Journal of Visualized Experiments

Strategies for optimisation of cryogenic electron tomography data acquisition

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62383R1
Full Title:	Strategies for optimisation of cryogenic electron tomography data acquisition
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Additional Information:	
Question	**Response**
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TITLE:

Strategies for Optimization of Cryogenic Electron Tomography Data Acquisition

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KEYWORDS:

cryogenic electron tomography, automation, high-throughput, PyEM, SerialEM

SUMMARY:

The increasing demand for large-scale data collection in cryogenic electron tomography requires high-throughput image acquisition routines. Described here is a protocol that implements the recent developments of advanced acquisition strategies aimed at maximizing the time-efficiency and throughput of tomographic data collection.

ABSTRACT:

Cryogenic electron tomography (cryoET) is a powerful method to study the 3D structure of biological samples in a close-to-native state. Current state-of-the-art cryoET combined with subtomogram averaging analysis enables the high-resolution structural determination of macromolecular complexes that are present in multiple copies within tomographic reconstructions. Tomographic experiments usually require a vast amount of tilt series to be acquired by means of high-end transmission electron microscopes with important operational running-costs. Although the throughput and reliability of automated data acquisition routines have constantly improved over the recent years, the process of selecting regions of interest at which a tilt series will be acquired cannot be easily automated and it still relies on the user's manual input. Therefore, the set-up of a large-scale data collection session is a time-consuming procedure that can considerably reduce the remaining microscope time available for tilt series acquisition. Here, the protocol describes the recently developed implementations based on the

SerialEM package and the PyEM software that significantly improve the time-efficiency of grid screening and large-scale tilt series data collection. The presented protocol illustrates how to use SerialEM scripting functionalities to fully automate grid mapping, grid square mapping, and tilt series acquisition. Furthermore, the protocol describes how to use PyEM to select additional acquisition targets in off-line mode after automated data collection is initiated. To illustrate this protocol, its application in the context of high-end data collection of Sars-Cov-2 tilt series is described. The presented pipeline is particularly suited to maximizing the time-efficiency of tomography experiments that require a careful selection of acquisition targets and at the same time a large amount of tilt series to be collected.

INTRODUCTION:

Cryogenic electron microscopy (cryoEM) methods are based on the imaging of biological samples by means of a transmission electron microscope (TEM) after their rapid vitrification, a sample preparation process that preserves the molecular and cellular structures of specimens in a close-to-native and hydrated state[1,2]. In cryogenic electron tomography (cryoET) a 3D model of the vitrified sample is achieved by acquiring a number of images of the same region of interest from different orientations, the so-called tilt series, followed by the computational reconstruction of the tomographic volume[3]. This advanced imaging technique has matured into a powerful method for the structural investigation of biological processes in the context of their native cellular environments[4–6].

In addition to the ultrastructural analysis of the vitrified sample, high-resolution reconstructions of macromolecular complexes that are present in multiple copies within the tomographic volume can be obtained by applying subtomogram averaging[5]. This reconstruction approach is based on the iterative alignment and averaging of sub-volumes containing the structure of interest and it is aimed at increasing the signal-to-noise ratio and the resolution of the final reconstruction[7,8]. Subtomogram averaging relies on the collection and processing of a large amount of data that often demands the acquisition of hundreds of tilt series by means of high-end TEMs with onerous operational running-costs.

Currently the setup of such automated cryoET sessions is a time-consuming process that usually relies on the user's manual input[9–11]. Typically, targets are identified by visual inspection of the mapped grid and subsequently set up for automated data collection. The user's efficiency in identifying acquisition points is often affected by the nature of the sample, becoming particularly challenging when analyzing purified macromolecules with suboptimal concentration or in the case of rare events within crowded cellular environments, implying the use of correlative approaches[12]. Furthermore, current workflows require the acquisition of images during set-up at various magnifications that will be later used for precise localization and centering of the target during automated acquisition[11,13,14]. These high-precision re-alignment steps are crucial for high-resolution applications, which demand the imaging to be performed at high magnification and require accurate centering steps for retaining the region of interest within the resulting small field of view. Altogether, several hours of each data collection session are committed for this time-consuming procedure during which the TEM is not engaged in tilt series acquisition. Therefore, depending on the amount of tilt series required, the identification and set-up of

acquisition points can have a considerable impact on the microscope time available for data collection during a cryoET session.

Described here is an optimized protocol based on the SerialEM software package[15] and the latest version the PyEM software[16] to map grids, map grid squares, select targets, and set up automated data acquisition for large-scale tilt series collection. The key concept of this approach is to provide SerialEM with computationally generated images by PyEM for each acquisition item, termed virtual maps, for the accurate localization and centering of the target. To gain actual acquisition time, the selection of the targets as well as the creation of the virtual maps are performed off-line using a second dummy instance of SerialEM, decoupling the selection process of acquisition targets from TEM operations. While not addressing how to increase the data quality[13,17] or the speed of tilt series acquisition[18,19], this protocol is primarily focused on strategies to optimize the time-efficiency of large-scale automated cryoET sessions setup. Therefore, the implementation of the presented protocol is meant for those scientists establishing cryoET data collection workflows that desire to maximize the yield of automated data acquisition by increasing the microscope time available for tilt series acquisition.

PROTOCOL:

The protocol described here is part of a more comprehensive document produced by the EMBL CryoEM Service Platform that includes thorough step-by-step instructions and screenshots illustrating the entire procedure of a typical cryoET session, including sample loading, grid mapping, microscope tuning, set up of acquisition points, and automated data collection. The full protocol can be downloaded at the following link: https://oc.embl.de/index.php/s/9OuTl8AazDkCNq0/download

1. Prerequisites

1.1. Install SerialEM version 3.8 and set up to control the microscope and detector (https://bio3d.colorado.edu/SerialEM/betaHlp/html/setting_up_serialem.htm).

