EPU–Automated Single Particles Acquisition Software

Tips & tricks: EPU workflow set-up & application examples

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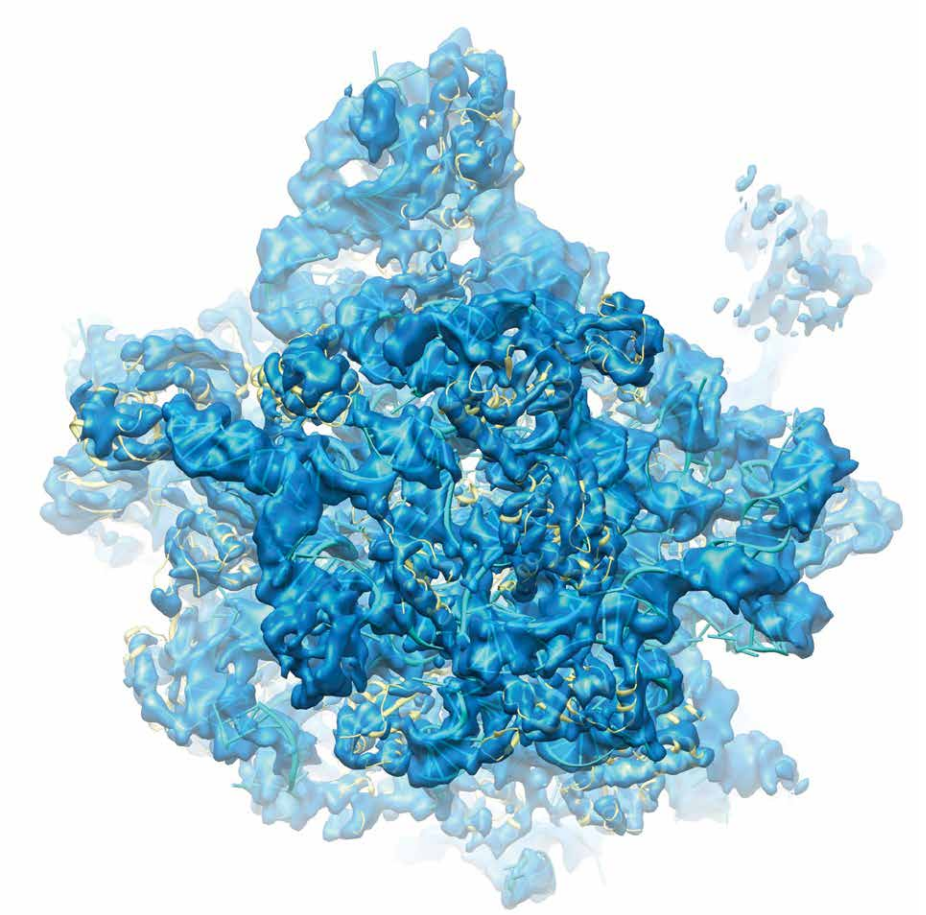
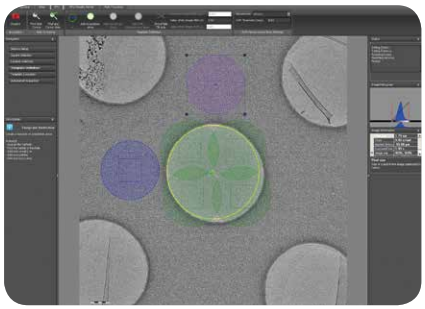
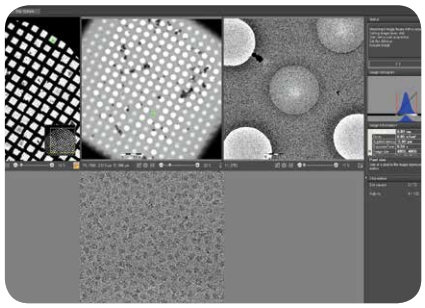
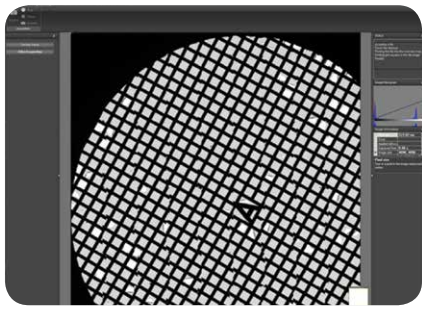








EPU stands for *E Pluribus Unum*—Latin phrase for "Out of many, One." It is the process of collecting many views of a biological macromolecule which are then combined computationally to build the corresponding three-dimensional structure. In structural biology this is referred to as single-particle analysis.

