Optimized cryo-EM data-acquisition workflow by sample-thickness determination

Jan Rheinberger, Gert Oostergetel, Guenter P. Resch and Cristina Paulino*

*Department of Structural Biology and Membrane Enzymology at the Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands, and Nexperion e.U. – Solutions for Electron Microscopy, Vienna, Austria. *Correspondence e-mail: c.paulino@rug.nl

Sample thickness is a known key parameter in cryo-electron microscopy (cryo-EM) and can affect the amount of high-resolution information retained in the image. Yet, common data-acquisition approaches in single-particle cryo-EM do not take it into account. Here, it is demonstrated how the sample thickness can be determined before data acquisition, allowing the identification of optimal regions and the restriction of automated data collection to images with preserved high-resolution details. This quality-over-quantity approach almost entirely eliminates the time- and storage-consuming collection of suboptimal images, which are discarded after a recorded session or during early image processing due to a lack of high-resolution information. It maximizes the data-collection efficiency and lowers the electron-microscopy time required per data set. This strategy is especially useful if the speed of data collection is restricted by the microscope hardware and software, or if microscope access time, data transfer, data storage and computational power are a bottleneck.

1. Introduction

In single-particle cryo-electron microscopy (cryo-EM), the thin layer of vitreous ice embedding the protein or macromolecule complex of interest is a key parameter in sample preparation and optimization. Obtained by rapidly plunge-freezing grids with holey support films into liquid coolant (Adrian et al., 1984), it preserves the structural integrity of the macromolecular complex of interest. The thickness of the vitreous ice layer has an impact on electron transparency and image formation and is a key criterion of image quality. Thicker layers experience a defocus gradient, which leads to a stronger dampening of higher frequencies, limiting the resolution of the final reconstruction obtained (Wu et al., 2016). Conversely, an ice layer that is too thin might not be sufficient to fully embed the target protein or molecule of interest. This might lead to its denaturation at the air–water interface, or push it towards the edge of the support film hole, limiting the number of copies in the field of view (Noble et al., 2018; D’Imprima et al., 2019). The importance and urgency of the optimization of cryo-EM sample preparation is reflected by the development of new techniques and devices in recent years (Dandey et al., 2018; Rubinstein et al., 2019; Ravelli et al., 2020; Tan & Rubinstein, 2020; Arnold et al., 2017; Kontziampasis et al., 2019; Mæots et al., 2020).

Determining the thickness of a specimen is nothing new and has been described previously (Cho et al., 2013; Yan et al., 2015; Rice et al., 2018; Suloway et al., 2005). Yet, it has been mostly calculated on high-resolution images and used to monitor sample thickness during an ongoing data collection or to select micrographs based on their sample thickness after
data acquisition. In contrast, our work focuses on using sample-thickness determination at low magnification to set up automated thickness-based hole-targeting, restricting data collection to optimal regions, similar to what is available in Leginon (Suloway et al., 2005; Cheng et al., 2021). Here we have successfully integrated this approach, available as open source, in the data-acquisition software packages SerialEM (Mastronarde, 2005; Schorb et al., 2019) and EPU (Thermo Fisher Scientific), and have tested it on 200 and 300 kV high-end electron microscopes, making this workflow now available for the vast majority of software packages used.

As previously described and implemented in Leginon (Rice et al., 2018; Suloway et al., 2005), two fundamentally different approaches to estimating the thickness from projection images exist, which depend on the configuration of the microscope. In the case of a microscope equipped with an energy filter, one can benefit from the thickness-dependent number of inelastic scattering events. Due to the energy loss, these electrons will be removed by the filter when operated in zero-loss mode.

The thickness $D$ is proportional to the natural logarithm of the ratio between the mean intensity without ($I$) and with the slit inserted ($I_a$):

$$D = \lambda_{\text{inel}} \ln \frac{I}{I_a} - C. \quad (1)$$

The apparent mean free path $\lambda_{\text{inel}}$ acts as a scaling factor and describes the average distance that electrons travel through the sample before an inelastic scattering event occurs. The correction term $C$, which was not present in the original description (Rice et al., 2018), is used by the scripts presented in this work to set the measurements in a hole over vacuum to zero.

