

KEK cryo-EM (**Titan Krios G4, Falcon 4i**)

Initial Training Textbook for External Users

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***Chapters I-III are for external users and chapters IV-V are for KEK staff.**

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I. Preparation of AutoGrids and loading them into EM

General notes on cryo-grid preparation

*Confirm that everything that touches the Grid is already cool. Wait until the bubbles coming out of the apparatus have transitioned from a state of intense boiling to a state of small bubbles before using it.

*Once a tool is chilled in LN₂, if you take it out of the LN₂/cold nitrogen gas area, it will frost over. If you put it back into the LN₂, the frost may transfer to the grid, which is not good. Therefore, do not take it out of the LN₂/cold nitrogen gas area, or use a new tool.

1. Cooling of the equipment, etc.

- Pour LN₂ into 1L pots x 4. Cover the spout with aluminum foil.

• Launch of Vitrobot

- Attach the humidifier (if not fitted correctly, it will collide with the stage and fail).
- Inject 50 mL of MQ through the tube on the bottom with a syringe
(pull back with the syringe plunger after injecting the water to set the proper water level).
- Turn on the Vitrobot (switch is on the back of the device, lower right hand side).
- See the Touch panel, set to 18°C (or desired temperature), 100%, humidifier **ON**.
- See the Touch panel, check **Use Footpedal**, **Humidifier off**, and **Skip grid transfer**.
- Step on the foot pedal 2-3 times to close the white lid on the bottom in the Vitrobot chamber.
- Wear gloves and set one piece of filter paper on each side.
(*the bend of the cut end faces outward)
(*Replace the filter paper with a new one after one hour)

• Assembly and cooling of styrofoam containers for Vitrobot

- Assemble styrofoam containers and cover it with a 500mL plastic beaker.
- Pour LN₂ into the surrounding moat and ethane cup (gold).
(*repeat 2-3 times, wait at least 10 minutes)
- Turn gaseous ethane into liquid ethane. Fill it as close to the edge of the cup as possible.

CAUTION: Liquid ethane is very dangerous. Always wear protective glasses and gloves. If liquid ethane gets into your eyes, you could go blind. Also, it is dangerous to hold the container directly, so carry it on a tray.

- Remove the spider (if left in place, it will freeze and the spider will not come off).
- Wait until the inner wall of the ethane cup turns white.

Note: Liquid ethane temperatures range from -89°C to -183°C. To make a good grid, you need to freeze it quickly below -160°C and freeze it in a vitrified state, so it is best to use as close to solidification as possible. If it solidifies, melt it with the butt of tweezers.

- Put the CryoGrid Box (light blue) on the metal stage in the styrofoam container and wait until it has cooled completely (no boiling of the LN₂ around it).

• **Assembly and cooling of the loading station**

- Assemble (use a 500mL plastic beaker to cover the LN₂ spout).



- Put AutoGrid clipping tool (iron) and autogrid container (white) in the loading station.



- Pour LN₂ through the LN₂ spout.
(*pack the LN₂ spout of the loading station with Kimwipes to prevent frost contamination)
- Repeat pouring LN₂ 2-3 times.
- Wait at least another 10 minutes after the LN₂ has stopped decreasing.

(*The LN₂ in this container runs out quickly, so add LN₂ frequently)

• **NanoCab Cooling**

- Pour LN₂ into NanoCab.



- Wait a little and the LN₂ will be reduced by half, so add LN₂ again.
- Repeat this several times.
- Wait at least another 10 minutes after the LN₂ has stopped decreasing.

• **Test run of PIB-10**

- Press green button to test run the PIB-10.

2. Preparation of cryo-grids

- Grids should be soaked in acetone overnight in advance (see page 27 in detail).
- Take the required number of acetone-treated grids with fine-tipped tweezers.
- Place the back side of the grid (shiny side up) on a slide glass.
- Hydrophilize the grids with PIB-10 (Hard, 1.5min → press green button to start).
- Remove from PIB-10, turn the grid over, and hydrophilize again (Hard, 1.5 min).
- Vitrobot settings (blotting time 5 seconds, blot force=15, etc.).

Step on the foot pedal several times to lower the tweezers holder.