1.2. Install a dummy instance of SerialEM version 3.8 (https://sphinx-emdocs.readthedocs.io/en/latest/serialEM-note-setup-dummy.html).

1.3. Install PyEM (https://git.embl.de/schorb/pyem).

2. Grid mapping

2.1. Load a cassette with grids into the microscope autoloader.

2.2. Set up imaging conditions suitable for mapping the entire grid. Do this at the lowest magnification possible taking into account the field of view on the detector used (EFTEM SA 2250x). Save the imaging conditions as a **SerialEM Imaging State** to make things convenient for later use.

2.3. Set up full montage

2.3.1. Open the SerialEM **Navigator** menu.

2.3.2. Select **Montaging & Grids**.

2.3.3. Select **Set up Full Montage**.

2.4. Start the script **Grid_Mapping**. Allow the script to wait for the autoloader to cool down; do an inventory and then map each grid that the inventory procedure found. Input an email via the **Tilt Series Menu** to allow the script to conveniently send an email when done.

2.5. Save **Navigator**.

2.6. Inspect all grid maps from the SerialEM Navigator and choose which grid to map further at higher magnification.

NOTE: Many TEMs equipped with an autoloader system will show a rotation of the grid when reloaded. It's best to remap any grid after reloading it. Alternative systems usually only suffer some shifts, which can be corrected in SerialEM with a **Shift To Marker** procedure.

3. Set up the SerialEM low dose

3.1. Set the magnification of SerialEM low dose **View** and **Preview** modes (this is required for step 6).

3.2. Use the Navigator, acquire a **View** image, and save it as map.

3.3. Use the Navigator, acquire a **Preview** image, and save it as map.

3.3.1. Set both the modes to the same binning (binning 4 is suggested). This allows saving the two maps into one image stack.

3.3.2. In the SerialEM **Navigator** window, change the label of the View map to **View** and the label of the Preview map to **Preview**.

3.3.3. Save and close the navigator file.

NOTE: No target is needed for the initial **View** and **Preview** images, PyEM will merely use their imaging settings.

4. Grid square mapping

4.1. Set up the imaging conditions to map grid squares. It is of great importance to be able to see the sample of interest and the fiducial bead distribution. To ensure optimal contrast for grid square maps, perform the following steps.

4.1.1. Insert an objective aperture.

4.1.2. If applicable, insert the energy filter slit.

4.1.3. Use a defocus of -100 µm.

4.2. Screen for good grid squares suitable for further mapping. After visual inspection of the squares from the grid map, take test images of the square with the imaging conditions to be used for grid square maps.

4.3. When a good square is identified, mark its center in the grid map image using the **Add Points** feature of the SerialEM Navigator.

4.4. Once all the points are added, in the SerialEM Navigator press **Shift + A** on the first point, then press **Shift + A** on the last point.

NOTE: All added points are now marked by an **A**, meaning they are all **Acquisition Points**.

4.5. Press **Shift + N** (create a new file at item) on the first point and then again on the last point. In the dialog that comes up, select **Montaged Images**.

4.6. When the montage dialog pops up, set up a montage size that covers one grid square. This depends on the magnification and the mesh size of the grids used and typically requires 2 x 2 to 4 x 4 montages. Give it a name with a number (e.g., squaremap_01.mrc) to allow the SerialEM to conveniently auto-number all the files per grid square.

4.7. Start the grid-mapping by opening the SerialEM **Navigator** menu and click on **Acquire At Items**.

4.8. In the pop-up menu, select the following options.

4.8.1. Select **Rough Eucentricity**.

4.8.2. Select **Acquire Map Image**.

4.8.3. Select **Close Column Valves at End**.

4.8.4. Select **Send Email at End**.

5. Selecting the targets

5.1. Open a dummy SerialEM instance. This can be set up on the SerialEM PC that controls the microscope or on a different (support) PC if both computers share a network connection.

5.2. Once the first grid square is mapped, use the SerialEM Navigator menu option **Merge** to see the montage in the dummy SerialEM instance.

5.3. Double click on the **Navigator** window to open grid square map.

5.4. Search the map and use the dummy SerialEM Navigator option **Add Points** to add image acquisition points on the target of interest.

5.5. When done and after mapping the new squares, save the **Navigator** file and merge the Navigator again; continue until all grid squares are mapped.

6. Generate virtual maps

6.1. Once again, merge the navigator file with the dummy SerialEM instance.

6.2. Run PyEM **Virtual maps** script from dummy SerialEM menu, **Tools** and select **Virtual Anchor Maps**. This may take some time depending on the size and amount of the grid square maps and the binning of the View and Preview maps.

NOTE: PyEM starts a command window that automatically closes when done, then the new navigator file can be opened. Per montaged map that contains selected points, PyEM writes a single merged map and all its View and Preview maps. Finally, it writes a new Navigator file called <**navigatorfilename**>**_automaps.nav**.

7. Microscope tuning

7.1. Check the microscope tuning. To assure proper microscope performance, use the same magnification and beam size setup for data acquisition, in the following order.