For the aperture-limited scattering (ALS) method (2), the sample thickness can be determined by comparing the mean intensity over vacuum ($I_0$) with the mean intensity over ice ($I$), utilizing the effect that the objective aperture removes part of the elastically scattered electrons. The number of scattered electrons is again proportional to the thickness (and content) of the sample (Angert et al., 1996; Cho et al., 2013; Rice et al., 2018). Here, instead of the mean free path, a scaling factor termed the ALS coefficient $\lambda_{\text{ALS}}$ is used, which equally depends on the acceleration voltage, the objective aperture size, the sample content (Rice et al., 2018) and the imaging mode (LM versus M/SA),

$$D = \lambda_{\text{ALS}} \ln \frac{I_0}{I} \quad (2)$$

Both $\lambda_{\text{inel}}$ and $\lambda_{\text{ALS}}$ can be determined experimentally by tomography or comparison between both methods (Noble et al., 2018; Rice et al., 2018). The values are apparent and representative of the measurements in this work but not of precise physical quantities.

In this work, we present our approach for an optimized data-collection workflow by targeting only holes with optimal sample thickness. Thereby, we substantially increase the data-collection efficiency by maximizing the output quality, while minimizing the number of images collected that do not contribute to the high-resolution information and which are discarded immediately or early on during image processing. This is achieved by either combining Digital Micrograph (Gatan) scripts with the Filter Ice Quality histogram implemented in EPU (TFS) for targeting or by using scripts that combine the entire procedure in SerialEM (Mastronarde, 2005; Schorb et al., 2019). Based on quality assessments, such as resolution estimation of the CTF fit (better than 4 Å), more than 90–95% of the data collected using this workflow provide high-resolution information.

2. Materials and methods

2.1. Microscopy

A Talos Arctica (TFS) equipped with a BioQuantum/K2 energy filter was operated at 200 kV in zero-loss mode (slit width 20 eV). At M910× magnification (calibrated pixel size 144.6 Å per pixel) the system was set to microprobe mode with a 50 μm C2 aperture at spot size 8, with gun lens 5 and the C2 lens at 100%, resulting in an electron flux of 12.9 e⁻ per pixel per second (10.7 counts per pixel per second). For LM690× magnification (calibrated pixel size 190.7 Å per pixel), the system was set to microprobe mode with a 50 μm C2 aperture at spot size 7, with gun lens 5 and the C2 lens at 65.0%, resulting in an electron flux of 12.9 e⁻ per pixel per second (10.7 counts per pixel per second).

A Titan Krios (TFS) equipped with a BioQuantum/K3 energy filter was operated at 300 kV in zero-loss mode (slit width 20 eV). At SA2250× magnification (calibrated pixel size 37.9 Å per pixel), the system was set to microprobe mode with a 50 μm C2 aperture at spot size 11, with gun lens 3 and an illuminated area of 40 μm, resulting in an electron flux of 22.6 e⁻ per pixel per second. For LM580× magnification (calibrated pixel size 174 Å), the system was set to microprobe mode with a 50 μm C2 aperture at spot size 8, with gun lens 3 and an illuminated area of 200 μm, resulting in an electron flux of 12.8 e⁻ per pixel per second.

To reduce the noise in the display of the thickness distribution, images were acquired with fourfold binning. They were further median-filtered in Digital Micrograph to reduce noise. The area of the median filter can be adjusted in the Global Tags (the default is 3 × 3 pixels).

2.2. Sample preparation for calibration

Aldolase from rabbit muscle was purified as described previously (Herzik et al., 2017). The size-exclusion chromatography peak fraction was concentrated to 10 mg ml⁻¹, flash-frozen in liquid nitrogen and stored at −80°C. For grid preparation, the protein was thawed and first diluted to 2 mg ml⁻¹ with 20 mM HEPES pH 7.5, 50 mM NaCl. The diluted solution was further mixed in a 1:10 ratio with 10 nm nano gold fiducial suspension, resulting in a final protein concentration of 1.8 mg ml⁻¹. 2.8 μl of the mixture was applied onto glow-discharged (5 mA, 20 s) R1.2/1.3 holey carbon gold grids (300 mesh, Quantifoil) at 22°C and 100% humidity. The grids were immediately blotted for 2 s and...
plunge-frozen in liquid ethane/propane using a Vitrobot Mark IV (TFS).

2.3. Calibration

To calibrate \( \lambda_{\text{inel}} \), the grids were loaded onto a Talos Arctica operated at 200 kV and equipped with a BioQuantum/K2 energy filter (Gatan) with slit width 20 eV. A grid square was adjusted to eucentric height and the microscope was set to the imaging conditions for the thickness measurement.