- Pinch the grid with the Vitrobot tweezers and secure it with the black clasp.
(shake it to make sure it doesn't fall off)



- Attach the tweezers on the Vitrobot.
(*with the carbon side (surface) facing your dominant hand)
 - Step on the foot pedal 3 times and move the tweezers up.
 - Pour enough LN₂ into the styrofoam container and place on the Vitrobot platform.
 - Step on the foot pedal once to lift the styrofoam container up.
 - Step once on the foot pedal to lower the tweezers to the sample application position.
 - Pipette 3uL of sample onto the grid
(*try not to cause bubbles; give up if there is a little solution remains on the tip).
 - When the foot pedal is stepped on, the grid is blotted and plunged into the liquid ethane.
 - Tweezers and styrofoam container come down.
 - Wait about 20 seconds for the fog to clear (pour more LN₂ while waiting).
 - Remove the tweezers by pushing with the belly of the thumb and
move the entire styrofoam container in front of the Vitrobot.
 - While the grid is still dipped in the ethane,
hold the tweezers below the black clasp and shift the black clasp up to unlock the tweezers.
 - Move grid quickly from the ethane cup to the LN₂.
 - Store the grid in the CryoGrid Box (light blue) (record what you put in which number).
 - Pour enough LN₂ into a styrofoam container.
- (Return to # and repeat subsequent operations for the number of samples)

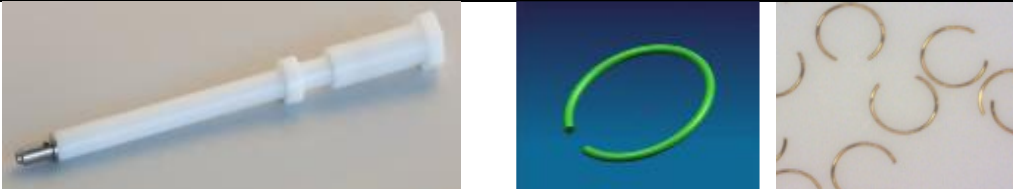
3. Clipping in AutoGrids

- Pour enough LN₂ into the loading station again.
- Fit the lid of the CryoGrid Box (light blue) into the lead holder (white pen-like tool).
- Cool down the lid and wait until the intense boiling is over.
- Close the lid of CryoGrid Box (light blue) and move it to the loading station.

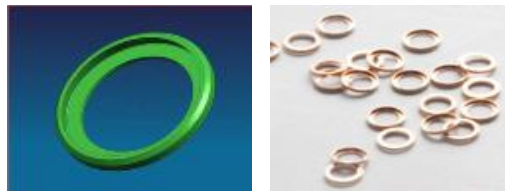
CAUTION: Be careful of static electricity when handling the lid. Remove sandals and ground yourself before operation. Or to reduce static electricity effects, we can use a conductive mat (like this one: <https://www.amazon.co.jp/Hozan-F-703-Conductive-Color-Green/dp/B004MXQ7TE/>).

- Remove the lid of the CryoGrid Box (light blue).
- Insert the C-clip into the C-clip insertion tool (the other white pen-like tool).

NOTE: If you use a warped C-clip, it will drop off in the cryo-EM, so throw away warped ones!



- Put C-clip rings into the loading station and let it cool.



- Allow the tapered tweezers to cool for about 30 seconds.
- Put a C-clip ring in the center of the AutoGrid alignment tool (iron).
- Place the grid so that it fits into the C-clip ring.
- Put the C-clip insertion tool with C-clip into LN₂ and wait until the intense boiling has finished.
- Snap the C-clip onto the C-clip ring (this state is the "AutoGrid").
- Confirm that it is properly assembled**

by e.g. dropping it from the edge of the AutoGrid alignment tool.

Note: If the AutoGrid is not assembled properly, the robotic arm will fail to grab it and debris may fall off inside the electron microscope or the Autoloader. If this happens, it will take several days of service if the accident was inside the Autoloader, or 1-2 weeks of service if the mishap was inside the optical tube. This will greatly interfere with the shared use of the microscope. Always make sure that the AutoGrid is well clipped!!!! You can use a magnifying glass if necessary.

- Store in AutoGrid container (white) (record what you put in which number).

4. Loading AutoGrids into EM with Cassette and NanoCab

- Pour more LN₂ into NanoCab.
- Go to the Titan, attach the NanoCab, and press the Undock button on the touch panel.
- Attach the NanoCab to the loading station.
- Move the cassette from the NanoCab to the loading station
(press the button next to the handle to grab it).



- Allow AutoGrid tweezers (cross-tipped tweezers) to cool for at least 1 minute.



