Video Article

Preparation and Observation of Thick Biological Samples by Scanning Transmission Electron Tomography

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Abstract

This report describes a protocol for preparing thick biological specimens for further observation using a scanning transmission electron microscope. It also describes an imaging method for studying the 3D structure of thick biological specimens by scanning transmission electron tomography. The sample preparation protocol is based on conventional methods in which the sample is fixed using chemical agents, treated with a heavy atom salt contrasting agent, dehydrated in a series of ethanol baths, and embedded in resin. The specific imaging conditions for observing thick samples by scanning transmission electron microscopy are then described. Sections of the sample are observed using a through-focus method involving the collection of several images at various focal planes. This enables the recovery of in-focus information at various heights throughout the sample. This particular collection pattern is performed at each tilt angle during tomography data collection. A single image is then generated, merging the in-focus information from all the different focal planes. A classic tilt-series dataset is then generated. The advantage of the method is that the tilt-series alignment and reconstruction can be performed using standard tools. The collection of through-focal images allows the reconstruction of a 3D volume that contains all of the structural details of the sample in focus.

Video Link

The video component of this article can be found at https://www.jove.com/video/55215/

Introduction

Since the early 1970s, tomography approaches in transmission electron microscopy (TEM) have been widely used for the structural characterization of biological specimens[1,2,3]. The appeal of transmission electron tomography was that it could be used to study a wide range of biological structures at the nanometer scale, from the architecture of cells and the ultrastructure of organelles to the structure of macromolecular complexes and proteins. Nevertheless, transmission electron tomography could not be used to study very thick samples (greater than 0.5 µm). Indeed, thick specimens produce too many scattered electrons, generating low signal-to-noise ratio (SNR) images. In addition, tomography involves the collection of images of tilted specimens, with the apparent thickness of the sample increasing with the tilt angle. Even though inelastic scattering can be filtered out using energy filters, the critical amount of electrons needed for high SNR images is barely reached in TEM. Hence, thick biological specimens have only been studied using sectioning[4].

Some samples cannot be sliced: some might degrade when they are cut, and others need to be studied in their entirety in order to understand their complexity. An alternative approach is to use TEM in scanning mode[5,6,7,8]. In scanning transmission electron microscopy (STEM), the optical path of the electrons is different from that in conventional TEM. Electrons passing through the sample without scattering can be collected on the optical axis with a bright-field (BF) detector[9], whereas those scattered elastically can be collected at a certain angle from the optical axis with a dark-field (DF) detector. The other advantage of STEM is that the focused electron beam is scanned at the surface of the sample, enabling the pixel-by-pixel collection of the images. Even though the electron beam broadens while passing through the sample[10], this particular collection scheme is less sensitive to inelastically scattered electrons than conventional TEM. Furthermore, there is no lens post-specimen in STEM, thereby avoiding the chromatic aberrations that can occur in TEM. The camera length can be adjusted so that the BF detector mainly detects unscattered electrons. Using the DF detector to study thick samples is not recommended because of multiple scattering, which produces inaccurate images. Instead, the BF detector can be used[11]. While STEM can produce high SNR images, it has a relatively low depth-of-field because of the relatively high beam convergence compared to TEM, decreasing the amount of in-depth information that can be recovered from thick specimens. In the case of aberration-corrected STEM microscopes, where the convergence angle can be as high as 30 mrad, the depth-of-field can be low enough so that the information that is in focus originates from a focal plane of only a few nanometers. Setting up the electron beam in parallel mode enhances the depth-of-field of the electron beam to the detriment of the resolution[12]. However, this setup is not always possible.