Using a default value for \( \lambda_{\text{inel}} \), apparent thickness values for different holes were calculated via the script. A tilt series was acquired at each of the positions at SA24000 \( \times \) magnification (calibrated pixel size 5.5 Å per pixel) with TOMO (TFS) using a tilt range from \(-60^\circ\) to \(+60^\circ\) with \( \pm 2^\circ \) increments (dose per tilt angle 0.6 e\(^{-}\) Å\(^2\)), total dose 36 e\(^{-}\) Å\(^2\)). IMOD (Kremer et al., 1996; Mastronarde, 1997) was used to reconstruct tomograms using the gold fiducials as markers to align the tilt series. From the cross section of the tomograms, the actual ice thickness can be determined. Comparison of the apparent with the actual thickness value under given conditions allowed the correct \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) values to be obtained, which are used by the Digital Micrograph and SerialEM script, respectively, when executed for the first time. For the 200 kV Talos Arctica, in the M/SA magnification range, \( \lambda_{\text{inel}} (200 \text{ kV, M/SA}) \) is 305 nm. Comparing thickness values at the M magnification with the same grid square at the LM magnification resulted in a respective \( \lambda_{\text{inel}} (200 \text{ kV, LM}) \) of 485 nm. For a 300 kV Titan Krios, the \( \lambda_{\text{inel}} (300 \text{ kV, SA}) \) of 435 nm for the SA magnification range was taken from previous work (Rice et al., 2018), which worked very well. The value for the LM range was determined as mentioned above and corresponded to an \( \lambda_{\text{inel}} (300 \text{ kV, LM}) \) of 805 nm. \( \lambda_{\text{ALS}} \) for the LM magnification range was determined by comparison with the filter-based method (600 nm for the TFS 200 kV Talos Arctica and 1750 nm for the TFS 300 kV Titan Krios). The values of \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) seem to be very stable and recalibration was not necessary at a later point using the same imaging condition. The fact that we could use the reported values of \( \lambda_{\text{inel}} \) from Rice et al. (2018) for the Titan Krios suggests that these values might actually be transferable and stable. However, we do not have sufficient data to ensure long-term stability and we therefore recommend checking and eventually recalibrating \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) after any major change in microscope configuration.

3. Results and discussion

3.1. Digital Micrograph and EPU

With the functions available through Digital Micrograph (version 2.2 or higher), we developed a script that allows the user to calculate and monitor the sample thickness of a grid square with a holey-carbon support film at low magnification. The respective holes are colored with a heatmap as markers to align the tilt series. From the cross section of the tomograms, the actual ice thickness can be determined. Comparison of the apparent with the actual thickness value under given conditions allowed the correct \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) values to be obtained, which are used by the Digital Micrograph and SerialEM script, respectively, when executed for the first time. For the 200 kV Talos Arctica, in the M/SA magnification range, \( \lambda_{\text{inel}} (200 \text{ kV, M/SA}) \) is 305 nm. Comparing thickness values at the M magnification with the same grid square at the LM magnification resulted in a respective \( \lambda_{\text{inel}} (200 \text{ kV, LM}) \) of 485 nm. For a 300 kV Titan Krios, the \( \lambda_{\text{inel}} (300 \text{ kV, SA}) \) of 435 nm for the SA magnification range was taken from previous work (Rice et al., 2018), which worked very well. The value for the LM range was determined as mentioned above and corresponded to an \( \lambda_{\text{inel}} (300 \text{ kV, LM}) \) of 805 nm. \( \lambda_{\text{ALS}} \) for the LM magnification range was determined by comparison with the filter-based method (600 nm for the TFS 200 kV Talos Arctica and 1750 nm for the TFS 300 kV Titan Krios). The values of \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) seem to be very stable and recalibration was not necessary at a later point using the same imaging condition. The fact that we could use the reported values of \( \lambda_{\text{inel}} \) from Rice et al. (2018) for the Titan Krios suggests that these values might actually be transferable and stable. However, we do not have sufficient data to ensure long-term stability and we therefore recommend checking and eventually recalibrating \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) after any major change in microscope configuration.