EPU is the latest product among the set of FEI® software applications released to increase the throughput and ease-of-use of FEI electron microscopes for biological research. EPU runs on Tecnai™ and Titan™ platforms and is fully compatible with all cameras and detectors which are fully integrated with FEI hardware.

EPU is a reliable, easy-to-use application that collects from tens of thousands up to millions of molecular views of a biological object in a quasi-automated fashion. Typically, it takes less than one hour to set up an EPU session that can run for days or weeks, depending on the microscope being used. For example, it's possible to collect more than one million particles in a four-day session on an FEI Titan Krios™ microscope. Thanks to the stability of the Titan platform and the high sensitivity of the FEI Falcon™ direct electron detector, a very large amount of extremely high-quality data can be collected in a short amount of time.

Before starting an EPU run

Check the documentation that came with your EPU software on the DVD. In particular, we strongly suggest reading the EPU Preconditions document to correctly align and set up the microscope and run an automated data acquisition in a reliable, reproducible way.

Detailed instructions include a step-by-step guide and checklists to ensure your microscope is ready for EPU.

Atlas acquisition

The Atlas acquisition relies exclusively on magnification calibrations, particularly those made in LM mode. If you are setting up an EPU run for the first time, it is strongly recommended to redo the magnification calibrations in both LM and SA modes with a cross-grating sample. Directions for microscope setup, regardless of type, are included; however, specific instructions are given for the Tecnai and Titan systems separately.

NOTE: Pay special attention to the alignments and calibrations sections. Most preconditions are also noted in Chapter 2 of the EPU User Manual.

EPU workflow: an overview

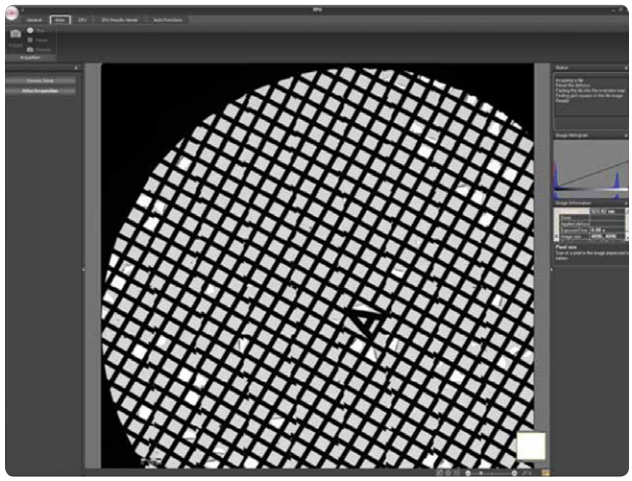
The EPU workflow consists of:

- Atlas acquisition (whole grid overview)
- Square selection
- Ice filter location selection
- Template definition
- Template execution
- Automated acquisition

At the end of this overview, you'll also find examples of application results as well as troubleshooting options.

Atlas acquisition

The Atlas acquisition relies exclusively on magnification calibrations, particularly those made in LM mode. If you are setting up an EPU run for the first time, it is strongly recommended to redo the magnification calibrations in both LM and SA modes with a cross-grating sample.



↑ **Figure 1.** Atlas acquired on a cross-grating sample; the 'A' represents the center of the grid.

- On a Titan Krios, the lowest available LM magnification is 40X, which may not allow the whole grid to be visualized in the Atlas (especially if it was acquired on a Gatan Orius CCD). That said, there are still hundreds of usable squares, and data collection will be possible for a few weeks, assuming that all the squares would be used for the automated high-resolution data acquisition.
- The LM magnification calibrations are done in focus, so correct the eucentric height and eucentric focus before the Atlas acquisition.
- The closer to focus, the better the "stitching" of the tiles in the Atlas (though the tiles are simply inserted in the Atlas view and not really stitched with an algorithm.) Note that this does not apply to the Titan column, provided that the beam is kept parallel in all settings.

- Before running the Atlas:
  - Choose the appropriate settings in the ribbon
  - Acquire a single preview in the EPU screen
  - Verify that the image is in focus. Remember to remove the objective aperture and disable the SA diffraction aperture as it may engage automatically, especially if the React to Mode Changes option is selected and active in the Aperture OCX control xpanel (main Tem UI).

Note: In EPU, you'll set up two separate sessions detailed as follows: one for the Atlas and one for the automated data acquisition. If you're using EPU for the first time, realize these are two separate workflow steps.

Session 1: Atlas acquisition

The Atlas acquisition relies exclusively on magnification calibrations, particularly those made in LM mode. If you are setting up an EPU run for the first time, it is strongly recommended to redo the magnification calibrations in both LM and SA modes with a cross-grating sample.