7.1.1. Run SeriaLEM **Coma-free alignment by CTF** (Zemlin tableau).

7.1.2. Insert and center an objective aperture.

7.1.3. Run SerialEM **Correct Astigmatism by CTF** (auto-stigmate).

7.1.4. Run **GIF Quick Tune** (i.e., only slit focus).

NOTE: As steps 7.1.1, 7.1.3, and 7.1.4 might require more dose rate, only the spot size should be changed; the beam size should not be changed as this causes beam tilt, which makes the

tunings incorrect. Steps 10.4.1, 10.4.2, and 10.4.3 are semi-automated in this publicly available script: https://serialemscripts.nexperion.net/script/47.

8. Set up Navigator

8.1. In SerialEM, open the new navigator file named **xxx_automaps.nav**.

NOTE: **V_xxxx** are the View maps and **P_xxxx** are the Preview maps. The preview maps are set as Acquisition Points.

8.2. In the SerialEM Navigator window, deselect all **A** points, select the first **V_xxxx** map, select **Collapse**, click on **A** twice, and deselect **Collapse**.

8.3. Select the first **V_xxxx** position, press **Shift + T**, then select the very last **V_xxxx** position, and press **Shift + T** again.

8.4. Choose **Single Frame Images** in the properties of the file to open dialog.

8.5. In the next File Properties dialog, select the desired parameters according to the imaging needs and the instrument setup.

8.6. When prompted, give a name with a number (e.g., TS_001.mrc) and click on **Save**.

NOTE: SerialEM will auto-number the filenames for all tilt series.

8.7. Set up the tilt series controller for the first TS position. When done, click on **OK** to set these parameters for all tilt series after this acquisition item. All Preview maps are now selected as **TS** (Tilt Series) with numbered file name **TS_xxx.mrc**.

NOTE: One can change the parameters manually later using the Navigator **TSparams** feature; changes will be applied for all items downwards on the list. The user has the option to run a custom script instead of the tilt series.

9. Set the focus/track positions

9.1. Set the focus/track distance for each target if needed (make sure that SerialEM **Low Dose** is switched on).

9.2. Double click on the **View Map** to load it.

9.3. Select the **Preview Map** in the Navigator list.

9.4. Select **Edit Focus** in the Navigator window.

9.5. In the **Low Dose Control** panel, deselect **Rotate inter-area axis** to position **Trial** and **Focus** along the stage tilt axis.

9.6. Click on the desired region in the loaded view map to set the focus/trial position for this tilt series.

9.7. Ensure that the Navigator item has **TSP** set now; repeat the procedure for all the items.

NOTE: Focus/track positions are automatically copied downwards in the Navigator. Therefore, if the focus/track position for the previous item is on the correct side and of the correct distance, there is no need to change it for the current item.

10. Set up additional scripts

10.1. Two scripts handle the Focus range: **pretomo** and **duringtomo**. The **pretomo** script runs before each tilt series and the **duringtomo** script during each tilt.

10.2. Edit the focus range in the script **pretomo**.

10.3. In the SerialEM Tilt Series menu, check **Run Script in TS** and select the script number of the **duringtomo** script.

11. Run

11.1. Check the nitrogen tank level.

11.2. Check whether the Autoloader turbo off is selected.

11.3. Check the data storage free space.

11.4. In the SerialEM file menu, deselect continuous saving for the log file and close any currently open logfile. Each tilt series will get its own log file.

11.5. In the Navigator menu, click on **Acquire at Items**.

11.6. Run the script **PreTomo**.

11.7. Select **Primary task Acquire tilt series**.

11.8. Select **Run script after PostTomo**.

11.9. Select **Close column valves at end**.

11.10. Select **Send email at end**.

11.11. Click on **GO**.

NOTE: SerialEM will send an email per tilt series, either successful or error; error can, however, also mean the full tilt range was not reached.

REPRESENTATIVE RESULTS:

This procedure was used to acquire the Sars-Cov-2 tilt series described in Turonova et al. 2020[20]; the whole dataset was produced using three distinct grids over three microscopy sessions at EMBL Heidelberg. The current study will focus on and describe the first 3-day (~72 h) session run with the first grid.

After the entire grid was mapped at low magnification (~10 min, see step 2), 71 suitable squares were selected on the grid map, and medium magnification maps (**square maps**) were acquired with settings (magnification, exposure, defocus) that allow for the direct visualization and identification of the sample of interest, coronaviruses in this case (see step 3) (**Figure 1A**). The acquisition time was ~3 min per square, 3 h 45 min in total.

As soon as the first square map was created, a dummy SerialEM instance (without any control on camera or microscope) was opened on a separate computer to visualize the square map and to add points on targets suitable for tilt series acquisition (see step 4) (**Figure 1B**). Newly acquired square maps were retrieved by merging the current dummy SerialEM navigator with the navigator from the acquiring SerialEM instance. After ~2 h of grid square acquisition and selection, 50 initial targets could be identified.

After the square map acquisitions had finished, SerialEM low-dose was set up and reference **View** and **Preview** images were taken and saved as maps (see step 5). The latter maps could then be used immediately on the dummy SerialEM instance to generate, from the corresponding square map images, the **Virtual View** (**Figure 1C**) and **Virtual Preview** (**Figure 1D**) maps of the 50 selected targets with the PyEM software suite, for a processing time of ~30 min (see step 6). This processing time on the dummy SerialEM session was used to perform final preparations of the microscope for acquisition: energy filter tuning, new camera gain reference image acquisition, astigmatism- and coma-free alignment of the objective lens.

Once microscope tuning was completed and virtual maps from the 50 initial targets generated, the actual SerialEM navigator to be used for acquisition was set up (see step 7), focus and track positions were set (step 8), and tilt series acquisition could be started (see steps 9 and 10). The **Virtual View** maps (**Figure 1C**) were used for an initial centering of the target (**Figure 1E**) followed by a final centering performed at the actual tilt series acquisition magnification (**Figure 1F**) using the **Virtual Preview** map (**Figure 1D**).