Whenever it is necessary to use a convergent electron beam, one must use techniques that enhance the depth-of-field of the electron beam when studying thick samples. Recent studies have reported the acquisition of multiple images at various focal planes throughout the sample to

recover the maximum amount of in-focus information[13,14]. The two studies describe different ways to handle the information from the different focal planes. Hovden *et al.* combined in Fourier space the images that were collected at various focal planes, and the final reconstruction was obtained directly from the 3D inverse Fourier transform[13]. In contrast, Dahmen *et al.* developed a convergent beam reconstruction engine to reconstruct in real space the 3D volume from the various focal planes[14]. Our laboratory also developed a method for imaging thick biological specimens. Our strategy was different from the two methods described above in that we merged the information that is in focus in the various focal planes and reconstructed the final 3D volume in real space using a parallel beam projector[15]. Our aim was to develop a method that can easily be performed in any electron microscopy laboratory. To this end, we aimed to collect the focal images in a limited amount of time, comparable to the time frame of conventional tomography experiments. In addition, our proposed method could be adapted for use with different types of alignment and reconstruction software.

In the context of our publication from 2015[15], we wanted to visualize and characterize the recovery of the depth-of-field, so we used a large convergence semi-angle of 25 mrad. Here, we present a step-by-step protocol for performing through-focal imaging in STEM according to the method developed in our laboratory in 2015[15], and we present how the data from 2015 was processed. This method recovers in-focus information from several focal planes throughout a thick (750 nm) biological sample and enables high-quality 3D reconstructions. Where relevant, the differences in this methodology versus the methods used by other groups are also presented.

Protocol

Caution: Consult the material safety data sheets (MSDSs) of the various reagents before using them. Several of the chemicals used during sample preparation are toxic, carcinogenic, mutagenic, and/or reprotoxic. Use personal protective equipment (gloves, lab coat, full-length pants, and closed-toe shoes) and work under a fume hood while handling the sample. Sectioning the sample with an ultramicrotome involves the use of sharp instruments, and careful use of these tools is mandatory. In the steps described below, the authors assume that the sample is a cell culture sample. The protocol may need to be modified according to the sample type, especially the centrifugation steps.

1. Preparation of Buffers and Mixtures

1. Prepare phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4).
2. Prepare solutions containing 30%, 50%, and 70% ethanol in PBS for sample dehydration. Perform sample dehydration by gradually incubating the sample in increasing ethanol concentrations.
3. Prepare epoxy resin by mixing bisphenol-A-(epichlorohydrin) (20 mL), dodecenyl succinic anhydride (9 mL), methyl-5-norbornene-2,3-dicarboxylic (11 mL), and 2,4,6-tris(dimethylaminomethyl)phenol (0.7 mL); other mixing ratios can be used depending on the desired hardness of the resin.
4. Prepare solutions containing 30%, 50%, and 70% epoxy resin in ethanol for sample embedding. Perform resin embedding by gradually incubating the sample with increasing resin concentrations.
   NOTE: The volumes of the prepared solutions depend on the type of sample that is studied. More solutions are needed for tissues than for cell cultures.

2. Fixation and Coloration of the Sample

1. Centrifuge the cell culture for 5 min at 5,000 x g. Perform all of the following centrifugations using the same conditions.
2. Discard the supernatant and fix the cell pellet by resuspending it in PBS (1 mL) with 4% paraformaldehyde and 2% glutaraldehyde. Incubate it at room temperature for 30 min (**Figure 1A**). Alternatively, fix the cells at 4 °C overnight or over the weekend.
3. After centrifugation, remove the supernatant and wash the pellet 3 times with PBS (1 mL) to completely remove the fixative agents.
4. Post-fix the sample with 1% OsO_4 in PBS (1 mL) for 30 min at room temperature.
5. After centrifugation, remove the supernatant and wash the pellet 3 times with PBS (1 mL) to completely remove the OsO_4. Deactivate the OsO_4 in oil and discard.
6. Add 2% uranyl acetate in PBS (1 mL) as a contrast agent and incubate the sample for 30 min at room temperature (**Figure 1B**).
7. After centrifugation, remove the supernatant and wash the pellet 3 times with PBS (1 mL) to remove the unbound uranyl acetate; uranyl acetate contains radioactive elements and should be disposed of in a bin dedicated to radioactive waste.
   NOTE: The incubation times can be modified according to the size of the sample. For example, fixation and staining of thicker samples, such as tissues samples, require longer incubation times. Longer incubation times may also be better for the dehydration and the embedding steps. Uranyl acetate can be replaced by a "uranyl-less" solution that contains Gadolinium salts.