With the functions available through Digital Micrograph (version 2.2 or higher), we developed a script that allows the user to calculate and monitor the sample thickness of a grid square with a holey-carbon support film at low magnification. The respective holes are colored with a heatmap as markers to align the tilt series. From the cross section of the tomograms, the actual ice thickness can be determined. Comparison of the apparent with the actual thickness value under given conditions allowed the correct \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) values to be obtained, which are used by the Digital Micrograph and SerialEM script, respectively, when executed for the first time. For the 200 kV Talos Arctica, in the M/SA magnification range, \( \lambda_{\text{inel}} (200 \text{ kV, M/SA}) \) is 305 nm. Comparing thickness values at the M magnification with the same grid square at the LM magnification resulted in a respective \( \lambda_{\text{inel}} (200 \text{ kV, LM}) \) of 485 nm. For a 300 kV Titan Krios, the \( \lambda_{\text{inel}} (300 \text{ kV, SA}) \) of 435 nm for the SA magnification range was taken from previous work (Rice et al., 2018), which worked very well. The value for the LM range was determined as mentioned above and corresponded to an \( \lambda_{\text{inel}} (300 \text{ kV, LM}) \) of 805 nm. \( \lambda_{\text{ALS}} \) for the LM magnification range was determined by comparison with the filter-based method (600 nm for the TFS 200 kV Talos Arctica and 1750 nm for the TFS 300 kV Titan Krios). The values of \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) seem to be very stable and recalibration was not necessary at a later point using the same imaging condition. The fact that we could use the reported values of \( \lambda_{\text{inel}} \) from Rice et al. (2018) for the Titan Krios suggests that these values might actually be transferable and stable. However, we do not have sufficient data to ensure long-term stability and we therefore recommend checking and eventually recalibrating \( \lambda_{\text{inel}} \) and \( \lambda_{\text{ALS}} \) after any major change in microscope configuration.

When executed, the script will collect two images: with and without the energy-filter slit inserted. From these two images a ratio image is calculated representing \( I/I_0 \) (1). In the LM magnification range, where it might be difficult to align the zero-loss peak to the higher magnification, we provided the option of a magnification-dependent energy shift to compensate for the offset. The user has to individually determine and provide these shifts in the Global Tags (default 0 eV). For the ALS method, the user needs to define the mean background intensity, which is obtained by measuring the intensity over vacuum within an empty hole (\( I_0 \), equation 2). This value needs to be determined only once per data-collection setup and is subsequently used to calculate the local thickness (2) within a grid-square image acquired using the same imaging settings. The switch between the filter-based and ALS methods can be activated by a parameter in the Global Tags.
The colored heatmap is obtained through a pixel-by-pixel calculation (equation 1 or 2) and the script assigns each pixel a thickness value. With respect to the user-defined threshold, it will allocate the respective color in an RGB image (too thin, red; optimal, green; too thick, no color; Fig. 1b). The local measurement uses the cursor position in the image and averages the value of the ratio image within a square around these coordinates to calculate the sample thickness. The script also checks the beam intensity to prevent coincidence loss that is too high (for example below 10 counts per pixel per second for a Gatan K2 camera), thereby ensuring consistent results. While detecting the used voltage, either 200 or 300 kV, it will use the respective set of $\lambda_{\text{inel}}$ and $\lambda_{\text{ALS}}$ needed for the calculations. Furthermore, it can distinguish between a K2 and K3 camera (Gatan) and handle the different ways of electron counting. Everything is finished with a GUI that allows relevant parameters such as the thickness range, as well as the exposure time and binning, to be quickly changed (Fig. 1a).

Further parameters can be changed in the Global Tags in Digital Micrograph. Since EPU only offers dedicated presets for data collection, and microscope control via Digital Micrograph is limited, we wrote an additional script in JScript within the TFS TEM scripting environment which allows the microscope parameters to be stored and recalled to run the thickness measurement reliably.

Once the sample thickness has been determined, the gained knowledge can be transferred into EPU. Here, the Filter Ice Quality threshold option during hole targeting is manually adjusted to mimic the hole selection obtained in Digital Micrograph (Fig. 1c). However, this only needs to be performed once for a representative grid square at the beginning when setting up data collection, and requires a total of only ~5–10 min of additional user input. The same parameters will be adopted for any other regions within the same grid and no additional steps are required during data collection. Hence, with the exception that on average fewer holes per grid square are selected and imaged, data acquisition itself is not slowed compared with a conventional setup.

