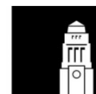

This is a repository copy of *Collection, pre-processing and on-the-fly analysis of data for high-resolution, single-particle cryo-electron microscopy*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/140170/

Version: Supplemental Material

Article:

Thompson, RF, Iadanza, MG, Hesketh, EL et al. (2 more authors) (2018) Collection, pre-processing and on-the-fly analysis of data for high-resolution, single-particle cryo-electron microscopy. Nature Protocols, 14. pp. 100-118. ISSN 1754-2189

https://doi.org/10.1038/s41596-018-0084-8

© 2018, The Author(s), under exclusive licence to Springer Nature America, Inc. This is an author produced version of a paper published in Nature Protocols. Uploaded in accordance with the publisher's self-archiving policy.

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

Supplementary Methods 1- Mirrored protocol for EPU set up with K2 summit direct electron detector.

EPU set up

Transfer of cryo-EM grids to the microscope (30 minutes)

1. Follow steps 1-11 of main protocol to load grids into microscope

Perform initial microscope checks (10 minutes)

2. Check that all required software is open (UI, FluCam viewer, TIA and EPU on Thermo Fisher PC, Digital Micrograph (DM) on Gatan PC).

3. Ensure you are in EFTEM mode (‘UI’ > ‘EFTEM’; ‘EFTEM’ mode should be yellow). Autozoom is off (UI’ > ‘Setup’ > ‘Beam settings’ > ‘Tab out’ > ‘Autozoom’; ‘Autozoom’ should be gray), ‘UI’ > ‘Autoloader’ > ‘Options’, check ‘Turbo Auto Off’). Check the UI for any error messages, as indicated by red icons with a black cross. If errors are present, consult with a member of the facility staff.

4. Check that the gun lens, high tension and extraction voltage in UI are set to the values recommended by your facility management (based on recent alignment of the microscope).

5. Check in UI software that vacuum values are green and all autoloader temperatures are colder than -170 ^{o}C (this may take > 10 minutes after loading specimens).

Gain reference and tune energy filter (45 minutes)

6. With no specimen blocking the beam (i.e. no grid on the column, or over an empty area) go to a magnification to be used for data acquisition, set the spot size to 2. Using the FluCam viewer ensure the beam is centred over the entrance to the Gatan Imaging Filter (GIF) (green circle - use FluCam viewer controls to view) and contract the beam so it is just larger than the entrance to the GIF.
7. In DM software, select ‘Centre ZLP’ (Zero Loss peak). *Troubleshooting*
8. In DM software, ‘Tune Filter’
9. At approximate beam settings for data acquisition, take an image with no specimen in the field of view. If you get a flat image you do not have to perform a gain reference. We perform a gain reference at least after every camera bakeout. In DM software, go Camera > Prepare gain reference. Follow the on-screen instructions, selecting ‘yes’ to ‘Update Hardware Dark’ and ‘Collect Super resolution gain reference’.

Critical- Make sure that the beam is sufficiently expanded so beam fringing is not observed in the gain reference image.

10. Take a counting mode image over vacuum and check the result is a featureless, flat image. If not, you will have to repeat step 8.

Identify grid for data collection (0.5-4 hours)

11. Start grid inventory (‘UI’ > ‘Autoloader’ > ‘Tab out’ > ‘Inventory’) and check whether the number and positions of the grids in the microscope correspond to those loaded. Once the inventory has finished, select the desired grid in the autoloader and select ‘load’. When complete, ‘cartridge successfully loaded’ will be displayed in the UI, the autoloader menu will not display anything in the log area, and the grid will be shown as located in the column if you hover the mouse over that slot in the ‘Autoloader’ menu. The slot position in the autoloader will be colored yellow.

12. Confirm that the grid is suitable for automated data collection, i.e. its appearance is consistent with your screening images. If the grid has been previously loaded into a microscope, assess the quality of the ice to ensure there is no increase in surface ice contamination or any evidence of devitrification.

Critical step- Here, we assume that the grids loaded have been prescreened and identified as suitable for data collection. The 'ideal' ice conditions vary dramatically for different samples; for more guidance, see refs [9, 10].

Determine beam setting pre-sets (Box 1) (30 minutes)

13. Navigate to an intact square which you are unlikely to use for data collection (a dry square works best, and areas of thick ice should be avoided). Set the eucentric height manually at a low magnification to bring the specimen roughly to eucentric height. In EPU, navigate to 'Preparation' > 'Acquisition Optics and Settings' > 'Presets' > 'Grid Square' and 'set' beam settings to the microscope. View on K2 live view in DM software, use the joystick to centre the image on a visible feature like ice contamination. Tilt the stage to 20° by navigating to 'UI' > 'Stage' > 'Set Alpha' (this becomes yellow when activated) and use the z-axis buttons on the right-hand panel to move the feature back to the center of the screen. Click 'Set Alpha' again to return the stage to 0° tilt. If this has been done correctly, the visible feature should still be centered. If not, repeat the process. process.