3. Sample Dehydration

1. After centrifugation, discard the supernatant and incubate the sample for 30 min at 4 °C in PBS (1 mL) with 30% ethanol (**Figure 1C**).
2. Repeat step 3.1 using solutions with gradually increasing ethanol concentrations (50%, 70%, and up to 100%). During these dehydration steps, maintain the sample at 4 °C for sample preservation purposes.
3. After centrifugation, discard the supernatant and resuspend the pellet in pure ethanol (1 mL); incubate overnight at 4 °C.

4. Resin Embedding

1. After centrifugation, discard the supernatant and resuspend the pellet in ethanol with 30% epoxy resin (1 mL) for 30 min at RT (**Figure 1D**).
2. Repeat step 4.1 using solutions with gradually increasing epoxy resin concentrations (50%, 70%, and up to 100%).
3. After centrifugation, discard the supernatant and resuspend the pellet in pure epoxy resin (1 mL); incubate overnight at RT. The presence of hardening DMP-30 might trigger the polymerization of the resin; if this happens, prepare two batches of epoxy resin, one with and one without DMP-30.

4. After centrifugation, resuspend the pellet in 200 µL of pure epoxy resin (containing the hardener) in a plastic capsule; the sample should be at the bottom of the plastic capsule.

5. Resin Polymerization

1. Incubate the plastic capsule containing the sample in an incubator set at 60 °C for 48 h; other kinds of resin might polymerize with different settings.
2. Remove the capsule from the incubator and verify that the resin has polymerized; the resin should be hard if pressed against a hard surface.
3. Add pure epoxy resin (containing the hardener) on top of the polymerized resin containing the sample; this extra layer of resin will make it easier to cut the sample. Incubate the capsule at 60 °C for 48 h.

6. Sample Sectioning

1. Mount the sample upside-down in a specimen holder so that the sample is towards the top and fix it on the trimming block of the ultramicrotome. Tightly lock the lever to secure the trimming block to the ultramicrotome.
2. Using a razor blade or a similar sharp cutting instrument, cut out the plastic capsule to separate it from the polymerized resin. Cut the resin so that the sample is contained in resin in roughly the shape of a pyramid. There is no need to remove the entire plastic capsule; only remove the part covering the polymerized sample.
3. Take the capsule and the specimen holder off of the trimming block and install them on the moving arm of the ultramicrotome.
4. Install a trimming knife on the knife block and accurately trim the faces of the pyramid. The use of binoculars is advised to give a perfect shape to the pyramid; the better the pyramid, the better the sections will be.
5. Take off the trimming knife and replace it with a histology knife that can be used to cut sections that are thicker than 0.5 µm.
6. After filling the cuvette of the histology knife with the correct amount of water, bring the cutting edge of the knife to the surface of the sample and use the binoculars to adjust the capsule pitch angle so that the knife will slice perpendicularly to the pyramid.
7. Adjust the cutting speed according to the desired section thickness to recover homogeneous sections.
8. Let the section float over the water and fish it with a loop.
9. Move the loop towards an electron microscopy copper grid that has been placed on top of filter paper.
   NOTE: The grid should have been treated previously to increase its hydrophilicity. This can be performed with a grid-coating pen.
10. Due to water absorption by the filter paper, let the section stick to the grid. Let it dry for few minutes before inserting it into the electron microscope.