3.2. SerialEM

A clear disadvantage when using EPU as data-collection software is that the sample thickness needs to be measured separately in Digital Micrograph first and subsequently transferred into EPU. Moreover, EPU is commercial software that is only available for electron microscopes from TFS. To avoid this, we were able to implement the entire workflow in the open-source acquisition software SerialEM (Mastronarde, 2005; Schorb et al., 2019) that is compatible with multiple TEM platforms. The newly developed scripts determine the sample thickness and use the acquired values to automatically target holes within the user-defined thickness range. This setup takes advantage of recently added software functions, such as hole finder, extended by hole combiner for acquisition in multiple holes via beam-image shift. It also requires some newly introduced script commands, which are all available in SerialEM version 3.9 beta1 or higher. Basic parameters (mean free path length, thickness thresholds, imaging settings, correction term and averaging radius) are set as variables in the script to minimize user interaction.

The script will first set the microscope to the desired imaging conditions and acquire an unfiltered and a filtered image of a selected grid square. This can be performed for multiple squares and be automatically executed via the Acquire at Items function. The newly implemented hole finder function then locates the position of each hole within the square. At each hole position the mean intensities in the unfiltered ($I$) and filtered image ($I_\lambda$) are extracted within a defined radius (1), which should be carefully chosen based on the magnification and binning used. This allows the sample thickness to be calculated on a hole-to-hole basis that is stored for each item in the Navigator window of SerialEM as a note (Fig. 2a, red box). Based on a user-defined sample thickness range, the calculated values are used to select entries for target acquisition (Fig. 2a, green box) and to color positions, respectively (too thick, blue; optimal, green; too thin, magenta; Fig. 2b). The acquisition of each grid square currently requires about 5 min, but the process can be automated thanks to the eucentric height routine in SerialEM such that the user only has to provide the locations of the squares of interest. To operate in the LM magnification range, a slightly adapted version of the script is available using the Search preset function for image acquisition. This might require an additional energy shift for the respective magnification. As this energy shift is only applied when the slit is inserted and it introduces an image shift, a correction function was implemented allowing the correct intensity values to be extracted from both the unfiltered and the filtered image. For systems without an energy filter we provide a separate script which can determine the sample thickness using the ALS method outside a Digital Micrograph implementation. For this, the user provides the reference intensity $I_0$ over vacuum within an empty hole, which is divided by the intensity $I$ extracted from a grid-square image (2).

The entire workflow is flexible, allowing the user to intervene at any point and make adjustments. If, for example, the initially targeted thickness range is not ideal, a second script allows the user to adjust the hole selection using the sample-thickness values stored in the Navigator note and repeat the threshold-based selection for all entries. This needs minimal user input and finishes within a minute for 5000 points.

3.3. Advantages and limitations

While in our experience, based on small membrane proteins, the optimal sample thickness is in the range 20–40 nm (Fig. 3), this should be tested and optimized for each project individually. A good starting point is the expected particle size $\pm$ 5–20 nm. For this purpose, on-the-fly preprocessing software packages such as FOCUS (Biyani et al., 2017), Warp (Tegunov & Cramer, 2019) or Appion (Lander et al., 2009) are useful to provide rapid feedback. Here, calculated parameters such as the resolution of CTF estimation, particle distribution or preliminary 2D classifications can be
used to assess data quality on the fly and correlate it with the measured sample thickness, allowing the user to define the optimal thickness range and adjust the targeting parameters if required.

While sample quality and thickness homogeneity can differ significantly between and within grids, the grid squares shown in Figs. 1 and 2 are, in our experience, representative. Here, conventional hole selection would have targeted the majority of all visible holes. Yet, more than 50% of the images would not meet the abovementioned quality criteria and would be discarded right after data collection or during early image processing. By contrast, our thickness-based approach restricts data collection to only a relatively small fraction of optimal holes present, with 90–95% of the data preserving high-resolution information. The remaining 5–10% of the micrographs are usually discarded due to contamination or mismatches between the position of targeting and acquisition. Compared with conventional data-acquisition workflows, this approach can thus lead to a twofold to fivefold increase in retained data.