14. In EPU software, each beam setting preset should be checked to ensure variables are optimal for collection, as these will vary experiment to experiment (Box 1, Supplementary Table 1 and 2). These are set in 'EPU' > 'Preparation' > 'Acquisition' and 'Optics Settings' > 'Presets' dropdown menu. For each setting, on the FluCam viewer ensure that the beam fully illuminates the entire detector, that the beam is parallel (this can be checked in 'UI' > 'Beam Settings' > 'Parallel', 'Spreading' or 'Condensing') and that you are using the dimmest beam needed to complete the Atlas, Grid Square and Hole/Eucentric tasks. This exposes your specimen to as little electron dose as possible prior to the data acquisition exposure. Small adjustments to variables such as the illuminated area can be made later in the session, but changes to spot size can mean repeating steps and can cause delays to data acquisition.

15. Check that the electron dose is appropriate for your detector mode choice and data collection goals (see Box 2 for information of detector dose and Table 1 for example parameters). To set the detector mode, in 'Preparation' > 'Acquisition and Optics Settings' > 'Presets' > 'Data acquisition' select either 'Counted' or 'Super-resolution'. To calculate electron dose, move to an area where there is no specimen, i.e. a broken grid square. Push the data acquisition beam settings to the microscope by clicking 'Set' in EPU. Press 'Eucentric focus' on the hand panel. With the FluCam viewer, check that the beam is centred, if not use the beam shift direct alignment to correct. The condenser apertures should be set in the way you intend to use them during data acquisition. In DM, take a 1 second image in counted/super resolution mode (depending on your mode of collection), the dose in electrons/physical pixel/second will be displayed in the bottom toolbar. For counting mode, we typically a dose between 5-10 e^-/physical pixel/second, and for super resolution mode between 1-3 e^-/physical pixel/second.

16. To work out the dose per Å^2, first calculate your Å^2, i.e. at 130 k magnification, in counting mode each physical pixel represents 1.07 Å (calibrated for ABSL Krios 2, K2 combination) at the magnification calibrated on our microscope, and 1.07 x 1.07 = 1.14 Å^2. Now divide your dose per physical pixel per second by the Å^2 to get e^-/ Å^2/second, e.g. if you have 5 e^- per pixel per second, 5/1.13= 4.42 e^-/ Å^2/second. To get your total dose, multiply this figure by the length of exposure in seconds, for example for a 10 second exposure the total accumulated does per exposure would be 4.42 x 10 = 42.2 e^-/ Å^2. If you need to, alter data acquisition parameters to achieve the desired dose. Be mindful to keep the illuminated area as small as possible to a) allow multiple exposures

per hole, if appropriate and b) to reduce the likelihood of the beam pre-exposing neighbouring acquisition areas. However, the beam should be sufficiently large so beam fringing cannot be seen in the image. Beam spot size, illuminated area and, if needed, condenser 2 aperture can be changed to fine-tune the desired dose. Set the number of fractions you require in the movie, We recommend a number of fractions that results in between 1 and 2 e^{-}/A^{2}/fraction, as this seems to represent a good trade-off between the number of signals in each frame for which to perform motion correction and the frequency of correction for beam-induced movement. Bear in mind the fractions need to be divisible by the physical frame rate of the K2.

Image shift calibrations (10 minutes)

17. Navigate to an intact grid square. Centre on a feature of interest that will be visible at the 'Atlas' and 'Data Acquisition' magnifications, for example a piece of ice contamination. Roughly set eucentric height as described in step 13. Run the autofunction 'set eucentric height by stage tilt'. *Troubleshooting.*

18. In EPU, 'Set' data acquisition beam setting pre-set to the microscope. On K2 live view check the feature is visible in the centre of the screen. Ideally centre a distinctive feature such as the point of an ice crystal. Press eucentric focus on the hand panel. In EPU navigate to Preparation > Calibrate Image Shifts > and click Start Calibration.

19. In EPU, an image at data acquisition magnification will appear with a marker in the centre. If the marker is placed on your recognisable feature, click 'proceed'. If you want to move it, double click in the image and click 're-acquire' until the feature is in the centre of the image.

20. The microscope will now shift to the next highest magnification in the beam presets and take an image. In the second image, double click so the same feature is centred identically to the first image and click 're-acquire'. Repeat until the feature is identically centred and then click 'proceed'. Repeat this step until it says 'image shift calibration finished successfully' in the EPU log on the right.

Atlas the grid (15 minutes)

21. Ensure the objective aperture is not inserted ('Apertures' > 'Objective' > 'none'). In EPU, navigate to 'Atlas' > 'Session Setup', click on 'Create new sample' and name your session. We recommend a format such as Username_myprotein_date_ATLAS. Save images in MRC format and store atlas directly onto the F3EC offload server (usually designated Z: in a standard Krios installation). Click on 'Acquire' to acquire the atlas.

Select square for data acquisition and define template (1-2 hours)

22. In EPU, navigate to 'EPU' > 'Session Setup' > 'New Session'. We suggest a session name such as Username_myprotein_date_EPU. Choose if you would like to write out your images gain normalised or un-gain normalised, and subsequently correct at the motion correction stage. Due to better image compression rates, we recommend writing out un-gain normalised images. Select 'manual selection', save images as either gain normalised or ungain normalised packet and save data directly to the Gatan PC RAID. If you choose a subdirectory data will be split between different directories. Choose the type of grid from the drop-down menu i.e. Quantifoil and the size of the holes, or Lacey grids, then click 'Apply'.