Starting with grid mapping at **9:30** in the morning, the acquisition of the tilt series for the 50 initial targets commenced at roughly **15:00**. With the settings used for tomographic acquisition (see the reference for details), a tilt series took ~20 min to acquire, with enough targets then to run

through the entire night. While the acquisition was running, the rest of the square maps could be inspected and more targets added, still off-line via the dummy SerialEM instance. 121 more targets were selected among the remaining square maps and added to the acquisition SerialEM navigator after virtual maps had been created for these new targets, enough to run until completion of the 72 h session.

This procedure (summarized in **Figure 2**) allowed, in a single working day, the setup of 171 targets for automated tomographic acquisitions for a 72 h (3 days) microscope session.

FIGURE AND TABLE LEGENDS:

Figure 1: Example of square map with representative virtual maps and corresponding acquisitions after centering. (**A**) Representative square map of a Sars-Cov-2 cryoEM grid used in Turonova et al.[20]. Four regions of interest are marked with a red cross. Microscope magnification is 2,250x. (**B**) Crop out of the square map highlighting the areas that were used to generate the **Virtual View** (orange) and **Virtual Preview** (yellow) maps for the selected target (red cross) (**C**) Virtual View map. (**D**) Virtual Preview map. (**E**) Actual View acquisition after centering using the **Virtual View** map as a reference. Microscope magnification is 11,500x. (**F**) 0° tilt acquisition from the tilt series after centering using the **Virtual Preview** map as a reference. Microscope magnification is 64,000x.

Figure 2: CryoET session workflow using SerialEM with PyEM tools.

DISCUSSION:

From a niche technique, cryoET has now matured into a widespread method to perform structural studies at the cellular and molecular level with unprecedented reachable resolution[21,22]. The ever-increasing demand for cryoEM imaging has put a strain on the limited resources available to access this technology. Despite the opening of a number of national cryoEM facilities and the efforts of scientific institutes to increase their TEM capacity to support the needs of the community worldwide, access to cryoEM instruments is still limited and the time available for data collection must therefore be efficiently used by the users to maximize the yield of each microscopy session. The need to acquire hundreds of tilt series combined with the limited time available for data collection called for novel image acquisition routines to achieve better throughput without compromising data quality. Recent developments in hardware and imaging workflows have considerably increased the speed of tilt series acquisition[18,19], thus resulting in a dramatic shift of the ratio between time spent to set up an acquisition point and time needed for the actual tilt series acquisition. Altogether, the procedure for setting up acquisition points is becoming one of the major bottlenecks for the achievable throughput of cryoET sessions.

The optimized protocol presented here enabled us to set up, in off-line mode, 171 positions for automated tomographic acquisition within the first day of a cryoET session while the microscope was actively engaged in other operations (e.g., square mapping, tuning, and automated tilt series acquisition), thus without affecting the microscope time available for data collection. In addition to maximizing the throughput of a cryoET session, this pipeline drastically reduces the amount of

time invested by the user in the preparatory phase of an automated data collection session. In the described protocol, the user is asked to browse the mapped grid squares to identify suitable regions of interest and add them to the Serial EM Navigator as acquisition points. All targets will then be automatically processed in batch within SerialEM by the PyEM tool for the production of the virtual maps[16]. The presented computational approach is therefore considerably faster than acquiring real anchoring maps by eliminating the waiting periods associated with stage movement, image acquisition, change of imaging conditions between View and Preview, and by the eventual reiteration of these steps while centering at high magnification. Additionally, as each acquired image leads to accumulation of electron dose on the object of interest[23], the use of virtual maps for the precise realignment of the targets reduces the radiation damage introduced in the preparatory phase of a cryoET session before the actual tilt series acquisition. The protocol described here makes use of both intermediate and high magnification virtual maps (Preview and View, respectively) for realignment of the target before tilt series acquisition. This procedure can easily be modified to only use the intermediate magnification View image when alignment accuracy is of less importance, for instance, in the case of large structures where ultimate target accuracy is of less concern[10] or for single particle analysis samples that are poorly spread on the cryo-grid requiring the user's manual selection of each acquisition point[24,25]. Finally, an approach based on the off-line use of a dummy SerialEM instance also facilitates the setup of acquisition points through remote connection by minimizing the need of the physical presence of the user at the microscope, thus enabling more flexibility in terms of operational organization of the facility.

The recent advances in technology and methods for cryoET have drastically improved the speed and reliability of automated data collection sessions. However, further developments are required to address the remaining rate-limiting steps of this method. Most notably, the initial step of grid and square mapping is now becoming one of the major bottlenecks of the session setup, thus generating the need for hardware improvements aimed at increasing the speed of microscope stage movements and of image acquisition by the direct electron detectors. Additionally, the development of machine learning approaches to fully automate the process of target identification will be crucial to eliminate the need of the users' visual inspection for selecting the regions of interest, a time-consuming procedure that relies on the users' expertise.

ACKNOWLEDGMENTS:

We acknowledge support from the Structural and Computational Biology Unit and the Electron Microscopy Core Facility at the European Molecular Biology Laboratory in Heidelberg, Germany. We are extremely grateful for the excellent support from the author of the SerialEM software package, Professor David Mastronarde. We also thank Herman Fung for the critical reading of the manuscript.

DISCLOSURES:

The authors declare no conflicts of interest.