An emerging technique to speed up data collection is the use of beam-image shift. It allows the acquisition of multiple images (for example a 3×3 pattern) by a combined beam and image shift instead of using stage movement (Cheng et al., 2018; Wu et al., 2019; Cash et al., 2020). The introduced beam tilt can be corrected directly using the aberration-free-image-shift (AFIS) correction in EPU (currently only available for the TFS Titan Krios) or the coma versus image shift calibration in SerialEM. Alternatively, it can be corrected later during image processing (Cash et al., 2020) by using the CTF refinement functions implemented in, for example, RELION or cryoSPARC (Zivanov et al., 2018; Punjani et al., 2017). Ideally, one would like to combine this approach with the selection of the best thickness areas. This is possible in SerialEM with the newly implemented hole combiner. It only considers Navigator entries that are selected for acquisition and groups them in a user-defined pattern (for example 3×3). All entries within a group not marked for acquisition (i.e. outside the defined thickness range) will be skipped. In EPU, with the fast acquisition mode activated, grouping is performed automatically while considering only selected holes.
within a group. Both setups provide a significant boost in throughput compared with acquisition by stage movement, while keeping the benefits of optimal area selection.

Another approach that aims to minimize beam-induced movement is the use of gold-coated support films (Russo & Passmore, 2016; Naydenova et al., 2020). We can confirm that our workflow also works well with commercially available UltraAuFoil grids (Quantifoil), in particular using the filter-based approach in SerialEM.

The thickness scripts described here have been developed and tested on TFS microscope systems, namely on a 200 kV Talos Arctica and a 300 kV Titan Krios, both equipped with BioQuantum K2/K3 energy filters. On these systems the energy filter-based method is the preferred option and is most accurate in the M or SA magnification range. This works well for a TFS Talos Arctica, as the lowest possible magnification (M910×) still includes a full square of 300- and 400-mesh grids and the majority of a 200-mesh grid square. However, for a TFS Titan Krios, the lowest SA magnification (SA2250×) covers less than a quarter of a 300-mesh grid square or about a third of a 400-mesh grid square. Alternatively, an LM magnification can be selected in combination with the filter-based approach. Although slightly less accurate, it provides a larger field of view that is able to cover an entire grid square for automatic targeting with SerialEM on a TFS Titan Krios. For systems without an energy filter, the ALS method has to be obtained within an empty hole (due to higher intrinsic stage drift, which requires longer waiting times, and due to the lack of a third condenser lens, which requires large beam sizes to maintain parallel illumination and does not allow multiple data acquisitions per hole), this new workflow has been demonstrated to be crucial. The approach has been routinely used in all our projects, proving its versatility and efficiency experimentally (Garayeva et al., 2018, 2019; Stock et al., 2018; Alvadia et al., 2019; Kalienkova et al., 2019; Arkhipova et al., 2020; Sikkema et al., 2020; Lam et al., 2021).

5. Data availability

The Digital Micrograph script can be found in the FELMI-ZFE DM-Script Database (https://www.felmi-zfe.at/dm_script/measure-thickness-in-eftem-2/). The script for setting the imaging conditions written in JScript is available on github (https://github.com/jrheinberger/SetThicknessMeasurementConditions). All SerialEM scripts have been deposited in the SerialEM Script Repository (https://serialemscripts.nexperion.net/script/63 and related scripts).

Further details regarding the installation and execution of the Digital Micrograph script can be found in the supporting information.

Acknowledgements

Testing on the Titan Krios was performed at the Netherlands Centre for Electron Nanoscopy (NeCEN) with the help of Wen Yang, Rebecca Dillard and Ludo Renault. We would like to specially thank David N. Mastronarde for providing SerialEM and his dedication to supporting new functionality, which was crucial to parts of this work, and Michiel Punter for IT support. Special thanks are also given to Radostin Danev, Craig Yoshioka, Mike Strauss and Nadav Elad for their helpful discussions on the preprint version of the manuscript.
Conflict of interest: GPR’s company Nexperion – Solutions for Electron Microscopy provides commercial services related to the SerialEM software discussed in this work.

Funding information
This work was funded by NWO Veni grant 722.017.001 and NWO Start-Up grant 740.018.016 to CP.

