. Therefore in electron tomography, resolution measurements are not based on the Rayleigh criterion, as used in light microscopy, but rather are self-consistency measurements of noisy electron microscopy datasets. In addition, compared to resolution measurement approaches developed for single particle electron microscopy, the situation for electron tomography is less straightforward. One of the recurring limitations is that tomography data is non-redundant, since there is only one projection available per tilt angle. Another limitation is that the data is incomplete, due to the missing wedge and the limited amount of angular increments leading to both anisotropic data coverage and resolution in the dataset.

Several methods and theories have been put forward to assess the resolution in cryo electron tomography, and all have their pros and cons. Some theoretical approaches are based on the estimation of the spectral signal-to-noise ratio (Penczek, 2002; Unser *et al.*, 2005). In practice, there are two criteria that can be used to assess the quality of a tomographic reconstruction (Cardone *et al.*, 2005).

The first method computes the Fourier shell correlation of two tomograms that are generated from the even and odd split images from one tilt series ($FSC_{e/o}$). This method resembles the resolution measurements that are applied in single particle electron microscopy. The disadvantage is that only half datasets are used for comparison and hence the signal-to-noise ratio is decreased. When the number of projections is the resolution limiting factor, the resolution is underestimated with a factor of two.

The other method is the noise compensated leave-one-out (NLOO) method that compares one of the original images

from the tilt series with the appropriate reprojection from the tomogram that is computed from the remaining images of the tilt series, using Fourier ring correlation. Since the noise levels in one individual image from the tilt series is different from the calculated projection from the rest of the tomograms (minus the one image) the noise levels have to be compensated for this. When the tomograms are noise limited both methods are mutually consistent. However NLOO provides a more reliable criterion when the tilt increment becomes a significant factor. The NLOO is computationally more expensive than the $FSC_{e/o}$ but has the advantage that the resolution can be assessed as a function of the tilt angle.

The prospects of future instrumentation

Currently, two promising instrumentation developments that potentially can have a vast impact on the resolution in cryo electron tomography are direct electron detectors and phase plates. The detectors used on electron microscopes are CCDs (charge coupled devices) that detect photons, which are generated by electrons that hit a scintillator on top of the CCD. The detection of electrons is indirect and less efficient. Direct electron detectors detect electrons more efficiently and will improve the signal-to-noise ratio per image.

Another development is geared towards the incorporation of phase plates in electron microscopes to overcome the resolution limiting effects described by the contrast transfer function. Their implementation into cryo electron microscopy has been reported, but is not commercially available yet. A phase plate is a device that shifts the phase of the scattered electron beam compared to the unscattered beam. Consequently, the intensity of the image is related to the phase of the wave. As a result, with a phase plate incorporated, images can be recorded in focus, phase changes due to the defocus of the objective lens are absent, and the contrast transfer over the whole resolution range is more uniform. The use of phase plates in tomography EM will allow imaging at low defocus values without the need of CTF correction resulting in images that contain both high-resolution information as well as low-resolution information that is required for the alignment of a tilt series (Danev *et al.* 2010).

Furthermore, resolution improvements can also be obtained by new single particle reconstruction approaches for studying single particles using cryo electron tomography (Zhang & Ren, 2012) and by improving the yield of tomographic acquisition by software automation (Sulloway *et al.*, 2009) in combination with suitable high-end electron microscopes. The prospect of imaging biological specimens with a combination of an energy filter, direct electron detector and a phase plate with current high end electron microscopes provides a positive prospect on improving the resolution in cryo electron tomography that will result in sufficient structural information to dock high-resolution models of macromolecular structures within cryo tomograms of cellular structures.

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