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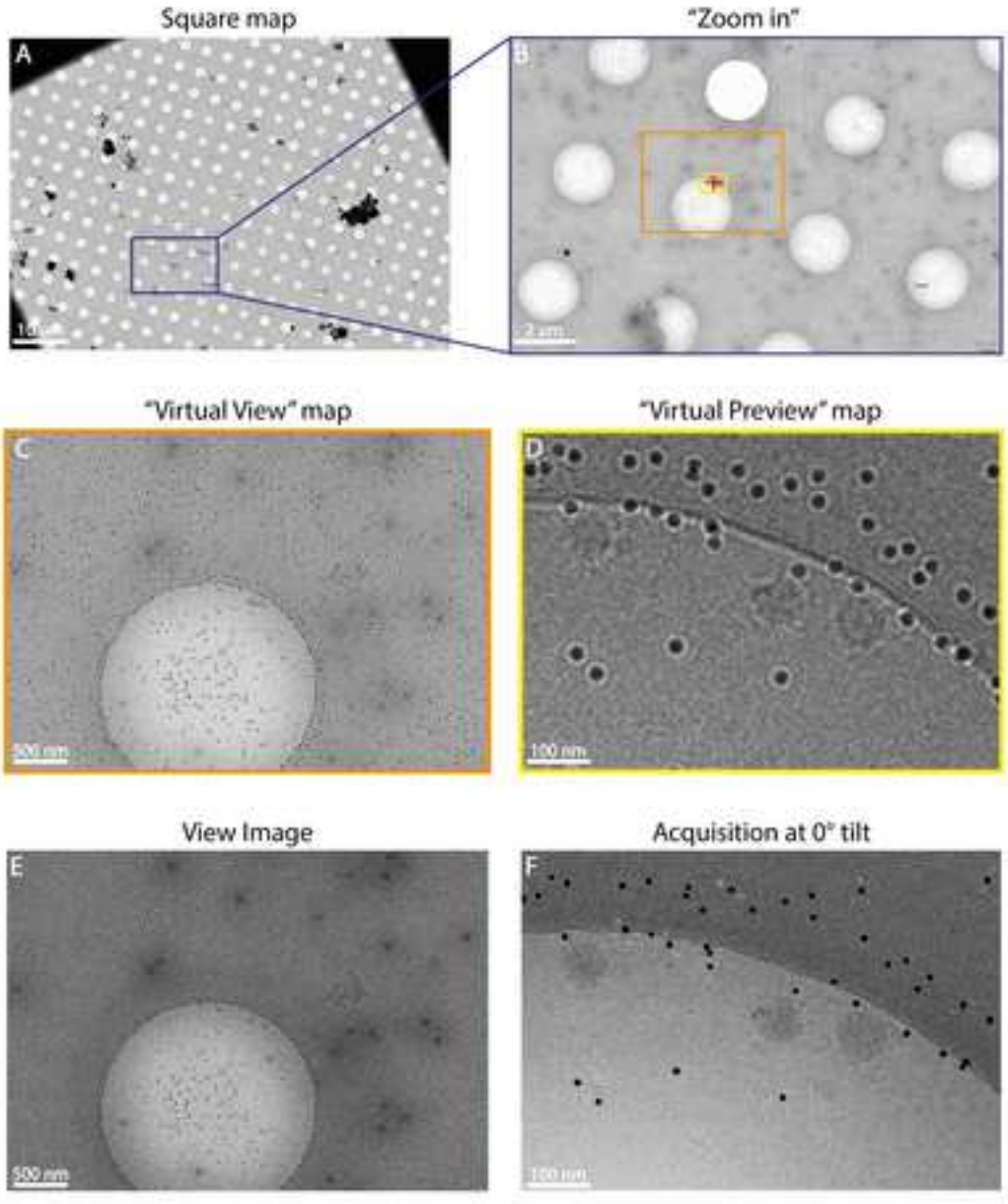