23. Go to 'EPU' > 'EPU' > 'Square Selection', if all squares are green, click 'Unselect all' in the top left. Open tiles (hover over atlas image > right click > 'open tile') and look to see which areas of the grid have appropriate ice thickness for data acquisition. For most specimens you can tell

at this low magnification if an area is dry (no ice) or the ice is too thick. When you have identified a square, select it by hovering over the square followed by right click > 'add', then right click > 'Move stage to Grid Square'.

24. Bring grid square to eucentric height by following step 13 for manual adjustment, then as step 17 for autofunction eucentric height by stage tilt) Press 'Eucentric focus'. Go to 'EPU' > 'EPU' > 'Hole Selection'; 'acquire'. This will save your x, y and z coordinates.

25. Set the template for automated acquisition, including areas and variables for autofocus, drift measurement and image acquisition. This process varies depending on the type of grid hole, which can be either regular such as Quantifoil ® and C-flat ™ (Option A) or irregular such as lacey carbon (Option B).

A) For grids with a regular array of holes such as Quantifoil ® and C-flat ™

i. Go to 'EPU' > 'EPU' > 'Hole Selection'; 'acquire'. The first time you do this, click 'Measure Hole Size'. Move and adjust the yellow circles so they match the hole size then click 'find holes'. Repeat this until the software finds the hole sizes correctly (use the zoom function to see more accurately). This only needs to be done once per session.

ii. The 'filter ice quality' histogram on the right can be adjusted by moving the red histogram lines in order to adjust and refine hole selection. Use the 'Select' tools menu at the top to fine-tune hole selection. For example, remove holes which are empty or in which the ice is too thick/thin/contaminated and holes that are close to the grid bar. *Troubleshooting*

iii. Go to 'EPU' > 'EPU' > 'Template Definition'. Click 'Acquire' and 'Find and Centre Hole'. *Troubleshooting*

iv. Change the 'Delay after Stage Shift' and the 'Delay after Image Shift' times to between 1-5 seconds, depending on the stability of the stage. 1 second is generally sufficient, but this time can be increased if unacceptable drift is observed (as measured during motion correction in step 41-50).

v. Click 'Add acquisition area', then click anywhere on the template image. The outer circle represents the illuminated area, the inner square represents the exposure area. On the top right, add your defocus range for the acquisition (add the defocus you would like, making sure it's a negative number if you wish to work in standard defocused mode imaging) see Table 1 for examples.

vi. Move the acquisition area to desired location. Depending on the sample, hole size and user preference you can do one exposure in the middle of the hole, ideally with the illuminated area covering the whole hole (this may help reduce the effect of charging), or add multiple exposures around the hole, taking care not to overlap illuminated areas into neighbouring exposure areas.

vii. If you want to add additional exposure areas, click 'Add Acquisition Area', click the template image and move the exposure to the desired location (double checking they have retained the defocus list). When choosing the number of acquisitions to take around a hole, bear in mind that the beam diameter shown in EPU can vary by +/- ~10 %, depending on the accuracy of alignment, so it's safer to leave some space between the acquisition areas or check the physical beam and the virtual beam in EPU coincide, by burning a hole on the carbon to confirm true size of the illuminated area.

viii. Click ‘add Autofocus Area’ and click anywhere on the image. Move the autofocus area to the carbon surrounding your hole. Standard practice is to autofocus every 5-15 µm, depending how large the variation in height is across the grid square (with more uneven grids we recommend to autofocus more often). Focus using the objective lens and ensure autostigmate is set to ‘no’.

ix. Click ‘add Drift Measurement area’. Perform a drift measurement once per grid square, set the threshold to 0.05 nm/s. If your microscope has known stage stability issues you may have to relax this threshold and/or perform drift measurement more often. The drift measurement area should overlap directly with the autofocus area. Make sure neither drift nor autofocus area overlap with an acquisition area either in this hole, or neighbouring holes.

x. Check the template layout by running the ‘template execution’ function. This is a good idea in order to see if you need to move your acquisition areas (e.g. too much/not enough carbon in images) or would like to assess particle distribution.

B) For irregular arrays of holes such as lacey carbon

i. In ‘EPU’ > ‘EPU’ > ‘Area Selection’, choose the spacing between acquisitions. It is recommended that you add ~ 300-400 nm to the data acquisition illuminated area (e.g. illuminated area 0.9 µm, choose spacing of 1.3 µm), to ensure the illuminated areas do not overlap. Click ‘View Pattern’. Use area selection tools to add or remove desired acquisition areas.

ii. Change the stage shift delay to 5 seconds. A shorter or longer time may be used depending on the stability of the stage. As collection on irregular carbon typically means a greater number of stage shifts, a longer stage shift delay is recommended compared with regular arrays.

iii. Set the autofocus recurrence to ‘after distance’. Depending on the height variation of your grid, every 8-15 µm is recommended. Focus using the objective lens, ensure autostigmate is set to ‘no’.

iv. In ‘Data Acquisition Area Settings’ add your defocus range for the acquisition (add the defocus you would like, making sure it’s a negative number if you wish to work in standard defocused mode imaging. You only have to do this for one grid square, it remembers for subsequent squares.

v. Use the bottom histogram (filter ice quality) on the right and the exposure area selection tools to optimise hole selection in order to exclude areas of sub-optimal/no ice.

Check direct alignments (30 minutes)

26. Perform direct alignments on the grid (Supplementary Methods 2).

! CAUTION: In many electron microscopy facilities, direct alignments are only carried out by Facility Staff. We provide a guide to performing the basic direct alignments in Supplementary Methods 2 but you should check local procedures in your EM Facility before attempting.

