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Scanning transmission electron microscopy through-focal tilt-series on biological specimens

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ABSTRACT

Since scanning transmission electron microscopy can produce high signal-to-noise ratio bright-field images of thick (≥500 nm) specimens, this tool is emerging as the method of choice to study thick biological samples via tomographic approaches. However, in a convergent-beam configuration, the depth of field is limited because only a thin portion of the specimen (from a few nanometres to tens of nanometres depending on the convergence angle) can be imaged in focus. A method known as through-focal imaging enables recovery of the full depth of information by combining images acquired at different levels of focus. In this work, we compare tomographic reconstruction with the through-focal tilt-series approach (a multifocal series of images per tilt angle) with reconstruction with the classic tilt-series acquisition scheme (one single-focus image per tilt angle). We visualised the base of the flagellum in the protist *Trypanosoma brucei* via an acquisition and image-processing method tailored to obtain quantitative and qualitative descriptors of reconstruction volumes. Reconstructions using through-focal imaging contained more contrast and more details for thick (≥500 nm) biological samples.

1. Introduction

In recent decades, tomographic approaches in transmission electron microscopy (TEM) have become invaluable tools for the structural study of biological specimens (Gan and Jensen, 2012; Harapin et al., 2013; Lucic et al., 2013), enabling global, three-dimensional understanding of cellular architecture and ultrastructure. In TEM, the spread electron beam cannot produce high signal-to-noise-ratio images of thick samples (≥500 nm) because electrons transmitted through various scatterings add important noise. Thus, sectioning thin cryopreserved or resin-embedded samples using an ultramicrotome or milling the sample using a focused ion beam (before placing the sample in the electron microscope) is required to circumvent this problem (Al-Amoudi et al., 2004; Muller-Reichert et al., 2010), limiting the application of TEM-based tomography to relatively thin samples.

In scanning transmission electron microscopy (STEM), the electron beam is focused to form a probe, allowing pixel-by-pixel scanning of the sample and enabling high contrast and high signal-to-noise-ratio images of thick specimens; a bright-field detector mainly captures unscattered electrons (Sousa et al., 2011; Sousa and Leapman, 2012). However, the depth of field of the STEM convergent electron beam is rather limited compared to that of the spread electron beam in TEM (Biskupek et al., 2010). Therefore, the image of a thick specimen is not entirely in focus, impeding the recovery of full-depth information.

Nevertheless, several studies have demonstrated the advantages of STEM over TEM for the analysis of thick samples (Aoyama et al., 2008 Wolf et al., 2014). In order to capture the full specimen depth, multiple images collected at various defocus values should be combined. Using through-focal imaging, additional in-focus information provided by the different focal planes in over-focused and defocused images is used to generate reconstructions that are more accurate than those obtained via single-focus acquisition (Hovden et al., 2011). Very recently, through-focal imaging and tomography have been combined to recover full 3D information for specimens at subnanometre resolution (Dahmen et al., 2014;

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Hovden et al., 2014). Although the acquisition methods used by the two groups were very similar, different algorithmic approaches for combining through-focal images were employed. Hovden et al. (2014) combined the region in focus in the original images and reconstructed the 3D volume using a parallel beam reconstruction algorithm, whereas Dahmen et al. (2014) took advantage of the complete through-focal dataset and reconstructed the 3D data using a convergent beam reconstruction algorithm. Although a parallel-beam projector does not perfectly reconstruct the 3D information coming from a convergent beam (because it considers pixel values to arise only from the central ray of the convergent electron beam; all other information is considered noise), for decades, many structural studies have been conducted using a convergent STEM beam and reconstruction algorithms based on parallel-beam projectors. This approximation is commonly used because it provides correct reconstructions and because algorithms that consider the geometry of the beam have only been developed recently. Thus, while use of a convergent beam projector leads to better reconstructed volumes with fewer projections, this approach is still not widespread.

In this work, we sought to characterise the advantages and limitations of through-focal tomographic reconstruction based on a STEM configuration similar to configurations used in published studies (a convergent-beam configuration). Precise comparison of acquisition methods and reconstruction algorithms must consider that: (i) images may not be from the same area of the sample; (ii) tilt angles can vary from one collection scheme to another; and (iii) the final alignment parameters of the tilt series are rarely the same. Therefore, we used a through-focal tilt-series acquisition procedure that intrinsically contained various possible acquisition schemes, as well as a data-processing methodology in which volumes reconstructed using different acquisition schemes could be directly compared pixel by pixel. For this purpose, we visualised the well-known structures of the base of the flagellum in the protist *Trypanosoma brucei*.