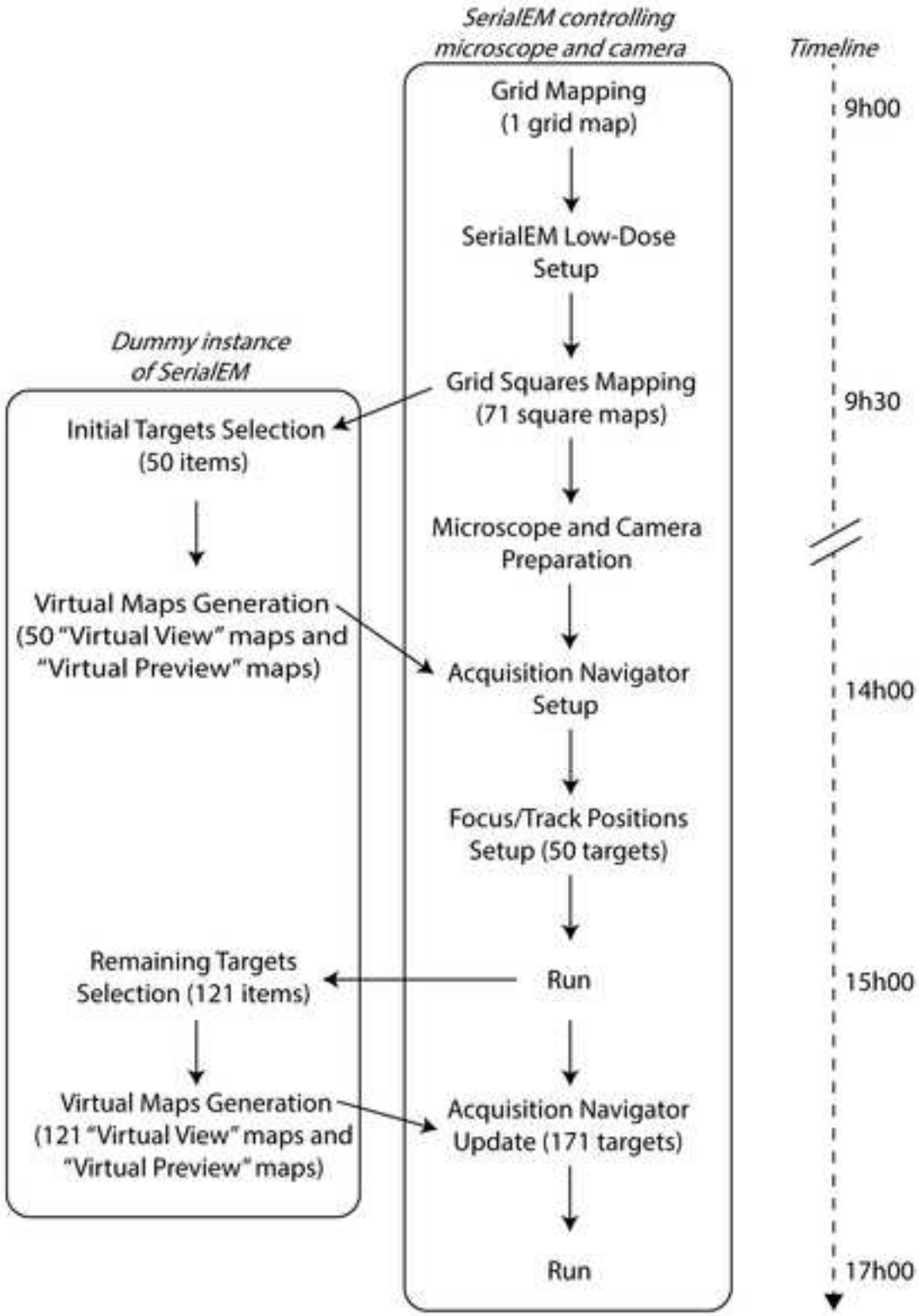

Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Transmission Electron Microscope			Our protocol is only based on computational workflows. T

he user will only need acess to a TEM of any kind

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11/02/2021

Revised manuscript "Strategies for optimisation of cryogenic electron tomography data acquisition" (Weis et al.)

Dear Dr. Iyer,

Thank you and your reviewers for the positive assessment of our manuscript. Please find attached a revised version of the manuscript which we reformatted according to your editorial requests. We addressed the comments made by the three reviewers and edited the text accordingly, as detailed within the reviewer comments below.

As requested, we highlighted the sections of the Protocol that include the essential steps for the production of the video.

Yours sincerely,

Simone Mattei

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes a more streamlined and updated version of the protocol previously published in Nature Methods (Schorb et al., 2019). The included scripts and tips will enable experienced users to speed up the initial phases of their cryoET sessions.
I want to emphasize that I did not have the opportunity to test the supplied software and scripts myself.
Overall, I think some steps of the protocol are aimed more at beginners while other steps already require prior experience. The manuscript would benefit from considering the target audience when deciding which steps to describe in detail and which not.

Minor Concerns:
1. Prerequisites
I think it is important to mention which version of SerialEM is required for use of the described protocol. Many institutions don't update SerialEM regularly. It should also be pointed out that the supplied scripts have to be imported.

The required version is SerialEM version 3.8, we have added this in the text.

2. Grid mapping

3. Grid square mapping
The defocus offset of -100 microns in view could also be higher depending on the sample visibility. Will a higher defocus offset cause problems when realigning to virtual maps later?

If needed to better identify the sample, a defocus higher than -100 could be used for grid square mapping. The amount of defocus used will not affect the efficiency of the realignment procedure when the microscope is properly aligned and the serialEM high-defocus mag calibration (calibration menu) has been performed for the appropriate used settings.

The function of "Shift + N" (Create new file at item) should be mentioned to avoid users blindly using shortcuts.

We have added this in the text.

"Acquire at Points" is actually called "Acquire at Items" in the software.

We thank the reviewer for pointing out this inconsistency, “Acquire at Points” was indeed the nomenclature used in older versions of SerialEM. We have changed this in the text.

4. Selecting targets
Does the navigator have to be saved before merging or will SerialEM keep track of other running instances?

Yes, it has to be saved. We have added this in the text.

5. Set up SerialEM low dose
It should be pointed out that no target is needed for the initial view and preview image and merely their imaging settings are used by pyEM.

We have added this in the text.

6. Generate virtual maps
The SerialEM tools tab needs to be setup properly for the PyEM options to be available. Is this a required step during PyEM setup? If not, it should be emphasized.

Setting up SerialEM tools is not strictly necessary however doing so greatly simplifies launching the procedure which would otherwise involve the use of the command line.

7. Setup Navigator
The option of running a custom script instead of the TS should be mentioned.

We have added this in the text as a note.

It says, "Don't change anything in the next File Properties dialog", but these settings depend on what a user set previously which was not described.

We have edited the text to encourage the user choosing the desired parameters based on their needs and setup of the instrument.

8. Set focus/track positions
I don't think this step is needed for normal cryoET collection unless the default focus area is obstructed in individual cases.

We think that this step is indeed necessary when acquiring tilt series for high-resolution tomography or for cellular tomography *on lamella*, in those cases there is less space around the region of interested and an accurate positioning of focus/track areas is often critical.

It should also be mentioned that the focus area should remain along the tilt axis.

We have added this in the text.