The first session is for the Atlas acquisition, which is usually performed on the local microscope PC in a common folder. The Atlas is saved separately and can be recalled (loaded) from the EPU UI at any time should a problem occur. FEI recommends archiving the JPEG overview, but once the whole data acquisition is finished, the Atlas data on the local microscope PC can be discarded.

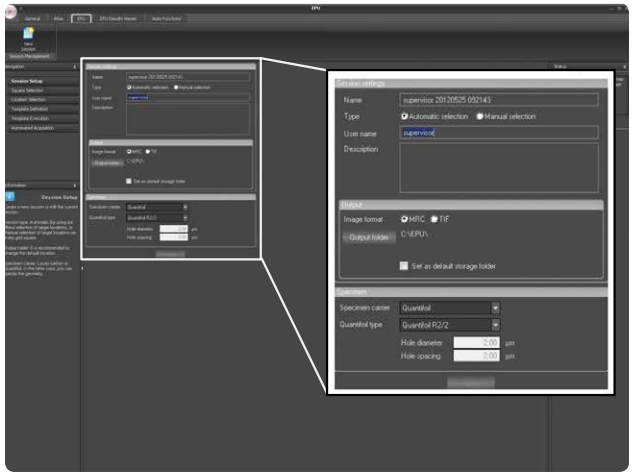
Grid square acquisition

This is also performed in the LM mode, but at a slightly higher magnification (depending on the microscope in use). On a Titan Krios, this would be around LM 165-320X. The idea is to have one grid square well visible in the field of view, so adjust the magnification depending on the grid type (200-, 300-or 400-mesh) and the CCD camera used.

Even though this acquisition is done in the LM mode, we strongly recommend inserting and centering the objective aperture to improve the results obtained by the ice filter (see following sections). On a Titan, make sure to set a parallel illumination area.

Image shift calibration

Before starting the session setup, run the image shift calibration (refer to the EPU user manual). It's critical that a feature "found" in the grid square acquisition (LM 165X) remains perfectly centered in the "Hole/Foil view" acquisition (SA 3800-5000X). See Troubleshooting Common Problems at the end of this document for more details.



↑ **Figure 2.** EPU session setup with MRC file format and holey carbon grid support from Quantifoil, type R2/2.

Session 2: Automated data acquisition

Setting up the EPU session

Once the Atlas acquisition is completed, set up an EPU session in the EPU tab and specify:

- File format
- Storage location (FEI suggests a file server due to the quantity of data collected over a typical 3-day run)
- Type of grid used (Quantifoil/C-flat, size/spacing of holes, etc.)

This is illustrated in **Figure 2**, with MRC as an output format and a Quantifoil R2/2 holey carbon support grid. Once the session has been defined, develop a unique protocol and follow the steps to ensure a smooth workflow. Think about the experiment beforehand, choose all the parameters appropriately in the ribbon view to avoid jumping back and forth between tabs as EPU may react slowly to sudden tab changes.

Square selection

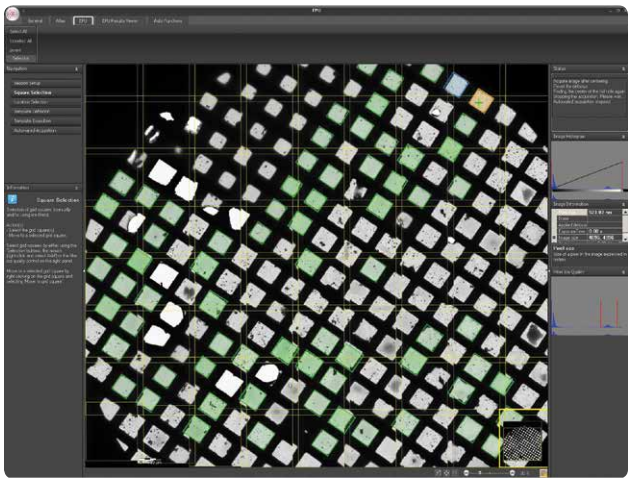
The Atlas will now appear in the main EPU UI and have all squares selected by default. To select specific squares:

- On the right side of the EPU UI, move the sliders in the histogram window to change square selection.

-or-

- Click Select None underneath the main ribbon and add squares manually. (This time-consuming and not really required on a well-prepared grid with regular ice thickness.)

NOTE: Pay special attention to the alignments and calibrations sections. Most preconditions are also noted in Chapter 2 of the EPU User Manual.



↑ **Figure 3.** The square selected with the histogram filter (on the right side) appears in real time on the Atlas itself.