7. Design of the Through-focal Tilt-series

1. Collect through-focal images at constant focus intervals.
   NOTE: The through-focal tilt-series consist of a set of through-focal images that are collected at each tilt angle of the tilt-series. The whole focus amplitude should be sufficient to cover the whole sample thickness.
2. Determine the value of the focus interval. If the interval is equal to the depth-of-field, collect the entire sample in focus; this setting represents the highest sampling possible. If the interval is greater than the depth-of-field, some parts of the sample will not be acquired in focus, meaning that some parts of the sample will not be collected in focus.
3. Calculate the depth-of-field of an electron beam from the electron's wavelength and the convergence semi-angle of the electron beam. Whereas the wavelength of electrons is directly linked to the acceleration voltage, use the convergence semi-angle provided by the electron microscope manufacturer or determine it experimentally from the diffraction pattern of a known specimen[16]. Because electrons accelerated at a wavelength λ hit the sample with a convergence semi-angle of α in an electron microscope, use the following equation to determine the depth-of-field (DOF):

$$DOF = 0.61 * \frac{\lambda}{\alpha^2}$$

4. Design the focal series so that the whole focus amplitude will cover the sample at its maximal apparent thickness (*i.e.,* at its highest tilt).
   NOTE: A 0.75 µm-thick sample will have an apparent thickness of 2.19 µm at a 70° tilt, so the focus amplitude should be greater than 2.19 µm. For this sample, if the focus interval determined above is equal to 50 nm, it will require 44 images (2.19 µm/50 nm) to cover the sample at its maximum apparent thickness. The whole focus amplitude is equal to the value of the focus interval multiplied by the number of focal steps. At the highest tilt (θ), the sample apparent thickness is defined as the following:

$$sample\ apparent\ thickness = nominal\ thickness * \frac{1}{\cos(\theta)}$$

8. Sample Imaging

NOTE: It is beyond the scope of this protocol to describe how to set up an electron microscope in STEM mode; most of this information can be found in the reference manual provided by the electron microscope manufacturer. However, note the following: i) the spot size should be chosen according to the desired magnification; ii) the Ronchigram must be well-aligned; and iii) in BF mode, the camera length must be adjusted to select unscattered electrons while maintaining good illumination of the detector. Resin sections deposited on electron microscopy copper grids should be illuminated before image acquisition to prevent shrinkage. Resin shrinkage and charging effects might be confounded during image acquisition. The use of an objective lens aperture might improve sample stability when charging effects occur. However, in the specific case of STEM setups, the objective aperture is not compatible with HAADF imaging mode, and very small objective apertures are not compatible with BF imaging mode.

1. Using low magnification (around 20,000X in STEM), browse through the sample and locate the region of interest.
2. Adjust the eucentric height (Z-axis) so that the region of interest does not drift away while rotating the sample.
3. Verify that the object of interest will remain visible throughout the entire tilt range.
4. Because of the substantial number of images acquired during a through-focal tilt-series, use an automatic collection software. Either use a commercial solution or design a custom data collection software if desired. Ensure that the software program is able to perform the three following steps:
    1. Track and focus as in a standard tilt-series collection scheme.
    2. Ensure that the through-focal images overlap with the Z-position of the sample.
    3. Automatically acquire a series of through-focal images at each tilt angle.
5. Give the software program the main parameters of the through-focal tilt-series (*i.e.,* focal intervals, focal step, minimum tilt angle, maximum tilt angle, tilt step, and scanning speed).
6. Start the image collection process and wait for the software program to finish.

9. Image Processing

NOTE: In this protocol, the through-focal tilt-series processing is performed using ImageJ plugins[17]. Supplementary Data 1 shows an ImageJ macro for the alignment step (Turboreg) and for merging in-focus information (Extended Depth of Field) for a through-focal tilt-series designed with three focus values.