9. Setup additional sripts
This step should also be optional. I was wondering why 2 scripts were necessary for this task until I saw the additional checks that 'duringtomo' takes care of.

We consider this step not optional for the successful application of the described protocol. Please, see below additional explanations regarding both scripts.

Pretomo script
This script runs eucentric height, backlashes the stage alpha tilt and then realigns to item. The SerialEM Acquire At Items dialog would do this in a different order which can lead to bad tracking behaviour for high-mag tomography data acquisition.
The script also takes care of a focus range over the tilt series by setting a persistent variable $TomoTargetDefocus which later is called by the during-tomo script: SerialEM does not have such functionality, so it would require setting a focus value for each tilt series manually in the Navigator window, a laborious process.

Duringtomo script

The SerialEM tilt series controller defines the defocus target value, by running this script each tilt angle, it overrides this value with the focus value set by pretomo script. This is key for subtomogram averaging data which needs a focus range to fill Fourier space properly.

REPRESENTATIVE RESULTS

The authors describe in a single sentence the microscope preparations that have been done. I think it is important to include this in the actual protocol. It might also be worth discussing which other alignments to check, when working in a non Wim-operated facility.

Standard procedures for microscope alignment and tuning are performed according to TFS manual and their thorough description is beyond the scope of this publication. A comprehensive and detailed set of instructions on how to setup and tune the microscope is included in the supplementary material with step-by-step operations and relative screenshots. The full protocol can be downloaded at the following link:
https://oc.embl.de/index.php/s/9OuTl8AazDkCNq0/download

The following has been added to the protocol as section "7" to ensure that best practice in microscope tuning will be followed. All details and screenshot can be found at the link above.

"To assure proper microscope performance, using the same magnification and beam size setup for data acquisition, in this order:

1. Run SeriaLEM 'Coma-free alignment by CTF' (aka Zemlin tableau).
2. Insert and centre an objective aperture.
3. Run SerialEM 'Correct Astigmatism by CTF' (aka auto-stigmate).
4. Run GIF Quick Tune (= only slit focus).

NOTE: As procedures 1, 3 and 4 might require more dose rate, only the spot size should be changed, beam size should not be changed as this causes beam tilt which makes the tunings incorrect.

Procedures 1, 2, and 3 are semi-automated in this publicly available script:
https://serialemscripts.nexperion.net/script/47"

I would be interested in hearing how successful the realignToItem routine is using high-magnification virtual maps. Does the high defocus offset in View cause problems? Are the generated coordinates by PyEM precise enough or does it usually need more than one round of realignment?

Multiple rounds of realignment serve the purpose to bring the starting beam-image shift down, it is not related to actual realignment accuracy. The generated coordinates are precise enough. The RealignToItem procedure described is done in two stages first using a low-magnification "View" image and then a high-magnification "Preview" image. High defocus is not problematic as SerialEM cross-correlation applies low-pass filtering and SerialEM has a Search + and - Scaling in Realign (Navigator - Options submenu) option.

DISCUSSION
It should be mentioned somewhere that target picking in dummy instances of SerialEM for SPA is also useful and already being used.

In the current version of the discussion such statement (with respective references) is already present: “This procedure can easily be modified to only use the intermediate magnification View image when alignment accuracy is of less importance, for instance in the case of large structures where ultimate target accuracy is of less concern[10] or for single particle analysis samples that are poorly spread on the cryo-grid requiring the user’s manual selection of each acquisition point.”

Figure 1
Headlines over the panels in the figure would make it faster to grasp.

We have added headlines over each panel as requested.

Figure 2
SerialEM Low-Dose Setup needs to be done before going to the dummy instance to make sure PyEM has the right imaging settings for the virtual maps. Microscope and camera preparations should also be included in the schematic.

We have now modified Figure 2 according to the suggestions.

Varia:
I am concerned that 'beam time' is a colloquialism in the cryoEM field and should be replaced with 'instrument time' or something similar.

We have changed “beam time” with “microscope time”.

Reviewer #2:
Manuscript Summary:
This manuscript provides a detailed yet digestible protocol for an improved method for selecting biological targets for downstream automated tilt series acquisition. This strategy takes advantage of scripting functionality in both PyEM and SerialEM packages that allows the user to select targets "offline" using "virtual" MAPS, all while the microscope is performing other required tasks for the data acquisition setup. As a result, the user maximizes efficiency during the laborious set-up process by simultaneously acquiring images and queuing targets, thereby significantly improving the overall throughput and efficiency of the tilt series data collection process. The authors capitalize on their recent efforts to collect large-scale datasets for structure determination of Sars-Cov-2 sample as a proof-of-concept for this improved approach.

Overall, the strategy described in this manuscript addresses an often overlooked but significant bottleneck in the cryo-electron tomography workflow, and I wholeheartedly

believe this protocol will be of substantial interest to many microscopists in the tomography community. It is extremely thorough, clear, and well-written, and is reminiscent of a typical in-house data collection protocol for a tomography lab. The authors are experts in this area, and this is reflected in the level of detail by which this protocol is described. I sincerely hope this article encourages other groups to publish similar, hands-on protocols for other aspects of the tomography workflow.

Major Concerns:
One aspect that is lacking from this manuscript is a section highlighting potential failure points of the procedure, with solutions and/or workarounds to address these issues. For example, are there situations in which the targeted "virtual maps" may not match up with the "real maps" used during subsequent tilt series data collection?