By moving move the sliders slowly, the square selection will be updated in the main screen in real time. You can see that broken squares, squares blocked by ice or those that are too dark will be excluded automatically and will not be visited during the automated data acquisition.

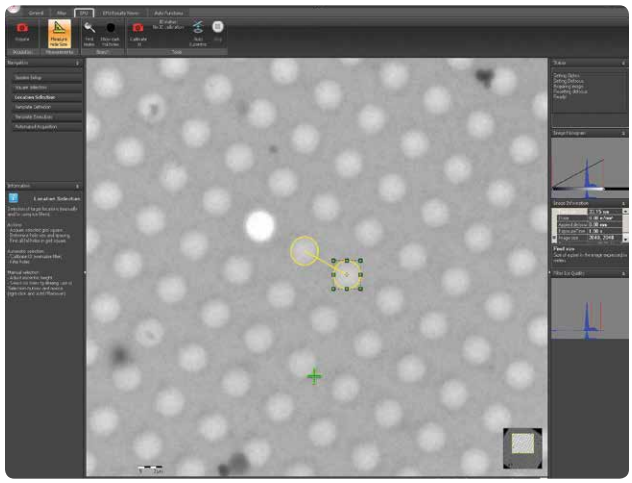
Don't be concerned about selected squares that don't seem to be fully visualized or are distorted in the Atlas view. If they are selected, they will appear normally in the appropriate later view. You can check by right-clicking on one such square and choosing the Move Stage to this Grid Square option. Wait for the stage position cross to appear in the aforementioned square, and then start a grid square acquisition preview in the appropriate tab.

Choose a "good" square, where you can see an intact support film. Ideally, select a square with a few empty holes and many others that are filled with homogeneous, thin ice. This will help the ice filter setup in the next step.

Ice filter location selection

In this critical step, the ice filter will be applied throughout the whole automated data collection.

- Begin with the location selection screen and start an acquisition using the red Acquire button. This should still be in the LM mode (e.g., 165-320X).
- Click Measure Hole Size. Two yellow circles will display in the main screen.
- If you selected the correct grid type during EPU session setup, and if the magnification calibration has been performed correctly, the size of the holes should be approximately correct. Their position, however, will not. Correct for both by using the mouse to drag them toward two continuous holes, as illustrated in **Figure 4**.

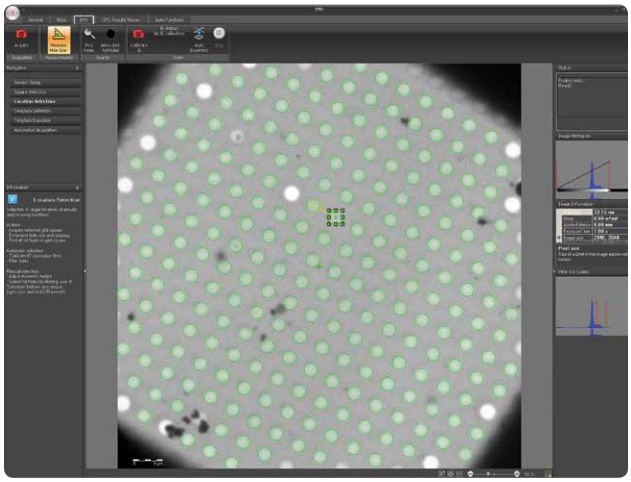


↑ **Figure 4.** Move and resize the yellow circles to match the size and position of the holes in your actual view.

- After positioning and resizing the yellow circles to match the current view, proceed by clicking Find Holes, which will highlight all the holes in the visible field of view, with a pattern following your initial preset. Note: The position of the other holes is thus calculated according to the initial preset; therefore, it is important to define the size and position precisely.
- Ideally, if the I0 calibration has been performed beforehand, choose the holes based on the intensity reading inside the circles (refer to the EPU user manual for this calibration).

This next step is crucial for success: the ice filter set-up, illustrated as follows. This example depicts a real experiment, similar to what would happen with cryo-EM samples.

The ice filter (**Figure 5**, right side, lower panel) shows the main histogram and the red sliders. The latter will have to be adjusted carefully to select holes with thin ice while avoiding those that are empty or too dark (i.e., bad ice or contamination).



↑ **Figure 5.** Move the ice filter sliders carefully to automatically deselect empty holes or holes that are too dark.

The ice filter works essentially with gray intensities and will perform quite nicely in the current field of view; however, if your grid contains a heavy ice thickness gradient, the ice filter settings chosen in the current view may not necessarily apply elsewhere in the grid, where the intensities may change substantially. EPU performs very well over a nice, homogeneous grid. If the grid is heavily contaminated, or bent, or contains a large variation of intensities, EPU performs moderately well. To adjust, you can monitor the run and change the ice filter settings accordingly. FEI aims to enable future EPU application versions to assign priorities to grid regions, thereby avoiding challenges due to large variations within one grid.