Critical Step- Some direct alignments cannot be adequately performed on UltrAuFoil® grids, and so if using these, perform direct alignments on a carbon grid or cross grating before starting data collection.

Final checks (10 minutes)

27. Before automated acquisition begins, perform a final check of variables that can affect data quality or prevent common mistakes. In 'UI' > 'Autoloader' > 'Turbo' > 'Options' ; click Turbo 'auto off'. Ensure Titan Krios enclosure is shut.

28. Check the microscopy parameters. Ensure that the aperture series is as desired, the beam is centred in the data acquisition beam settings and no beam fringes appear in the image. Check that you are collecting fractions, if desired. Ensure that the disk you are writing to has sufficient space available for the entire planned data collection.

Start automated collection (5 minutes- data typically collects for 24-72 h)

29. In 'EPU' > 'EPU' > 'Automated Acquisition' > 'Start' the EPU run. We suggest you now move on to start data organisation and on the fly image processing steps, then come back to select more grid squares as steps 24-25, but note the template only needs to be set once for each data collection. *Troubleshooting*

30. Fill out a data acquisition report (Supplementary Table 4).

31. Continue with data transfer and organisation as in main text, steps 42 onwards.

Supplementary Methods 2- Direct alignments

Caution: In many facilities direct alignments are only carried out by Facility Staff. We provide a guide to performing basic direct alignments but please check local procedures in your facility before attempting.

Note: Some direct alignments cannot be performed on UltrAuFoil® grids, and so if using these perform direct alignments on a carbon grid or cross grating before starting data collection.

Note: Here we assume the microscope is in a well aligned state, including condenser aperture series before starting this procedure.

Note: In the UI > Direct alignment menu, there is an 'autohelp' tickbox. When ticked, it automatically brings up a help menu describing the alignment procedure, and can be helpful for inexperienced users.

Note: If you have AutoCTF installed on your system this can be used to perform Coma-free alignment and stigmate the objective lens.

Direct alignments for F3EC integrating mode

1) Find an area with intact carbon film that you do not want to use for data acquisition. Set eucentric height and press eucentric focus on the hand panel.

2) In EPU, 'Set' data acquisition beam settings and press 'eucentric focus' on hand panel.

3) Condense beam to form a point on the FluCam Viewer. In UI, go 'Direct alignments' > beam pivot points x, perform correction and then repeat for beam pivot point y. The aim is to adjust so only 1 spot is visible, using multifunction X (and Y if a phase plate is not installed on the system).

4) Set 'data acquisition' settings. Make sure the objective aperture is out. Do the coma-free alignments on the Ceta camera by bringing up a live image and FFT (Camera > BM-Ceta > search). Look at the image in TIA with a live FFT. Do not use an integration time of more than 0.3 s. If the FFT is too noisy, condense the beam and make sure the binning of the camera is set to 1 and read out the full chip is selected. Make sure you are over some carbon. Apply -0.5 to -1.5 μm defocus. In UI, select Direct Alignments > Coma-free alignment x. Observe the FFT – the goal is that the Thon rings in the FFT do not change at all (i.e. have the same defocus) between the two changing images. Use a marker on the screen to help see if the rings are moving. Use multifunction X to minimise defocus change. Do the same for the coma-free alignment y, again using multifunction X to adjust. This should only require very small alterations. If AutoCTF is installed, this can be used to perform the coma-free alignment. If this alignment is far off, rotation centre may need to be performed first. Set 'data acquisition' settings again and do rotation centre direct alignments on the FluCam Viewer or Ceta using live view. In order to do so, observe a feature in the centre of the screen and use the multifunction buttons to make the feature as still as possible. Do not iterate between rotation center and coma free alignment as they aim to align the same thing but in different ways.

5) Re- 'set' data acquisition beam settings and do the beam shift direct alignment (It is important to perform the beam shift calibration at the same imaging parameters that will be used for data acquisition), using multifunction X and Y to centre the beam to the 40 mm circle on the FluCam viewer. Typically, the beam diameter at Data Acquisition beam settings is wider than the FluScreen, so to ensure beam is appropriately centred, with the FluScreen down change magnification until the whole beam is visible (typically two clicks lower), then perform beam shift alignment. Ensure 'autozoom' is off before attempting this (Step 13, main text).

6) In EPU, 'get' these beam setting presets into autofocus and drift measurement presets (so all three presets are the same).

7) Insert the 100 μm objective aperture.

8) Check objective aperture is centered. With the FluScreen down, go into diffraction mode on the right hand control panel. EPU will produce an error, drag this window out of view. Click on the FluCam Viewer and use the mouse scroll wheel to adjust the image histogram until you can see the shadow of the objective. In UI > Apertures, click 'adjust' and use MF X and Y to centre the bright spot in the shadow of the objective. Once done, click 'adjust' to stop adjusting, on the hand panel press diffraction mode to go out of diffraction and press 'retry' in EPU error window.