1. Align the images collected at the same tilt angle (*i.e.,* the through-focal images). Since images do not have the same in-focus information, they do not harbor the same features for alignment; perform alignment by aligning neighboring images two by two (*i.e.,* following a pyramidal hierarchy, with the closest focus values first) using Turboreg[18], an ImageJ plugin.
2. Consistently verify the alignment of the through-focal images at each tilt angle. Stack the aligned through-focal images to improve visual inspection.
   NOTE: Only the zone that is in focus should move while browsing through the stack. Accurate alignment is the most important thing, since the following step consists of the recovery of the information that is in focus from different images.
3. Merge the information that is in focus using Extended Depth of Field[19], an ImageJ plugin; this will eventually generate a single image from the stack of images. Several settings can be used during the depth-of-field recovery, but use the highest settings to preserve the image information.
   NOTE: The tilt-series is now composed of a single image per tilt angle, each image containing the in-focus information recovered from the through-focal images.
4. Process the data.
   NOTE: Standard tools for alignment and reconstruction can be used since the data has now been reduced to a standard tilt-series. In this protocol, TomoJ is used for data alignment and data reconstruction[20,21]. More details about how to use TomoJ can be found in the original publication[22].
5. Perform fine alignment of the tilt-series.
   NOTE: A good strategy is to use gold beads as fiducial markers to accurately track shifts between images. Local minima can also be used if gold beads cannot be added to the sample.
6. Perform a 3D reconstruction of the aligned data; several algorithms are available for performing the reconstructions in real space or Fourier space, each with advantages and limitations.
7. If the final alignment of the tilt-series is poor, make some improvements by optimizing the initial steps. Reiterate the alignment steps if the final 3D reconstruction appears blurred or deformed; in practice, the greater the number of collected focal planes, the better the contrast and details of the 3D reconstruction.
   NOTE: Several software programs can be used to process the through-focal tilt-series. However, the tools used in this protocol were chosen because, in our experience, they performed the best. Turboreg[18] performed better in terms of aligning the images when a gradient of focus was present. More information can be found on the dedicated website[23]. The Extended Depth of Field plugin[19] performed very well in terms of recovering the in-focus information from different images, whereas other tools resulted in visible pixels modifications. More information, especially regarding script writing, can be found on this dedicated website[24].

Representative Results

In our study, electrons were accelerated to 200 kV in the field emission gun transmission electron microscope. Images were collected in STEM BF mode using a 20-µs dwell time. Regarding the design of the through-focal tilt-series, we found that focus intervals of 150 nm gave satisfactory results for the ultrastructural study of a 750 nm-thick biological sample-even though the depth-of-field of the electron beam was only 3 nm-since we used a very large convergence semi-angle of 25 mrad.

The sample analyzed in this report is *Trypanosoma brucei*, a unicellular eukaryote whose flagellum is intensively studied because this organelle is remarkably conserved from protists to mammals[25]. Cells were treated as presented in the sample preparation protocol, and we focused the structural analysis on the flagellar pocket of *T. brucei* (**Figure 2A**). When the sample is highly tilted (70° tilt) in the electron microscope, the collection of a series of images at various focus values (as it is performed during through-focal imaging) induces a shift in the part of the sample that is in focus (**Figure 2B**, **C**, and **D**). On the collected images, the zone that is in focus appears thin, and most of the image is blurred because of the limited depth-of-field. The merging of the in-focus information is performed on the collected images and produces a single image, where the in-focus zone is much wider (**Figure 2E**).

The merging of the in-focus information can be better visualized by highlighting the high frequency areas of the through-focal tilt-series images (white pixels in **Figure 2F**, **G**, and **H**). The ImageJ command "Find Edges" was used in the present study. This command uses a Sobel filter to detect changes in neighboring pixel intensities. The displacement of the detected high-frequency area can be observed from one image to the other. On the image where the in-focus information has been merged, the high frequencies span most of the image (**Figure 2I**). This method permits the verification of the good processing of the through-focal tilt-series.

Using through-focal tilt-series, extra in-focus information is collected and used during the 3D reconstruction process. This produces greater contrast and SNR and reduces the blurring effects observed in the 3D reconstructions of depth-of-field-limited images[15]. In Fourier space, extra information is recovered compared to a classic tilt-series collection scheme, where only on the focal plane is collected[13]. Overall, the quality throughout the whole 3D reconstruction is more isotropic compared to a classic tilt-series collection scheme.