- Use AutoGrid tweezers to move the AutoGrids to the cassette (record the number).
(*Insert the AutoGrids so that the C-clip ring faces in opposite direction of the handle)
- Hold the AutoGrid tweezers vertically and press down lightly to be sure to confirm that all grids are properly inserted.**
- Return the cassette to the NanoCab (press the grab button on the handle).

Note: If the cassette is difficult to detach from the handle, release the button once it is in the NanoCab, and move the handle by turning the wrist so that the Cassette is pressed against the underside. So that the Cassette remains in the NanoCab. Be careful not to jostle it too much here, or the AutoGrids will disassemble and fall apart.

- Detach the NanoCab from the loading station.
- Pour more LN₂ into NanoCab.
- Confirm that the NanoCab pin is raised
(if not, push the cassette down with the backside of a tweezer to make sure that it is properly inserted and/or pinch the pin up with your fingers).
- Go to the front of the Titan, set NanoCab, and press the Dock button on the touch panel.
(If NanoCab is not recognized, turn the pin a little and it may be recognized.
Be careful not to turn it too much or the pin may come off!)

II. Automated data collection

*If you have finished all the preparations shown in Chapter IV, you can simply perform the operations described in Chapter II.

*TEM User Interface is version 3.13.1 and EPU is version 3.1.0.4506REL.

1. Inventory

(*See the center monitor and use the TEM User Interface, hereinafter called TUI)

• See TUI>Autoloader tab>Temperature Control

- When docking the NanoCab is finished, wait until all temperature displays are green.
- Then, wait until all temperature displays are below -160°C (*takes several minutes).

• See TUI>Autoloader tab>Autoloader>Option

- Click Inventory button.
- If all the grids you put in are correctly recognized, it is OK.
(If it is not recognized correctly, click Inventory again. If that does not work, call FEI.)
- When the inventory is completed up to (last grid) + 2, click Stop Inventory button.

***1. KEK save the data collected by Falcon4i as EER format. Since a gain reference file is needed to analyze the data in RELION, when you start automated data collection, copy the most recent file named "Date_Time_EER_GainReference.Gain" in OffloadData/ImagesForProcessing/EF-Falcon/300kV/ to the Directory of your collected data in OffloadData.**

***2. If you saved as EER format, the number of frames in the movie is the value of Frames (Nr) in EPU>Preparation tab>Data Acquisition>Exposure Settings. Divide this value by 50 and enter the value in the EER fractionation field under Motion Correction in RELION to obtain 1 e⁻/Å² frame. (For example, if the EPU shows 1220 frames, divide it by 50 and enter 24 in the EER fractionation field.)**

2. Automated data collection by EPU

• See **center monitor**: TUI>Autoloader tab>Autoloader

Click on the number of the grid you want to see and click button.

(*takes 2-3 minutes)

• See **center monitor**: TUI>Setup tab>Vacuum

Click button to open the column valve.

• See **right side monitor**: EPU>Preparation tab

See Presets in the upper left corner. Select Atlas from the pull-down menu.

Confirm that C2=50, Obj=none.

See the top right corner. Click to take a preview image.

(*If it is completely dark, the column valve is closed and should be opened. If the ice is too thick, it is almost completely dark, but you should be able to faintly see the grid square)

• See **center monitor**: TUI>Search tab

See Stage>option>Set tab. Enter 120um for z and click button.

• See **right side monitor**: EPU>Atlas tab

Click , click , click , click (record file name).

Click , check the number of the current Grid (lit green), and click to prepare Atlas image (*Click to see the image).

(*wait for a while as the whole image is taken in 4x4)

***KEK-Titan provides the following three conditions for automated data collection by EPU.**

Condition	VPP	mode	Mag.	Exp. time	AFIS	purpose
#1	Yes	Counted	x130k (0.96Å)	~3.1 sec	No	Screening
#2	No	Counted	x165k, 215k, 270k (0.75, 0.58, 0.46Å)	~3.1 sec	No	Data collection (initial measurement or when the particle distribution is non-uniform in the Hole)
#3	No	Counted	x165k, 215k, 270k (0.75, 0.58, 0.46Å)	~3.1 sec	Yes	Data collection (when the particle distribution is uniform in the Hole)

Condition #1: With phase plate, Counting, x130k, ~3.1 sec**• See EPU>EPU tab**

Click **Session Creation**, click **New Session**, and click **Yes**.

Click **Session Setup**, _____ (Record the Session name)

(*1. Session type: Manual, Acquisition mode: Accurate)

(*2. Check Use Phase Plate for measurement condition #1)

(*3. choose the same folder as Atlas to save to), click **Apply**.