9) To correct objective astigmatism, start 'search' acquisition on the Ceta camera in the UI and change the integration time to 1-2 s. Observe the FFT in TIA, going very close to focus (turn the focus knob until the number of rings in the FFT is reduced to 1-2). The rings in the FFT should be round (you may need to take a longer exposure to confirm). If not, go to UI > Stigmator > Objective. Set the step size to 3. Use the multifunction x and y buttons to carefully make the Thon rings in the FFT round. Click 'None' in the Stigmator tab when you are done, so that the 'Objective' button is not yellow anymore. Note- if you aren't experienced at this, make a note of the numbers in stigmator so you can reload if needed.

<u>F3EC Counting mode direct alignments</u>

Notes- in counting mode, beam is very dim, so doing some alignments is much harder, hence a different protocol to direct alignments for an integrating mode session.

1) Follow above steps to end of step 3.

2) Set 'autofocus' settings (or standard integrating mode beam settings with a brighter spot). Make sure the objective aperture is out. Do the coma-free alignments on the Ceta camera by bringing up a live image and FFT (Camera > BM-Ceta > search). Look at the image in TIA with a live FFT. Do not use an integration time of more than 0.3 s. If the FFT is too noisy, condense the beam and make sure the binning of the camera is set to 1 and read out the full chip is selected. Make sure you are over some carbon. Apply -0.5 to -1.5 μm defocus. Go to Direct Alignments > Coma-free alignment x. Observe the FFT – the goal is that the Thon rings in the FFT do not change at all (i.e. have the same defocus) between the two changing images. Use a marker on the screen to help see if the rings are changing. Use multifunction X to minimise defocus change. Do the same for the coma-free alignment y, again using multifunction X to adjust. This should only require very small alterations. If AutoCTF is installed, this can be used to perform the coma-free alignment. If this alignment is far off, rotation center may need to be performed first. Set 'data acquisition' settings again and do rotation centre direct alignments on the FluCam Viewer or Ceta using live view. In order to do so, observe a feature in the centre of the screen and use the multifunction buttons to make the feature as still as possible. Do not iterate between rotation center and coma free alignment as they aim to align the same thing but in different ways.

3) Insert the objective aperture.

4) Check objective aperture is centered. With the FluScreen down, go into diffraction mode on the right-hand control panel. EPU will produce an error, drag this window out of view. Click on the FluCam Viewer and use the mouse wheel to adjust the image histogram until you can see the shadow of the objective. In UI > Apertures, click 'adjust' and use MF X and Y to centre the bright spot in the shadow of the objective. Once done, click 'adjust' to stop adjusting, on the hand panel press diffraction mode to go out of diffraction and press 'retry' in EPU error window.

5) To correct objective astigmatism, start 'search' acquisition on the Ceta camera in the UI and change the integration time to 1-2 s. Observe the FFT in TIA, going very close to focus (turn the focus knob until the number of rings in the FFT is reduced to 1-2). The rings in the FFT should be round (you may need to take a longer exposure to confirm). If not, go to UI > Stigmator > Objective. Set the step size to 3. Use the multifunction x and y buttons to carefully make the Thon rings in the FFT round. Click 'None' in the Stigmator tab when you are done, so that the 'Objective' button is not yellow anymore. Note- if you aren't experienced at this, make a note of the numbers in stigmator so you can reload if needed.

6) Re- 'set' data acquisition beam settings and do the beam shift direct alignment. The beam is very dim, so (ensuring autozoom is off) go to a lower magnification until you can see the beam on the screen (usually 2 magnification clicks using the magnification knob on the right-hand control panel). Apply beam shift (using Direct Alignments > Beam shift, Multifunction X and Y to centre to the 40 mm circle on the FluCam viewer) and go back to acquisition magnification.

7) 'Set' autofocus beam settings then 'Get' these settings to drift measurement to ensure they are identical

K2 Direct alignments

1) Find an area with intact carbon film that you do not want to use for data acquisition. Set eucentric height. See notes at bottom if you are unsure.

2) In EPU, once you are sure you are at eucentric height, 'Set' data acquisition beam settings and press 'eucentric focus'.

3) Condense beam to form a spot on the FluScreen. Do direct alignments > beam pivot points x and y.

4) Set 'data acquisition' settings. Make sure the objective aperture is out. Do the coma-free alignments on the K2 camera by bringing up a live image and FFT (DM > start view > process > live > FFT). Do not use an integration time of more than 0.3 s. If the FFT is too noisy, condense the beam and make sure the binning of the camera is set to 1 and you read out the full chip. Make sure you are over some carbon. Start the coma-free alignment x procedure and observe the FFT – the goal is that the Thon rings in the FFT do not change at all (i.e. have the same defocus) between the two changing images. Use a marker on the screen to help see if the rings are changing. Use multifunction X to minimise defocus change. Note: you may have to apply a small defocus to be able to do the alignment. Use the smallest defocus you can. Do the same for the coma-free alignment y, again using multifunction X. If AutoCTF is installed, this can be used to perform the coma-free alignment. If this alignment is far off, rotation center may need to be performed first. Set 'data acquisition' settings again and do rotation centre direct alignments on the FluCam Viewer or Ceta using live view. In order to do so, observe a feature in the centre of the screen and use the multifunction buttons to make the feature as still as possible. Do not iterate between rotation center and coma free alignment as they aim to align the same thing but in different ways.

5) Re- 'Set' data acquisition beam settings and do the beam shift direct alignment.

6) Now 'Get' these settings into autofocus and drift measurement presets (so all three presets are the same).

7) Insert the objective aperture.