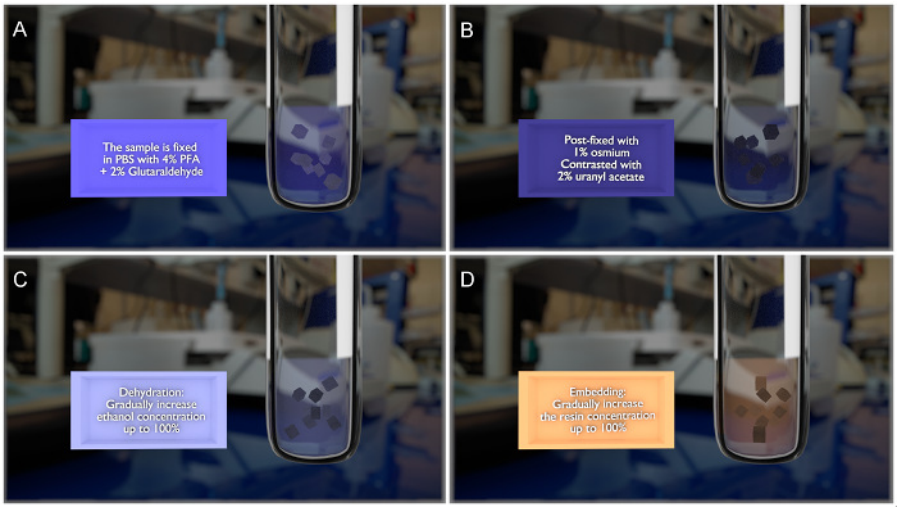

Figure 1: Images presenting the different steps of the biological sample preparation protocol. The sample preparation protocol can be divided into four mains steps. The sample is **A**) fixed in paraformaldehyde and glutaraldehyde, **B**) post-fixed in osmium tetroxide and contrasted with uranyl acetate, **C**) dehydrated in ethanol, and **D**) embedded in resin. Please click here to view a larger version of this figure.

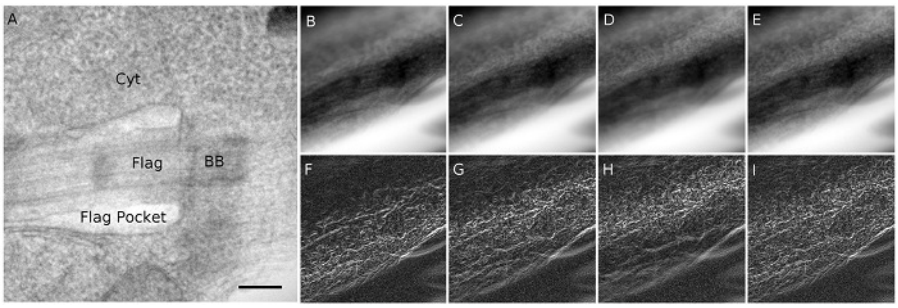

Figure 2: Verification of the depth-of-field recovery. The recovery of the depth-of-field can be verified on images of a tilted specimen. **A**) At 0° tilt, several details can be observed in the flagellar pocket (Flag pocket) of the resin-embedded *T. brucei* cell. The flagellar pocket is located in the cytoplasm (Cyt), and the basal-body (BB) anchors the flagellum (Flag) to the flagellar membrane. **B**, **C**, and **D**) Three images of the same flagellar pocket, tilted at 70° in the electron microscope, were acquired. They were collected with focus intervals of 300 nm. **E**) This image was generated by the Extended Depth of Field ImageJ plugin and contains the in-focus information of B, C, and D. **F**, **G**, **H**, and **I**) These images were computed with the "Find Edges" command in ImageJ in order to highlight the high-frequency information contained in B, C, D, and E, respectively. White pixels represent high frequencies, corresponding to in-focus information. The scale bar is 250 nm. Please click here to view a larger version of this figure.

Supplemental File 1. Please click here to download this file.

Discussion

In this article, we present a conventional sample preparation protocol along with a step-by-step guide for performing 3D analysis on thick biological samples using STEM through-focal tomography. Resin embedding of biological specimens has been used for decades[26], and alternative protocols that are adapted to different kinds of samples can be found throughout the literature[27]. In contrast, imaging thick specimens in STEM using through-focus methods is a new undertaking, and the methods will most likely be improved as it becomes more widespread.