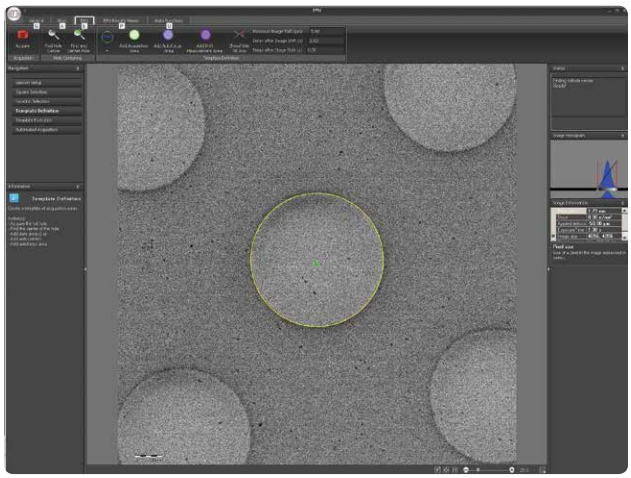
Template definition

Now it's time to define a template for the automated data acquisition. At this point, if settings were correct in the main ribbon tab, the microscope should switch to the SA mode.

NOTE: FEI strongly suggests redoing the magnification calibrations for the SA mode as well.

Choosing magnification

- Choose a magnification that will provide a field of view with one hole (clearly visible) and a few others also present, but not necessarily fully visible (see **Figure 6**, as follows).
- On a Titan Krios, make sure your beam is parallel and set the magnification between SA 3800X and SA 5000X.



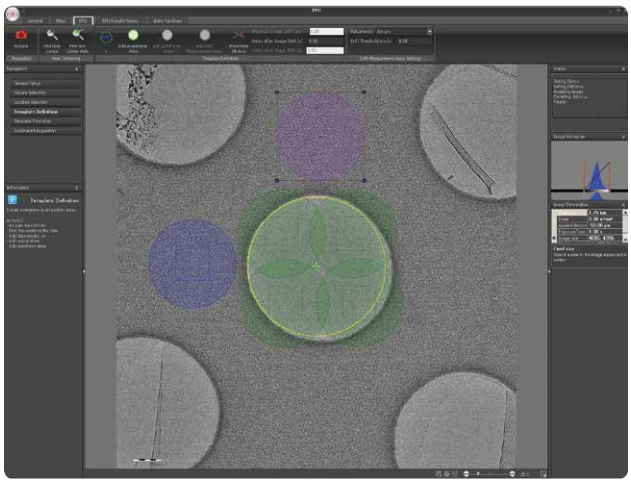
↑ **Figure 6.** After the acquisition, the find hole center algorithm will select the hole closest to the middle and center it. A new preview is acquired when Find and Center Hole is clicked.

Finding hole center

- The find hole center algorithm will find the hole closest to the center, regardless of what is inside or whether it's empty.
- If a good hole with thin ice is within the field of view, you can also right-click on that filled hole, center that feature and click Acquire again. The Find Hole and Center button will then keep the hole you selected in the center, as illustrated.

Inserting areas for acquisition

- Proceed to insert areas for acquisition using the green button in the ribbon, or choose an automatic selection pattern (may be of limited use). You could start with the automated pattern and then move the circles manually with the mouse.
- The size of the green circles depends on the settings you have selected in the main ribbon under the Data Acquisition option. Although it is not advisable, you can return to the main tab and change those settings while the EPU session setup is still running.
- Depending on your project needs, you can switch to a different setting to increase the number of acquisitions within one hole. Depending on your desired outcome (e.g., pixel size), the magnification must be adjusted accordingly. In **Figure 7**, we first selected Microprobe TEM, nominal magnification of 59000X, and a parallel illumination area of about 1.2 microns. With these settings, you can position four acquisition areas next to each other. Note: While the beam area slightly overlaps and touches the carbon foil (to avoid charge-induced drift), the areas exposed and captured by the detector are fully inside the hole (green squares inside each green circle).