8) Check objective aperture is centered. With the FluScreen down, go into diffraction mode on the right hand control panel. EPU will produce an error, drag this window out of view. Click on the FluCam Viewer and use the mouse wheel to adjust the image histogram until you can see the shadow of the objective. In UI > Apertures, click ‘adjust’ and use MF X and Y to centre the bright spot in the shadow of the objective. Once done, click ‘adjust’ to stop adjusting, on the hand panel press diffraction mode to go out of diffraction and press ‘retry’ in EPU error window.

8) To correct objective astigmatism, start view on K2, using integration time of 0.5 s. Observe the FFT in DM, going very close to focus (turn the focus knob until the number of rings in the FFT is reduced to 1-2). The rings in the FFT should be round. If not, go to UI > Stigmator > Objective. Set the step size to 3. Use the multifunction x and y buttons to carefully make the Thon rings in the FFT round. Click ‘None’ in the Stigmator tab when you are done, so that the ‘Objective’ button is not yellow anymore. Note- if you aren’t experienced at this, make a note of the numbers in stigmator so you can reload if needed.

Supplementary Methods 3 - Using a Volta Potential phase plate.

This guide is to be used in conjunction with the main protocol (main text for F3EC and Supplementary Methods 1 for K2 summit) and identifies steps where the set up with a phase plate will differ.

Important points

- We assume that the phase plate is operational (For our set up, heater on, power supply set to 6V and 0.025 A- the actual readings will be approx. 4.4V and between 0.022A – 0.027A)
- This procedure does not cover full alignment of the phase plate. For single particle applications the full alignment should remain stable over long periods and is generally performed by Facility Staff when required. If preforming full phase plate alignment cross grating grid is required.
- Retract the phase plate before loading/unloading grids to as this can cause contamination on the phase plate.
- Use 50 μm C2 aperture to have maximum parallel beam range.
- Each phase plate has 4 rows of 19 positions (76 positions in total). The middle 2 lines are the best for data collection as they are in the centre of the window. It is best practice to set up on positions 1 -19 and then move to 20 before you start the data collection.
- When taking gain reference or aligning energy filter, make sure phase plate is retracted.
- We recommend tracking the use of different phase plate windows as it is still unclear how long the phase plate takes to 'regenerate' or how long the Volta Potential takes to dissipate from used areas. Additionally, phase plate behaviour over time can be monitored.

Setting up a Phase Plate EPU session

1. Start with the phase plate retracted and follow the protocol until 'beam setting presets'.
2. When defining beam setting presets, GridSquare and Hole/Eucentric pre-sets should both be set so the beam is spreading (UI > Beam settings > illumination > 'spreading'). This can be achieved by using a larger than usual illuminated area and a brighter spot to compensate. Data acquisition, autofocus and drift measurement settings should be a parallel as normal.
3. Continue the protocol until 'select square for data acquisition and define template' set up and proceed as described with the following alterations (4-6);
4. On session set up, select 'phase plate'.
5. On EPU > EPU > Template Definition, set stage shift to 0 so beam isn't deflected to avoid satellite (parasitic) Volta Potential points on the phase plate. Autofocus every time (you will be working closer to focus than usual and this is to reduce possibility of over focused images). Set defocus range will be closer to focus than usual but will depend on your data collection scheme. Standard protocol is to use a single value of about -0.5 μm.
6. In EPU > EPU > Automated Acquisition, select phase plate and choose to change phase plate position after a certain number of images has been taken. Use prior information about the speed with which the phase plate produces given phase shifts (20-120° is useful) as a starting point. You can then monitor using micrograph analysis scripts described in the main text and Supplementary Note 1 and adjust the periodicity and activation time of the phase plate. You can also use AutoCTF (check the electron counting and phase plate inserted options are clicked) to perform a test and see how long the phase plate takes to achieve a given shift and how it builds over time. On our system with illumination settings for K2 summit, we typically use no activation time and a periodicity (exposures) of 40-60, but optimal settings will vary (dramatically) phase plate to phase plate.
7. For the direct alignment step (Supplementary Methods 2), with the phase plate retracted, perform beam shift, beam tilt PP, rotation centre (for a stable system this does not normally need to be adjusted).
8. Insert a phase plate (UI > Apertures > Objective > choose Phase Plate 1-6 from drop down menu.

9. Activate the phase plate (UI > Phase Plate > tab out, 'Active') and wait for 5 minutes for settling.

10. Perform on plane alignment which brings the beam to crossover (smallest point) on the phase plate.

i. Over an empty area find the 'blow up point' by imaging a ronchigram (a projection pattern on the diffraction plane). This can be done on the FluScreen initially (you can adjust the histogram to improve contrast so you can see the ronchigram). In order to fine tune the image, use a live view (i.e. K2 of Ceta camera).
ii. Increase the dose on by moving to a 150 μm C2 aperture if you need to (do not change illuminated area or spot size).
iii. Select UI > Phase Plate > tick 'MF-Y Fine Focus in back focal plane' in phase plate menu and adjust to find blow up point. As you adjust features will move towards or away from you. The aim is to find the point where the features have come towards you to the greatest extent (i.e. the point between when they move towards you then away again). Adjust condenser stigmation so rings are radial on the FluScreen and you have a flat field image on the K2/Ceta camera. You will have to iterate this process. NOTE on plane alignment is dependent on beam settings intensity and spot size- if you change any of these for data acquisition you will have to redo the alignment.
iv. It is useful to take note of the C2 and C3 lens values once you get close to/are at the blow out point.