The aim of this work was to describe a sample preparation protocol and, more specifically, imaging conditions for performing through-focal tilt-series acquisition in STEM with equipment that is currently available and in a reasonable amount of time. For this reason, large focus intervals were used. Indeed, there is no consensus about how close the focal planes should be for imaging a thick specimen with STEM for through-focal tilt-series. It usually takes up to several seconds to collect STEM images, and an entire conventional tilt-series can take hours to complete. As a proof-of-concept, it is possible to collect hundreds or even thousands of images. However, it is clear that long collection times are not suited for

everyday use. Beam stability and sample drift must be taken into account when designing such long experiments. Further, long collection times raise the question of accumulated electron dose. Thus, for the time being, STEM through-focal tilt-series is only suitable for the study of beam-resistant samples, such as resin-embedded biological samples or inorganic materials, mainly because of the substantial electron dose required. Because of the trade-off between acquisition time and quality of the final 3D reconstruction, we recommend using few through-focal intervals in projects where high resolution is not required, and we recommend using a greater amount of through-focal intervals in projects where high resolution is required.

There is a lack of consensus about the specific steps in this method because of its novelty. However, it is clear that several steps are critical for high-quality reconstructions from STEM through-focal tilt-series datasets. First, the alignment of the focal images at each tilt angle needs to be carefully executed, as pointed out in the three original articles describing through-focal image collection[13,14,15]. Even though images collected at various focal values do not share common information, Turboreg remains a good solution for the accurate, automatic alignment of images. Second, the merging of the focal images, as performed in this protocol, has the advantage of generating a single image per tilt angle, which is easily handled by any tilt-series alignment and 3D reconstruction software program. The Extended Depth of Field plug-in was initially designed for light microscopy images; nevertheless, it appears to be the best solution for merging the in-focus information from electron micrographs[28]. An alternative solution is to consider the entire set of images as a single tilt-series dataset with several images per tilt angle, similarly to what was done by Hovden *et al.*[13] and Dahmen *et al.*[14]. This requires the use of Fourier-based reconstruction algorithms[13] or Ettention, which was developed by Dahmen *et al.*[29] and which is the only 3D reconstruction software that can reconstruct 3D volumes from images collected at various focal values in real space. Notably, such alternative solutions will necessitate an extra alignment step in the focal direction (Z-axis), which might produce confusing results that are inconsistent with the defocus intervals chosen during image collection, as discussed in Dahmen *et al.*[14]. The merging of the in-focus information, as presented in this protocol, has the advantage of generating an image as if the data were collected using a parallel, thereby avoiding this extra alignment step in the Z-direction.

Through-focal tilt-series methods have been developed to study thick samples (around 1 µm). The main limitation of these methods is that they cannot be used to study samples thicker than several microns, since the electron beam cannot penetrate such samples. This limitation comes from the mean free path of the electrons, which is dependent on the acceleration voltage. Even though very high-voltage *(i.e.,* 1,000-kV) electron microscopes can be used for imaging eukaryotic cells[30], very thick samples still require the use of other techniques, such as focused ion beam or serial block-face scanning electron microscopy[31,32]. However, these methods generate anisotropic reconstructions that can be considered stacks of images because the Z-axis, which is defined as the cutting depth of the method, is not equivalent to the X- and Y- axes. These techniques cannot be used for high-resolution studies.

In 2014 and 2015, three original articles reported different methods for imaging thick samples[13,14,15]. Since then, only a few studies have used any of these methods to study thick samples[33]. Improvements in the methods will help to generalize through-focal imaging in STEM. Particularly in life sciences, through-focal methods need to be optimized for observing samples under cryogenic conditions. This is a major challenge, since cryogenic samples are particularly sensitive to electron dosage, and through-focus methods require many images.

Disclosures

We have no conflict of interest to disclose.

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