Click **Square Selection**: Click **Unselect All** and use mouse-wheel to enlarge.

Right-click on #1 thin, #2 medium, or #3 thick ice square to **Select**.

Right-click on #1, then select **Move stage to grid square** to move to #1.

(Screenshot: 000)

Click **Hole Selection**: Click **Acquire** button. If it is a good square, go to the following.

If not in the center of the square, right-click to move to the center of the square.

(If the hole is not visible in the center of the square,

use **Move stage here to move to a position where the Hole is visible.)**

See Auto Functions tab. Click **Auto-eucentric by beam tilt.**

See Presets. Select Hole/Eucentric and click **Start with **C2=50, Obj=none**.**

Return to EPU tab and click **Acquire.**

(If not in the center of the square, return to the center and click **Acquire.)**

Measure Hole size, **Find Hole, **Unselect All**.**

Right click and select **Add** to select 3 holes **(Screenshot: 001)**.

Return to **Square Selection** and select 3 holes each for #2 and #3 as well.

(*After selecting all squares/holes, save it as a Screenshot, a record of where you selected.)

Click **Template definition**: **Acquire**, **C2=50, Obj=PhP1**, and click **Find and Center Hole**.

Add Acquisition Area: Defocus = -1um.

(The two bottom corners of the green square are placed so that they touch the yellow circle.)

Add Autofocus Area: After distance, 10um, Objective lens.

Add Drift Measurement Area: once/gridsquare, 0.20nm/s.

See **TUI>Autoloader tab>Aperture>Option>PhasePlate**. Click **Next**.

Click **Automated Acquisition**: Click **Start Run**.

In condition #1, the correlation between the brightness of the hole (ice thickness) and the presence or absence of particles, etc. were examined.

(*If the skip rate is too high, confirm the hole size, height, and alignment.)

Condition #2: No phase plate, Counting, x165k/215k/270k, ~3.1 sec, No AFIS

• See EPU>EPU tab Click Session Creation, click New Session, and click Yes.

Click Session Setup, _____ (Record the Session name)

(*1. Session type: Manual, Acquisition mode: Accurate)

(*3. choose the same folder as Atlas to save to), click Apply. (Screenshot: epu)

Click Square Selection: Click Unselect All, use mouse-wheel to enlarge.

Right-click on good-looking squares based on the screening session and Select.

Right-click on the 1st Square and select Move stage to grid square.

Click Hole Selection: Click Acquire button. If it is a good square, go to the following.

If not in the center of the square, right-click to move to the center of the square.

(If the hole is not visible in the center of the square,

use Move stage here to move to a position where the hole is visible.)

See Auto Functions tab. Click Auto-eucentric by beam tilt.

See Presets. Select Hole/Eucentric and click Start with C2=50, Obj=None.

Return to EPU tab and click Acquire.

(If not in the center of the square, return to the center and click Acquire.)

Measure Hole size, Find Hole, adjust Quality Bar value if necessary.

(To select multiple squares, click Prepare all Squares.)

(If necessary, select holes or squares with different ice thickness.)

Use Selection Brush to erase near grid bar and around contamination.

After selecting holes, move to the carbon tear (check with Preparation>GridSquare).

See EPU>Preparation tab. Select Data acq and click Set. Confirm that **C2=50, Obj=None**.

EER, No, No, Dose: ~50, fraction: --, magnification: select x165k/215k/270k.

See Dose Rate. Click measure and Get x4. (Screenshot: dose)

Click R1 to insert the fluorescent screen and confirm that the beam is centered.

See EPU>Auto Functions tab>Auto Zero-loss. Change Presets to Zero loss and click Start.

Click Square Selection: Right-click on the 1st square and select Move stage to grid square.

Click Hole Selection: Right-click on a hole for Temp. Def. and select Move stage to grid square.

Click Template definition: Acquire, **C2=50, Obj=None**, and click Find and Center Hole.

Add Acquisition Area: Defocus = -0.8 - -2.0 or -1.0 - -2.5 um.

(Place it in appropriate position based on the screening session) (Screenshot: defocus)

Add Autofocus Area: Always, Objective lens.

Add Drift Measurement Area: Always, 0.20nm/s.

Click Template Execution: Click Preview. (*Confirm that the procedure set above works)

Click Automated Acquisition: Confirm that Auto Zero loss=Yes/20hrs, click Start Run.