We did not experience any failure of the described procedure. Running the workflow described in this protocol does not come with any additional risk of failure compared to any other automated acquisition routine. Automated routines that rely on finding back selected acquisition areas could fail if the specimen undergoes a considerable change (e.g., when the support film or the cellular lamella break). To minimise such risks the user has to accurately choose the regions of the grid square that are selected for image acquisition during the session set-up, for example by staying away from regions that present cracks in the support film or that are nearby prominent contaminations. However, in the case of failed realignment procedure, the software will acquire a tomogram in the resulting wrong region but then it will proceed to the next position.

One potential concern with "bulk" set-up procedures is that an upstream error in image registration may affect all downstream targeting. In the worst case, this error may only be detected upon initiation of tilt series collection, after the user has spent a great deal of time queuing hundreds of targets. It would be extremely valuable to briefly describe these potential problematic scenarios, and importantly, steps the user could take to fix and/or mitigate potential issues.

We have not suffered such scenarios and hence are unaware how to deal with these. The only thing we are aware of is that SerialEM has a Shift-To-Marker procedure to shift all navigator positions by some X and Y shift.

Minor Concerns:
This is a minor concern and likely out-of-scope for this manuscript, but I believe some follow-up describing these steps as it relates to the acquisition of cryo-focused ion beam (cryo-FIB)-milled lamella would be of substantial interest to the cellular tomography field. Although I anticipate that many of the steps outlined in this current protocol can be amended to target on cryo-lamella, there are unique challenges (determining lamella pre-tilt, careful selection of focus position, etc) that may require significant optimization and therefore may merit separate instruction. If the authors have already optimized some of these parameters, I believe it would be important to include in this manuscript. Otherwise, this information would be extremely useful for the field and may merit its own separate protocol for a future

publication.

As the reviewer pointed out, the described protocol is perfectly applicable to cryo-FIB-milled lamellae and does not need any variation/optimization. At the moment we haven't produced any additional protocol specifically designed for on lamella tomography and this would be out of scope for the presented protocol, however we fully agree on the relevance of such protocols. The EM manual here provided as supplementary material is publicly available and it will be regularly updated with future adaptations and implementations.

Reviewer #3:
Manuscript Summary:
The manuscript provides practical guidance on a novel routine that expedites the selection and set up of acquisition positions in cryo-ET and permits these operations to be done virtually, without the need for microscope access. A conventional approach uses hierarchical mapping of a cryo sample grid at four increasing magnifications: (I) low magnification grid map, (II) square map, (III) view map and, finally, (IV) preview map captured at acquisition magnification. Capturing view and preview maps is the lengthiest operation and contributes most of the "pre-acquisition" radiation damage to a sample within this workflow. Author's PyEM software package generates the later maps and their coordinates from grid square maps and passes them into the microscope-controlling SerialEM software thereby avoiding the need for their physical acquisition of these images. Furthermore, the microscope can continue sample imaging during such virtual maps generations and positions set-up enabling up to several folds improved throughput. Published in 2019 pyEM-focused paper (ref16) has facilitated many cryo-ET projects and has already attracted impressive 90 citations. The current submission is a much needed follow up providing users with practical protocol and exemplifying it with a large dataset collection case study. Furthermore, manuscript's concise protocol is supported by its comprehensive and well-illustrated version available for download. I, therefore, enthusiastically recommend that the submission be accepted for publication with minor changes.

Important changes:
1. After the introduction of " dummy serialEM" session, it is important to specify if the operation is done in "dummy" or "microscope-operating" instances of Serial EM. For example, step 5 "Set up serialEM low dose" switches from "dummy" to "operating" SerialEM without declaring that switch.

We encourage users to install the two SerialEM instances on two different computers: the "operating" one on the SerialEM computer that controls the microscope and the dummy instance on a second machine. Therefore, all operations that involve actual image acquisition or microscope setup must be carried out on the SerialEM instance on the microscope computer.

We have added "dummy SerialEM" to all operations that have to be performed in the dummy instance of SerialEM.

Figure 2 also graphically illustrates what are the steps to be performed on the different instances.

2. Keep the names of operations consistent between the protocol and the flowchart in Figure 2.

We have carefully double-checked the consistency of the names of operations between the protocol and the flowchart in Figure 2.

3. in "6.2. Run PyEM "Virtual maps" script from SerialEM menu, Tool." explain what outputs are being generated so they do not suddenly appear at the next step.

We have added a “note” explaining what is generated:
“NOTE: PyEM starts a command window which automatically closes when done, then the new navigator file can be opened. Per montaged map that contains selected points, PyEM writes a single merged map and all its View and Preview maps. Finally it writes a new Navigator file called <navigatorfilename>_automaps.nav.”

Suggestions:
1. "2.2. Set up imaging conditions suitable for mapping the entire grid. This should be done at the lowest magnification possible taking into account the field of view on the detector". I suggest to also provide the actual parameters (in this case magnification) that will be used in the demo video.

We have added this information (EFTEM SA 2250x)

2. "2.3. Set up full montage
2.3.1. open the SerialEM "Navigator" menu
2.3.2. select "Montaging & Grids"
2.3.3. select "Setup Full Montage"
For better readability, I suggest reorganising this GIU option selection operation into an in-line description. For example, "In Serial EM menu, open the "Setup Full Montage" dialogue window (Navigator->Montaging &Grids-> Setup Full Montage), check the parameters and click OK.)".

Although we agree with the reviewer’s comment, we think that the current version better reflects the following JoVE “Author instructions”: “The protocol must be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol.”

We would be happy to change this according to the editor’s comments.

3. The flowchart in Figure 2 would also benefit from indicating what type of key maps or metadata are generated/acquired in each step (when applicable).

We have modified Figure 2 adding more detailed information about the maps generated in all steps.