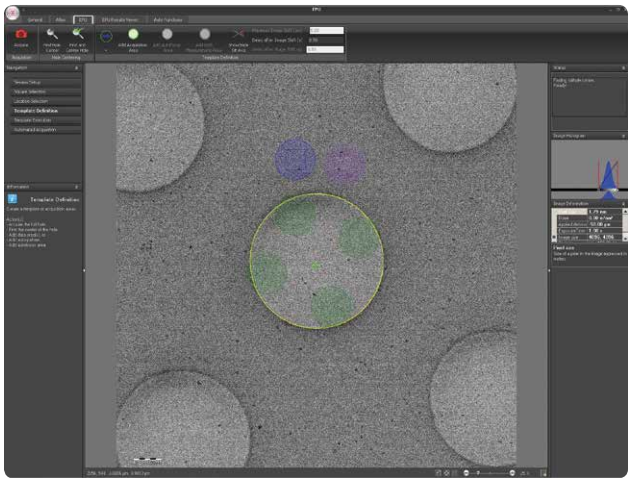
↑ **Figure 7.** Move the areas after insertion, position them as you wish. An example for Microprobe TEM is illustrated in this figure.

Performing drift measurement

- An auto-focus area (blue circle) and a drift measurement area (magenta circle) can be positioned around the acquisition hole as illustrated. Be careful to move the auto-focus area close enough to the hole without pre-exposing the sample.
- Usually the drift measurement area is placed on top of the auto-focus one to overlap perfectly. This helps the drift measurement because the auto-focus procedure will burn away the thin ice layer that may be present on the carbon foil. The drift threshold can be set to a strict drift rate; 0.05 nm/sec or even lower is often used. (Typical drift rates measured during the automated run are on the order of 0.01-0.03 nm/sec on a Titan Krios.) This should guarantee high-resolution results in terms of acceptable drift rates for single particles acquisition.

Additional notes

- In EPU version 1.2.X, it's possible to set up more than one acquisition area for the same field of view (i.e., to perform a focal pair). It's also possible to define a different defocus value for several acquisition areas within the same hole, or across several holes.
- To increase throughput on a Titan Krios, you can easily switch to Nanoprobe TEM imaging mode. [This will not work with Tecnai's Nanoprobe (i.e., small probe mode for EDX or STEM)]. This forms a small parallel beam to increase the number of exposure areas within one hole, as illustrated in **Figure 8**. Depicting a routine setup used to acquire high-resolution single particles data, we switched to TEM Nanoprobe on the Titan Krios, and selected 96000X nominal magnification (corresponding to 0.88 nm pixel size on the FEI Falcon direct electron detector installed at NeCEN) and a parallel illuminated area of 650 nm. As shown in **Figure 8**, the auto-focus and drift measurement areas were set up exactly the same.



↑ **Figure 8.** Move the areas after insertion and position them as desired. Note how much smaller the green acquisition areas are (as compared to Figure 7), thus the allowing for the setup of as many as eight per hole with these settings. Only four are shown for clarity.

Template execution

In this step, you'll see in real time what EPU can do in an automated fashion.

- Click Preview and watch EPU perform a few auto functions and collect the images as defined in the template.
- If something is amiss, you'll have an opportunity to make adjustments before launching the automated run.
- Eucentric height should be roughly corrected already, but you can run the eucentric height procedure again in the EPU Auto-Functions tab by using the stage tilt or beam tilt.
- Check the beam tilt pivot points and the rotation center at this point (eucentric focus) before launching the run.
- Perform 'stand-alone' auto-focusing and drift measurement, if desired.

Automated acquisition

The first step of the automated run moves the stage to the first square that was chosen in Square Selection (**Figure 3**) and performs the auto-eucentric height procedure using the beam tilt. If this fails, EPU moves to the next square and repeats the procedure.

- Set the eucentric height manually at this point. If the procedure keeps failing, stop the run and check it again.
- When the eucentric height procedure succeeds for a given square, auto-focus begins. The bottom panel always displays a cross-correlation peak when these procedures are run. If the peak is well visible and a small shift can be calculated (i.e., the peak is not too far from the center), the beam settings are good and EPU will likely continue to the next task. The top central panel is reserved for the grid square view, and it will appear only if the auto procedures were completed successfully.
- During the automated run, the main EPU UI will show the Atlas in the top left corner, the Grid Square view in the top-middle panel and the Foil view on the top right panel (**Figure 10**). A status log is temporarily displayed in the top right corner, while the bottom panel is reserved for the acquired images. The information on the image being acquired can be read on the right side, below the histogram, but only until the next image appears (which may have different defocus settings, for instance). The information panel keeps track of the number of squares visited over the total number of selected squares. The number of holes refers to the current square (top middle panel) and this is determined by the ice filter settings applied each time a new Grid Square view is acquired.