Condition #3: No phase plate, Counting, x165k/215k/270k, ~3.1 sec, with AFIS

• See EPU>EPU tab Click **Session Creation**, click **New Session**, and click **Yes**.

Click **Session Setup**, _____ (Record the Session name)

(*1. Session type: Manual, Acquisition mode: **Faster**)

(*3. choose the same folder as Atlas to save to), click **Apply**. (Screenshot: epu)

Click **Square Selection**: Click **Unselect All**, use mouse-wheel to enlarge.

Right-click on good-looking squares based on the screening session and **Select**.

Right-click on the 1st Square and select **Move stage to grid square**.

Click **Hole Selection**: Click **Acquire** button. If it is a good square, go to the following.

If not in the center of the square, right-click to move to the center of the square.

(If the hole is not visible in the center of the square,

use **Move stage here to move to a position where the hole is visible.)**

See Auto Functions tab. Click **Auto-eucentric by beam tilt.**

See Presets. Select Hole/Eucentric and click **Start with C2=50, Obj=None.**

Return to EPU tab and click **Acquire.**

(If not in the center of the square, return to the center and click **Acquire.)**

Measure Hole size, **Find Hole**, adjust Quality Bar value if necessary.

(To select multiple squares, click **Prepare all Squares**.)

(If necessary, select holes or squares with different ice thickness.)

Use **Selection Brush** to erase near grid bar and around contamination.

After selecting holes, move to the carbon tear (check with Preparation>GridSquare).

See EPU>Preparation tab. Select Data acq and click **Set**. Confirm that **C2=50, Obj=None**.

EER, No, No, Dose: ~50, fraction: --, magnification: select x165k/215k/270k.

See Dose Rate. Click **measure** and **Get x4**. (Screenshot: dose)

Click **R1** to insert the fluorescent screen and confirm that the beam is centered.

See EPU>Auto Functions tab>Auto Zero-loss. Change Presets to Zero loss and click **Start**.

Click **Square Selection**: Right-click on the 1st square and select **Move stage to grid square**.

Click **Hole Selection**: Right-click on a hole for Temp. Def. and select **Move stage to grid square**.

Click **Template definition**: **Acquire**, **C2=50, Obj=None**, and click **Find and Center Hole**.

Add Acquisition Area: Defocus = -0.8 - -2.0 or -1.0 - -2.5 um.

(Place it in appropriate position based on the screening session) (Screenshot: defocus)

Add Autofocus Area: **After Centering**, Objective lens.

Add Drift Measurement Area: **Always**, 0.20nm/s.

Click **Template Execution**: Click **Preview**. (*Confirm that the procedure set above works)

Click **Automated Acquisition**: Confirm that Auto Zero loss=Yes/20hrs, click **Start Run**.

3. Troubleshooting

III. Cleaning up

• Shutdown of Vitrobot

- Close the door and press **Exit** on the touch panel (it will ask you Yes/No, press **Yes**).
- After a while, the screen darkens and you can switch it off.
- Remove Humidifier and discard water inside (shake well and discard).
- remove and discard filter papers.
- Keep the door of chamber open to facilitate drying of the chamber.

• Styrofoam container for Vitrobot

- Separate all parts and lay them out on a Kim Towel.
- Leave it in place to dry.

(*If you want to do the next experiment immediately, dry the metal parts with a hair dryer, and completely drain the styrofoam parts with a Kimwipe or similar.)

• Loading station

- Separate all parts and lay them out on a Kim Towel.
- Leave it in place to dry.

(*If you want to do the next experiment right away, use another well-dried Loading station.)

• NanoCab

- Leave the LN₂ in and the lid on, and leave it in place until the next day.

(*If you want to do the next experiment immediately, use another well-dried NanoCab.)

Note: Before leaving the lab, be sure to check the following **three points**.

- Close the **column valves** of the electron microscope (if measurement is in progress in the EPU, press the "close after measurement" button).
- When the electron microscope is not in use, pull out the **phase plate** (if the vacuum blows out with it in, the phase plate will tear).
- Close Valve on **ethane gas cylinder**.

IV. Alignment before screening or automated data collection

1. Import alignment files (daily)

- See EPU. Click the x in the upper right corner and click **Yes** to shut down EPU (wait about 30 seconds for EPU to shut down completely).
- See TUI>Setup tab>Filter.
- Confirm that **EFTEM** button is yellow.
(If it has not turned yellow, click **EFTEM** button to turn it yellow.)
- Launch the EPU software (once launched, move Window to the right side monitor).