References
Adrian, M., Dubochet, J., Lepault, J. & McDowall, A. W. (1984). Nature, 308, 32–36.
Alvadia, C., Lim, N. K., Clerico Mosina, V., Oostergetel, G. T., Dutzler, R. & Paulino, C. (2019). eLife, 8, e44365.
Angert, I., Barmester, C., Dinges, C., Rose, H. & Schröder, R. R. (1996). Ultramicroscopy, 63, 181–192.
Arkhipova, V., Gusakov, A. & Slotboom, D. J. (2020). Nat. Commun. 11, 998.
Arnold, S. A., Albiez, S., Bieri, A., Syntychaki, A., Adaixo, R., McLeod, R., Poter, C. S. & Carragher, B. (2018). J. Struct. Biol. 204, 270–275.
Cheng, A., Negri, C., Bruhn, J. F., Rice, W. J., Daliakyan, S., Eng, E. T., Waterman, D. G., Potter, C. S. & Carragher, B. (2021). Protein Sci. 30, 136–150.
Cho, H.-J., Hyun, J.-K., Kim, J.-G., Jeong, H. S., Park, H. N., You, D.-J. & Jung, H. S. (2013). J. Anal. Sci. Technol. 4, 7.
Dandey, V. P., Wei, H., Zhang, Z., Tan, Y. Z., Acharya, P., Eng, E. T., Rice, W. J., Cheng, A., Negro, C. J., Shapiro, L., Rapp, M., Eng, E. T., Rice, W. J., Cheng, A., Negro, C. J., Shapiro, L., Kwong, P. D., Jeruzalmi, D., des Georges, A., Potter, C. S. & Carragher, B. (2018). eLife, 7, e34257.
Funding information
NWO Start-Up grant 740.018.016 to CP.
This work was funded by NWO Veni grant 722.017.001 and NWO Start-Up grant 740.018.016 to CP.

Conflict of interest: GPR’s company Nexperion – Solutions for Electron Microscopy provides commercial services related to the SerialEM software discussed in this work.

Funding information
This work was funded by NWO Veni grant 722.017.001 and NWO Start-Up grant 740.018.016 to CP.

References
Adrian, M., Dubochet, J., Lepault, J. & McDowall, A. W. (1984). Nature, 308, 32–36.
Alvadia, C., Lim, N. K., Clerico Mosina, V., Oostergetel, G. T., Dutzler, R. & Paulino, C. (2019). eLife, 8, e44365.
Angert, I., Barmester, C., Dinges, C., Rose, H. & Schröder, R. R. (1996). Ultramicroscopy, 63, 181–192.
Arkhipova, V., Gusakov, A. & Slotboom, D. J. (2020). Nat. Commun. 11, 998.
Arnold, S. A., Albiez, S., Bieri, A., Syntychaki, A., Adaixo, R., McLeod, R., Poter, C. S. & Carragher, B. (2018). J. Struct. Biol. 204, 270–275.
Cheng, A., Negri, C., Bruhn, J. F., Rice, W. J., Daliakyan, S., Eng, E. T., Waterman, D. G., Potter, C. S. & Carragher, B. (2021). Protein Sci. 30, 136–150.
Cho, H.-J., Hyun, J.-K., Kim, J.-G., Jeong, H. S., Park, H. N., You, D.-J. & Jung, H. S. (2013). J. Anal. Sci. Technol. 4, 7.
Dandey, V. P., Wei, H., Zhang, Z., Tan, Y. Z., Acharya, P., Eng, E. T., Rice, W. J., Kahn, P. A., Potter, C. S. & Carragher, B. (2018). J. Struct. Biol. 202, 161–169.
D’Imprima, E., Floris, D., Poppe, M., Sánchez, R., Grininger, M. & Kühlbrandt, W. (2019). eLife, 8, e42747.
Garaeva, A. A., Gusakov, A., Slotboom, D. J. & Paulino, C. (2019). Nat. Commun. 10, 3427.
Garaeva, A. A., Oostergetel, G. T., Gati, C., Gusakov, A., Paulino, C. & Slotboom, D. J. (2018). Nat. Struct. Mol. Biol. 25, 515–521.
Herzik, M. A., Wu, M. & Lander, G. C. (2017). Nat. Methods, 14, 1075–1078.
Kalienkova, V., Clerico Mosina, V., Bryner, L., Oostergetel, G. T., Dutzler, R. & Paulino, C. (2019). eLife, 8, e44364.
Kontziampasis, D., Klebl, D. P., Iadanza, M. G., Scarff, C. A., Kopf, F., Sobott, F., Monteiro, D. C. F., Trebbin, M., Muench, S. P. & White, H. D. (2019). IUCrJ, 6, 1024–1031.

Conflict of interest: GPR’s company Nexperion – Solutions for Electron Microscopy provides commercial services related to the SerialEM software discussed in this work.

Funding information
This work was funded by NWO Veni grant 722.017.001 and NWO Start-Up grant 740.018.016 to CP.