Examples: application results

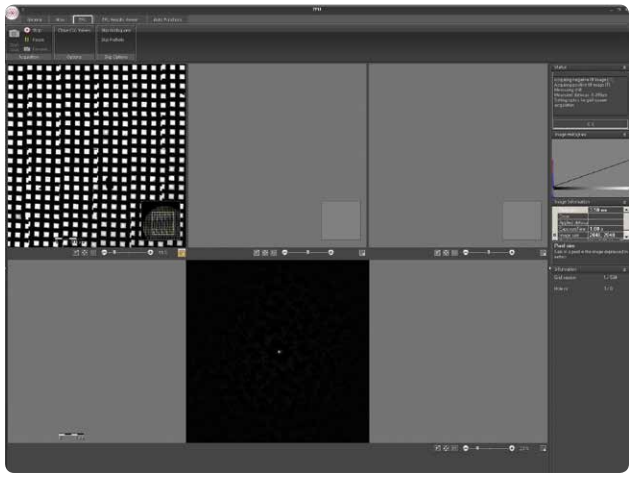
Results obtained using the FEI EPU application include:

- Lumbricus terrestris hemoglobin (Dr. Sacha De Carlo and Dr. Gert Oostergetel, NeCEN, Netherlands)
- 50S ribosomal subunit from Methanothermobacter thermautotrophicus (Dr. Daniel Böhringer, ETHZ, Switzerland)

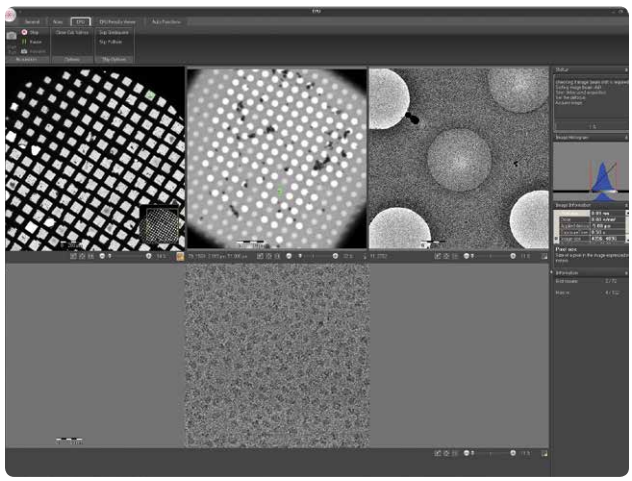
Application example #1: worm hemoglobin

Approximately 40,000 particles were collected in one overnight session on the NeCEN Titan Krios I using the Nanoprobe TEM setting explained in **Figure 8**, with defocus values ranging from -2.0 to -3.5 µm. The dataset then was separated into defocus groups. For illustration purposes, only the high-defocus data collected on the FEI Falcon direct electron detector are shown.

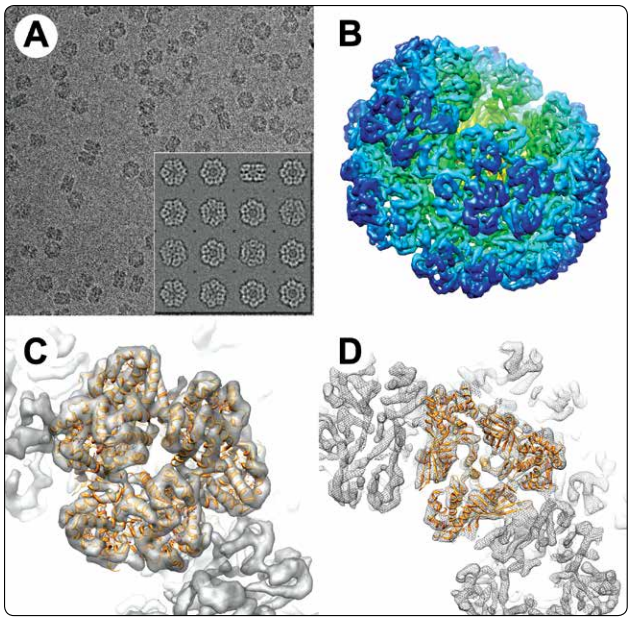
Fully-automated CTF correction on the full dataset and further image analysis was performed with IMAGIC-5 4D image processing package in collaboration with Prof. Marin van Heel (NeCEN).