11. Retract the phase plate and move to an area over carbon. Perform coma free alignment (Supplementary Methods 2).
12. Insert phase plate (UI > Apertures > choose Phase Plate 1-6 from drop down menu and wait 5 mins.)
13. Stigmate objective lens (using AutoCTF if you have it) (note- this will often change upon moving to a different phase plate position but as this is corrected for during CTF correction it is not of great concern).
14. Check over an empty area that there is still a flat field image (i.e. that on plane alignment is still correct)
15. Continue with EPU set up and OTF processing guide (main text). The micrograph analysis script will automatically plot phase shift to allow monitoring of phase shift.

Supplementary Note 1 - Description of scripts

All scripts are available on GitHub repository https://github.com/Leeds-ABSL/ABSL_pipeline

Raid2offload.py

This script is written in PowerShell. It pulls data from Gatan RAID every 10 minutes. This currently pulls .mrc files retaining the directory structure used by EPU.

ABSL_OTF.sh

This script for copying and organising data is specific to the ABSL file system. Most implementation of this protocol will require a built-for-purpose script which performs these tasks in the user's specific file system structure. To run this script, you must have access to the location the data is written by the direct electron detector and where you would like to store the data.

The script is run by the user from the users processing directory to pull raw data from the microscope offload servers. Pulling the data ensures the correct file permissions for the user's data. The user specifies a run duration and the script runs continually with 30 seconds break between cycles. ABSL_OTF.sh first copies (using the rsync command) the data from the offload server associated with the specified Titan Krios (i.e offload1 for Krios 1 or offload2 for Krios 2) to the dedicated GPFS storage system (i.e /absl/Equipment/KRIOS1 for Krios 1 or /absl/Equipment/GATAN for Krios 2). The data is placed in a directory with the users username and a subdirectory with the date and user specified project name. The script then creates symbolic links to the data in a 'Raw_data' directory in the processing directory where the script is run (i.e. /absl/SCRATCH/Users). Finally, the script runs the two further scripts described below, EPA_CC_threshold.py and micrograph_analysis.py to make diagnostic plots to assess the quality of the data.

The creation of symbolic links to the data in the processing directory allows users full access to their data but provides a safeguard that prevents inexperienced users from accidentally modifying or deleting the data. Additionally, this allows the user to rename the links if required, which is useful when importing the data into some programs.

Script usage:

```
sh OTF.sh <runtime in mins> <which microscope (krios1 or gatan)>
<project name> <name of EPU directory>
```

e.g. `sh OTF.sh 2880 krios1 project_name my_protein_EPU`

ABSL_EPA_CC_threshold.py

The original version of this script was kindly provided by Rado Danev.

Gctf , version 1.06, estimates the resolution of a micrograph as the resolution at which the cross correlation coefficient (CCC) between the eqi-phase average and actual micrograph power spectrum falls to 0. We feel this overestimates the resolution and prefer to use a CCC cutoff of 0.5. EPA_CC_threshold.py reads the log files created by gCTF and then replaces the _rlnMaxResolution column in RELION's file micrographs_ctf.star with values determined using the 0.5 CCC value.

The script is run automatically by OTF.sh. To run the script on its own use the command:

```
./ABSL_EPA_CC_threshold.py <micrographs_ctf.star>
```

Use the optional flag –CCC_cutoff <n> to change the CCC cutoff from the default of 0.5, where “n” is the CCC cut off you would like to use.

ABSL_micrograph_analysis.py
This script reads RELION’s micrographs_ctf.star file and create diagnostic plots for rapid assessment of the dataset quality, showing histograms for defocus, astigmatism and estimated resolution of all micrographs and, if present, phase shift information, and plots of these statistics over the length of the data collection (Figure 4).

The script is run automatically by OTF.sh and outputs a file named microgrpahs_analysis_0.png, but can be run on its own with the command:

```
./micrograph_analysis.py --i <micrographs_ctf.star>
```

Add the optional flag --f to generate a new micrographs_ctf.star file (called culled_micrographs_ctf.star) with low-pass filters based on defocus, resolution, and/or astigmatism applied to the data.

Supplementary note 2 - Modifications of RELION for on-the-fly processing

Modification of RELION 2.1 for on the fly processing

The standard RELION installation will crash if used on the fly for datasets of several thousand micrographs. We think this is due to how RELION decides a job is finished and starts the next job. The program looks for the output file it expects at the end of the job, waits 10s and then starts next job in schedule. When the output file contains 1000's of lines the time taken to write this file out can exceed 10s. If this happens the next step will run on a list of files which is empty - which crashes the pipeline.

The workaround used in the OTF modification of RELION is to change the 10s wait time to 60s - this allows for the file to finish writing before the next process begins. In the src directory of RELION line 648 in the file pipeliner.cpp should be changed to:

```
"
            // Now wait until that job is done!
            while (true)
            {
                if (!exists(fn_check))
                {
                    fn_check_exists = false;
                    break;
                }

                sleep(60);
                checkProcessCompletion();
                if      (processList[current_job].status      ==
PROC_FINISHED)"
```

After this change is made in the source code the program must be recompiled and installed as detailed in the RELION guide.