- See TUI>Setup tab>Vacuum. Click **Col. Valves Closed** button.
- Confirm that the fluorescent screen is retracted (*If not, use **R1**).
- See EPU>Atlas tab. Right-click on the carbon tear and select **Move stage here**.

- See EPU>Preparation tab. Confirm that **Acquisition and Optics Settings** is selected.
- See EPU>Preparation tab>Presets. Select Grid Square and click **Preview**.
- Confirm that the position is in a carbon tear in the grid.
- Press **R1** to insert the fluorescent screen.
- Confirm that Flucam Viewer is set to **EF** (=display the center of EF mode), **Natural**, **High Resolution**.

- See EPU>Preparation tab. Click **Import** and open the following .xml file.
D:/Preparation/EC_215k_230117.xml (=0.58Å/pix)
- See TUI>Align tab>Alignments>Option>File tab.
- Select **300** and move everything in **Available** to **Selected**, and click **Apply**.
- See TUI>Align tab>FEG registers.
- Select **300kV-nP_EFTEM_kek** from the list and click **Set**.

2. Centering of C2 aperture (daily)

- Since this is a continuation of the previous page,
the position is in a carbon tear in the grid and the fluorescent screen should be down.
- See EPU>Preparation tab>Presets. Select Grid Square and click **Set**.
- See control panel and press **Eucentric focus** (*Obj lens=6.9276% at x580).**
- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- See control panel and press **Eucentric focus** (*Obj lens=80.6499% at x215k).**
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
(*C1=2000 and C3=1000 do not need to be touched at all. Mag should be x215k.)

(If there are two red squares, one large and one small, drawn on the Flucam Viewer screen, operation shown in this frame is unnecessary.)

- See Flucam Viewer and draw a 0.4 x 0.4um red square.
- Move the red square with the arrow keys to circumscribe the green circle (= center of EFTEM mode).
- See Annotation Properties on the right and change the size of red square to 0.8 x 0.8um.
- See Flucam Viewer and draw a 0.4 x 0.4um red square, again.
- Move the red square with the arrow keys to circumscribe the green circle (= center of EFTEM mode).
- See Annotation Properties on the right and change the size of red square to 2.2 x 2.2um.

1st round

- Turn Intensity dial to the left to narrow the beam to about 0.8um red square.
- See the lower right corner of TUI. Select **Direct Alignment** and click **Beam shift**.
- Use Multifunction dial to move the beam to the center.
- Turn Intensity dial to the right to spread the beam until it reaches 2.2um red square.
- See TUI>Autoloader>Apertures. Click **Adjust** on the right side of C2.
- Use Multifunction dial to move aperture so that it is circumscribed by 2.2um red square.
- Click **Adjust** next to C2 again.

2nd round

- Narrow the beam again to about 0.8um red square.
- Use Multifunction dial to move the beam to the center.
- Turn Intensity dial to the right to spread the beam until it reaches 2.2um red square.
- See TUI>Autoloader>Apertures. Click **Adjust** on the right side of C2.
- Use Multifunction dial to move aperture so that it is circumscribed by 2.2um red square.
- Click **Adjust** next to C2 again.

(*Repeat the above several times so that the beam and aperture is centered.)

- Click **Done**. (*It is OK if the circle of light spreads out almost concentrically.)

3. Pivot point adjustment (daily)

- The beam position can be left as it was when centering the C2 aperture, and the fluorescent screen can be inserted.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click .
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
- See the lower right corner of TUI. Click from Direct Alignment.
- Use **Multifunction-X** to fine-tune the overlap of the two flashing lights.
- See the lower right corner of TUI. Click from Direct Alignment.
- Use **Multifunction-X** to fine-tune the overlap of the two flashing lights.
- Click .

4. Rotation Center (daily)

- Press to retract the fluorescent screen.
 - See EPU>Atlas tab. Right-click on a contamination and select .
 - See TUI>Search tab>Stage>Option>Set tab and move z=120um.
 - See EPU>Preparation tab>Presets. Select Grid Square and click .
 - Right-click on a contamination and select .
 - See EPU>Preparation tab>Presets. Select Hole/Eucentric and click .
 - Right-click on a contamination and select .
 - See EPU>Auto Functions tab. Click .
 - See Presets. Select Hole/Eucentric and click .
- (*Tilt axis runs horizontally to the screen.)