2. Materials and methods

2.1. Resin-embedded T. brucei cells

T. brucei cells derived from strain 427 were cultured in flasks in SDM79 medium supplemented with haemin and 10% foetal calf serum, as previously described (Brun and Schonenberger, 1979). The *FLAM3*^{RNAi} strain was used in non-induced conditions (Rotureau et al., 2014). *T. brucei* cells were fixed directly in the culture with 2.5% glutaraldehyde for 30 min, exhaustively rinsed in phosphate-buffered saline, post-fixed in 1% OsO_4 for 30 min, dehydrated in baths of increasing ethanol concentrations, and embedded in Epon (Lacomble et al., 2009). Sections with three thicknesses (250, 500, and 750 nm) were prepared using an ultramicrotome and were mounted on electron microscopy copper grids for observation.

2.2. STEM through-focal tilt series

Prior to image acquisition, we irradiated the specimens (~30 min depending on sample thickness) in order to prevent shrinkage and drift during data collection. Tomographic tilt series were acquired using the bright-field STEM detector (camera length: 60 cm; magnification: 150,000×; probe size: 1.5 nm; convergence semi-angle of the beam: 25 mrad; collection semi-angle of the detector: 6.667 mrad) on a JEOL 2200FS field emission gun 200 kV electron microscope (JEOL LTD®) following a Saxton scheme (Saxton et al., 1984) from −70° up to 70° with tilt increments varying between 1° (at the highest tilt angles) and 2° (at the lowest tilt angles around 0°). For clarity, a series of five images (with a defocus range from −300 nm to +300 nm using 150-nm increments) were acquired at each tilt angle, giving rise to five brackets (JEOL denomination) on the Recorder software (JEOL Ltd.®). With these microscope settings, we found that a total defocus range of 600 nm by 150-nm increments was sufficient to cover the total thickness of the sample. Dynamic focus would have been useful to minimise the number of collected images and to reduce the final acquisition time; however, modification of the focus value would have generated distortions in the collected images, preventing accurate alignment of tomographic datasets.

2.3. Data processing

In order to process data from a through-focal tilt series, two alignment steps that are not necessary in a classic tomography reconstruction workflow must be performed: (i) alignment of the through-focal images collected at the same tilt angle and (ii) combination of the aligned through-focal images in order to discriminate the information in focus from the information out of focus. ImageJ (Schneider et al., 2012) plugins Turboreg (Thevenaz et al., 1998) and Extended Depth of Field (EDF) (Forster et al., 2004) both yielded very satisfactory results for accurately aligning images collected at the same tilt angle and for combining the information at focus for the five aligned images. EDF provided the highest fidelity (visually assessed by the preservation of structural details after combination) compared to two other algorithms implemented as ImageJ plugins: Stack Focuser (Umorin, 2006) and Depth From Focus (Ferreira de Lucena and Rogerio de Oliveira Hein, 2010). To process the through-focal tilt series, alignment and focus combination were automated using a macro based on the Turboreg and EDF ImageJ plugins (Supplementary Fig. 1).

Image processing (Fig. 1) consisted of the following workflow. First, two steps were carried out: (i) correction of small XY shifts induced by focus changes (from −300 nm to +300 nm) during the collection of the five through-focal images at the same tilt angle (bracket 3, which was collected at 0 nm defocus, was used as a reference for this alignment step), and (ii) combination of the regions in focus in these shift-corrected images to compute a single-focus combined image at each tilt angle (Supplementary Fig. 2). Second, final tilt-series alignment was computed from these combined images. The use of this combined tilt series was very important because alignment of out-of-focus images cannot be accurate enough for sharp 3D reconstruction. Fine XY shifts and in-plane rotation alignments were performed using the local minima algorithm of TomoJ v2.28 (Messaoudii et al., 2007; Sorzano et al., 2009). Third, final alignment parameters (fine XY shifts and in-plane rotation) were imposed on the images and 3D reconstruction was achieved with the OS-SART-GPU module (iterations: 100; relaxation coefficient: 0.01; volume update: every four images; parallel illumination projector) of TomoJ v2.28.

2.4. Comparison of tomographic acquisition schemes

Our data-processing procedure was designed so that the through-focal dataset intrinsically contains various tilt-series acquisition schemes: (i) the through-focal scheme, with five through-focus images per tilt angle (similar to Dahmen et al., 2014); (ii) the combined scheme, which combines the information in focus from the five through-focus images (similar to Hovden et al., 2014); and (iii) the classic scheme, with a single image per tilt angle (the 0-nm defocus image represented by the third bracket). In our process, all bracket images from the same tilt-angle were aligned using the 0-nm defocus image as reference. Therefore, the classic, combined, and through-focal tilt series have exactly the same alignment parameters and 3D reconstructions of the three