A copy of this file with the change already made is included in the git repository containing the other scripts described in this manuscript (https://github.com/Leeds-ABSL/ABSL_pipeline).

Supplementary Tables 1-4

Preset	Bin	Readout	Exp. Time (s)	Nanoprobe (NP) /Microprobe (MP)	Mag	Mode	Defocus (µm)	Spot	Ill. Area (µm)	Beam parallel
Atlas	1	Full	1	MP	100 x	Linear	-180	7	900	Yes
Grid square	1	Full	1	MP	520 x **(Varies on grid mesh size)**	Linear	-160	7	250	Yes
Hole/ eucentric	1	Full	0.2	NP	8700 x **(Varies on hole size)**	Linear	-60	Ideally as data acquisition	8	Yes
Autofocus	2	Full	0.5	NP	As data acquisition	Linear	na	As data acq.	As data acq.	Yes
Drift measurement	2	Full	0.5	NP	As data acquisition	Linear	-3	As data acq.	As data acq.	Yes
75 k- Data acquisition	1	Full	2	NP	75k x **(Varies on desired parameters)**	Linear	Typically -1 to -3.5 varies on sample	5	1.3	Yes

Supplementary Table 1- Example EPU beam setting presets for F3EC integrating mode. Please note these numbers are a rough guide and starting point. Due to variations in different gun settings, aperture series and illumination area calibration these will require optimization for each system.

Preset	**Bin**	**Readout**	**Exp. Time (s)**	**NP/MP**	**Mode**	**Mag**	**Defocus (µm)**	**Spot**	**Ill. Area (µm)**	**Beam parallel**
Atlas	1	Full	1	MP	Linear	100 x	-180	7	900	Yes
Grid square	1	Full	1	MP	Linear	520 x **(Varies on grid mesh size)**	-160	7	250	Yes
Hole/ eucentric	1	Full	1	NP	Linear	8700 x **(Varies on hole size)**	-60	Ideally as data acq. Or Autofocus/drift measurement	8	Yes
Autofocus	2	Full	0.5	NP	Linear	As data acquisition	na	4	1.3	Yes
Drift measurement	2	Full	0.5	NP	Linear	As data acquisition	-3	As Autofocus	1.3	Yes
Data acquisition	1	Full	60	NP	Counting	75k x	Typically, -1 to -3.5 varies on sample	9	1.3	Yes

Supplementary Table 2- Example EPU beam setting presets for F3EC counting mode. Please note these numbers are a rough guide and starting point. Due to variations in different gun settings, aperture series and illumination area calibration these will require optimisation for each system. In these settings, as the spot size changes dramatically between integrating mode and counting mode, some instabilities will be created in the optics due to C1 lens changes. As an alternative, autofocus and drift measurement can be carried out at the same settings as data acquisition, but this will produce a further decrease in acquisition speed.

Preset	Camera	Bin	Readout	Pre- exp time (s)	Pre-exp pause time (s)	Exp. Time (s)	NP/MP	Mag	Defocus (µm)	Spot	Ill. Area (µm)	Beam parallel	Insert slit (eV)
Atlas	K2	2	Full	0	0	1	MP	275 x	-500	8	500	Yes	No
Grid square	K2	1	Full	0	0	1	MP	740-1200 x	-500	7	200	Yes	No
Hole/eucentric	K2	1	Full	0	0	1	NP	15000-19500 x	-60	Ideally as data acq.	6-12	Yes	No
Autofocus	K2	2	Full	0	0	0.5	NP	As data acq.	Na	As data acq.	As data acq.	Yes	Yes (20 eV)
Drift measurement	K2	2	Full	0	0	0.5	NP	As data acq.	-3	As data acq.	As data acq.	Yes	Yes (20 eV)
Data acquisition	K2	1	Full	0	0	10	NP	130k x	-4 (add range)	7	1.1	Yes	Yes (20 eV)

Supplementary Table 3- Example EPU beam setting presets for K2 counting mode. Please note these numbers are a rough guide and starting point. Due to variations in different gun settings, aperture series and illumination area calibration these will require optimization for each system.

Date:

Hardware	
Microscope	*Krios 1*
Detector (mode)	*Falcon 3 (integrating)*
Accelerating voltage (ke-V)	*300*
Pixel size (Å)	*1.065*
TEM server version	*2.9.1*
EPU version	*1.9*
C_s	*2.7*
Data acquisition parameters	
Nominal magnification	*75,000 x*
Spot size	*5*
Illuminated area	*1.2 µm*
Data acquisition dose parameters	
Square pixel (Å^2)	*1.13*
Dose per physical pixel per second	*61.69*
Dose per Å^2/sec	*54.6*
Exposure time (seconds)	*1.2*
Total dose (e/Å^2)	*65.5*
Number of fractions	*47*
Dose per fraction (e/Å^2)	*1.4*
EPU parameters	
Defocus range (-µm)	*-1 to -3.3*
Autofocus	*Every 8µm, using objective*
Drift measurement	*Once per grid square, 0.05nm/s*
Delay after stage shift	*5s*
Delay after image shift	*5s*
Exposures per hole	*5*
Apertures (size in microns)	
C1	*2000*
C2	*70*
C3	*2000*
Objective	*100*
Energy filter	
Slit (eV)	*na*

Supplementary Table 4- Data acquisition report with example values.