- Press to insert the fluorescent screen.
(If the contamination is not in the center of the green circle, use the joystick to center it.)
- See EPU>Preparation tab>Presets. Select Data Acquisition and click .
- See TUI>Autoloader tab>Apertures. Set C2=150 (to widen field of view), Obj=none.
(*The orientation of the image is different for EPU and the fluorescent screen.)
- Turn the Magnification dial to the right to change magnification to x350k.
- See the lower right corner of TUI. Select and click .
- Use Multifunction dial to fine-tune the contamination so that it does not move.
(*Later, AutoCTF is used to remove coma aberration, so it is good if it is not so correct.)
- Click .

5. Focusing on phase plate (daily)

- Confirm that the phase plate heater is turned on. (*wait for about 2 hrs after turning it on)
- Press **R1** to retract the fluorescent screen.
- See EPU>Atlas tab. Right-click on a carbon tear in the Grid and select **Move stage here**.
- See EPU>Preparation tab>Presets. Select Grid Square and click **Preview**.
- Confirm that the position is in a carbon tear in the Grid.

- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- Press **R1** to insert the fluorescent screen.
- See TUI>Autoloader tab>Apertures. Set **C2=150, Obj=PhP1**.
- See Flucam Viewer. Change its display mode to Linear (it's easier to see).
- See the right side of TUI. Select only the peaks at the histogram to see the haze.

- See TUI>Search tab>Beam Settings>Option>TEM tab.
 - Check **MF-Y Fine focus back-focal plane**.
- (*In the case of Titan, even if intensity dial is turned, parallel illumination is maintained, and the focus position does not change.)
- Turn Multifunction-Y left and right to produce stripes.
- (*Turn Multifunction-Y to change the image **from hazy to vertical-stripe to white to horizontal-stripe to hazy**, so turn left/right to find the place where you can see stripes.)
- See the lower right corner of TUI. Select **Stigmator** and click **Condenser**.
 - Then use Multifunction-X,Y to widen the stripe to a mesh.
- (*It takes a certain amount of skill, but it works well if you do it as if you were widening the space between the stripes. The stripes are visible because the beam is elliptical.)

Repeat the operations in the frame above, it becomes from **hazy to white to hazy**.

- When finished, stop the Multifunction-Y dial where the image disappears (=Just focus).
 - Uncheck MF-Y Fine focus back-focal plane**.
 - Click None under Condenser**.
- See TUI>Autoloader tab>Apertures>Option>PhasePlate. Click **Next**.
- See Flucam Viewer. Change its display mode to Natural.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none, Mag=x215k**.
- See EPU>Preparation tab>Presets. Click Get x4 from Data Acquisition to Zero loss.**
- Return Presets to Data Acquisition.

6. Centering of objective aperture (daily)

- Perform after focusing to phase plate. (*Illumination area=800nm is OK)
- Press **R1** to retract the fluorescent screen.
- See EPU>Preparation tab>Presets. Select Hole/Eucentric and find a contamination image.
- Right-click on the vicinity of a contamination and select **Move stage here** to move it.
- See EPU>Auto Functions tab. Click **Auto-eucentric by stage tilt**.
- See Presets. Select Hole/Eucentric and Click **Start**.
- See EPU>Preparation tab>Presets. Select Hole/Eucentric and click **Preview**.
- Right-click on a contamination-free carbon film and select **Move stage here**.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.

- Press **R1** and make sure to insert the fluorescent screen!!!**
(*Pressing the Diffraction button without the fluorescent screen may damage the camera.)

- See control panel and press **Diffraction** button.
- See TUI and confirm that D=6.4m, etc.
- See TUI>Autoloader tab>Apertures. Change to **Obj=100**.
- See Flucam Viewer. Confirm that its display mode is HDR.

*The position of objective aperture should be almost right,
 so it's safer not to do the three points in the box below, unless they are very far off.

- See TUI>Autoloader>Apertures. Click **Adjust** on the right side of Objective.
- Use Multifunction dial to adjust the center of bright light with the center of blurred light.
- Click **Adjust** next to Objective again.

- Press **Diffraction** button again to exit from diffraction mode.**
 (*FluCam Viewer should automatically return to Natural display mode.)

7. Energy filter adjustment (daily)

- Press **R1** to retract the fluorescent screen.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
- See EPU>Atlas tab. Right-click on a carbon tear in the Grid and select **Move stage here**.
- See EPU>Preparation tab>Presets. Select Grid Square and click **Preview**.
- Confirm that the position is in a carbon tear in the Grid.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.