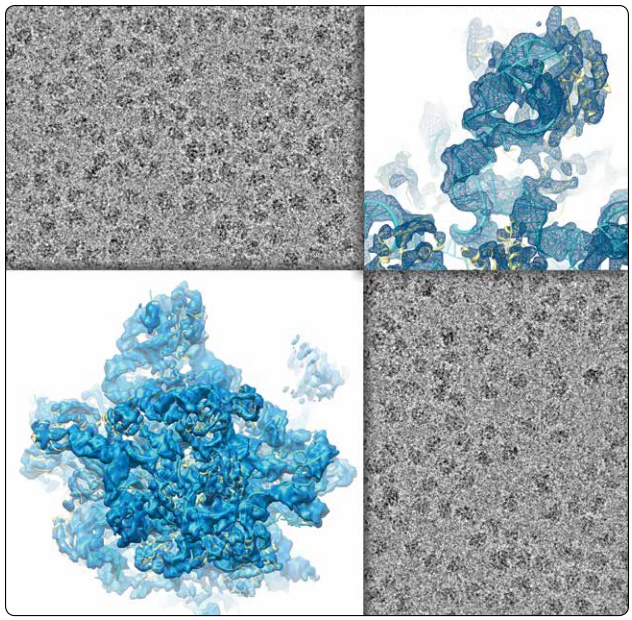
↑ **Figure 9.** Bottom panel displays cross-correlation peak during auto-functions (i.e., eucentric height, auto-focus).



↑ **Figure 10.** During the automated run, the task selection panel can be hidden with a mouse click, and the main EPU UI will look like the one above. (Top left) Atlas; middle: Grid Square (current view gets updated); (Top right) Foil view (gets updated). Bottom panel: current image acquired).



↑ **Figure 11.** (A) Typical view of worm hemoglobin as observed in cryo-EM. (Inset) a few representative class-averages from the data shown in (A). (B) Surface rendering of the worm hemoglobin 3D reconstruction showing secondary structure elements at about 6.5Å resolution. (C & D): Slabs through the hemoglobin subunit (1/12th) viewed from the 3-fold axis.



↑ **Figure 12.** During the automated run, the task selection panel can be hidden with a mouse click, and the main EPU UI will look like the one above. (Top left) Atlas; middle: Grid Square (current view gets updated); (Top right) Foil view (gets updated). (Bottom panel) current image acquired.

Application example #2: ribosome

The 50S ribosomal subunit from Methanothermobacter thermautotrophicus is presented as follows at 5.8 Å resolution (using the FSC0.5 criterion). The data was provided by Dr. Daniel Böhringer from ETH in Zürich, Switzerland. One thousand images were collected on Falcon in a one-night EPU session. 265,000 particles could be picked, 170,500 of which were used for the 3D reconstruction in SPIDER.

Troubleshooting

The following are workaround solutions to some of the common application problems encountered that are not covered in the EPU user manual. If you have a problem that is not listed here, refer to the troubleshooting section of the EPU user manual.

ISSUE	WORKAROUND
Focus stigmators calibrations	If you are running EPU for the first time, this calibration must be done once.
Image shift calibrations	In the EPU scheme for automated data acquisition, the microscope switches from LM to SA mode frequently, and it's important that the LM and SA modes are aligned with respect to each other. A feature that is well-centered in the field of view at the lowest LM magnification (Atlas) should stay centered when switching to a higher LM magnification, as well as all the other SA magnifications used for finding the hole, performing the auto-focus and acquiring the image. This should be checked every time an EPU run is planned. For specific alignment procedures, refer to the TEM UI online help or the EPU user manual. In addition to these basic calibrations, an additional image shift calibration is also present within the EPU user interface. This is typically done for every run to make sure that all features of interest will remain well-centered when switching modes.
Atlas	If the Atlas acquisition fails to start in the predefined stage position, then tiles will be placed in the Atlas view in the wrong position and will not be tiled correctly. A simple work around is to set the optics ready for the Atlas (in the main EPU UI ribbon) then take a simple preview image with EPU. That will set the Atlas mode to the proper setting; usually the Atlas acquisition itself can be restarted and the stage goes to the correct default position. If this does not help, make sure you have corrected the eucentric height and focus. On a Tecnai, make sure you are close to focus (use the focus wobbler if needed); on a Titan use a parallel illumination setting. Otherwise, you'll need to redo the LM magnification calibrations.
EPU run on FEI Falcon direct electron detector	If you set up EPU to collect data on the FEI Falcon detector, make sure that you choose illumination conditions that are compatible (safe) for the Falcon Protector software. Check to see that the eucentric focus preset is correct in the microscope alignments; otherwise, the Falcon protector may be activated during the EPU run and stop it completely. This can happen when the eucentric height procedure (or the auto-focus procedure) within EPU changes the objective lens excitation values outside of the safe range for the Falcon protector software. Another important setting to check before starting the automated EPU run is the illumination conditions of the exposure mode (data acquisition in the main ribbon), especially the total screen current when the sample is present in the hole. Make sure the current reading is the same as noted above while the beam is in the empty hole, and start the gain reference procedure (refer to the best practice document for the Falcon gain reference). This will ensure that all data collected in holes with ice will have the proper gain reference.

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