- Press **R1** to insert the fluorescent screen.
- If the beam is not centered, see the lower right corner of TUI, use **Direct Alignments**>**Beam Shift** to move the beam to the center, and click **Done**.
- Confirm that the beam covers the entire region of the green circle.
- Press **R1** to retract the fluorescent screen.

- Normally, the Sherpa software should already be running, so see the bottom of the monitor, click the gear symbol, and bring up Sherpa window. (If it is not running, see Microscope Software Launcher>Tools and select Sherpa.)
- See Sherpa window>the left side. Click **Energy Filter** button.

- See Sherpa window>the lower left corner>Settings.
- Confirm that EF-Falcon, Bin=1, Exp time=0.5sec, and check Electron counting.**

- See Sherpa window>the upper left corner> Controls.
- See Zero loss. Click **Center** button to adjust (wait 1-2 minutes).

- See Isochromaticity. Click **Tune** button to equalize the energy on the camera.
- After a while, a red-green-blue image will appear.
Wait a few minutes until the colors of this image become uniform in green.

- See Geometric and Chromatic Distortions. Click **Tune Magnification** button.
- See Geometric and Chromatic Distortions. Click **Tune Distortions** button.
- See Zero loss. Click **Center** button to adjust again.

8. AutoCTF (daily)

- See EPU>Preparation tab>Presets. Select Hole/Eucentric and find a contamination image.
- Right-click on the vicinity of a contamination and select **Move stage here** to move it.
- See EPU>Auto Functions tab. Click **Auto-eucentric by stage tilt**.
- See Presets. Select Hole/Eucentric and click **Start**.
- See the bottom of TUI and confirm that Defocus is 0um.
- See EPU>Preparation tab>Presets. Select Hole/Eucentric and click **Preview**.
- Right-click on a contamination-free carbon film, and select **Move stage here**.

- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
- Press **R1** to insert the fluorescent screen.
- If the beam is not centered, see the lower right corner of TUI, use **Direct Alignments>Beam Shift** to move the beam to the center, and click **Done**.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set** again.
- Press **R1** to retract the fluorescent screen.

- See Sherpa window>the left side. Click **AutoCTF** button.
- Select the camera type to EF-Falcon.
- For EC mode, so check **Electron counting** box.**
(**Exp. Time=2, Binning=2, Readout=Full, Auto-focus-to=checked -1.0**)
- See Objective Stigmation. Click **Measure**** (*move a little if there is a mark) .
(If the thon ring does not pick up, turn Focus dial to apply -800 nm Defocus.)
- If thon ring is picked up, click **Correct**.

(It removes astigmatism of the obj lens. If the light is too dark, it will not work. Do not stop in the middle of the process; if you do, it will stop in a strange state in the middle of the process.)

- When "Completed" appears in the lower right corner, click **Correct in Coma**.
- When "Completed" appears in the lower right corner, leave **C2=50, Obj=none**, and click **Correct in Objective Stigmation** again.
(*If AutoCTF does not work well, something is wrong. Let's start over.)
- When finished, minimize Sherpa's Window.

9. Measure dose in EPU (daily)

- See EPU>Atlas tab. Right-click on a carbon tear in the Grid and select **Move stage here**.
- See EPU>Preparation tab>Presets. Select Grid Square and click **Preview**.
- Confirm that the position is in a carbon tear in the Grid.

- See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.

- See EPU>Preparation tab>Exposure Settings.
- Set them to EER, No, No, Dose=**~50**, Fractions= (1).
- Change magnification to x165k, x215k, or x270k as needed.
- See EPU>Preparation tab>Dose Rate. Click **Measure** button.
 - x165k: Exp. time= ~ 3.1 sec (about 8.28 e/px/s)
 - x215k: Exp. time= ~ 3.1 sec (about 5.10 e/px/s)
 - x270k: Exp. time= ~ 3.1 sec (about 3.24 e/px/s)
- See EPU>Preparation tab.
 - Select **Data Acquisition** from Presets and click **Get**.
 - Select **Autofocus** from Presets and click **Get**.
 - Select **Drift Measurement** from Presets and click **Get**.
 - Select **Zero loss** from Presets and click **Get**.
- See EPU>Preparation tab>Presets. Select Data Acquisition again.

10. Gain reference (if necessary)

*Because the settings in this text do not change the beam diameter when the magnification is changed, the brightness will change when the magnification is changed. In this case, gain will also change because CMOS has poor linearity. So it is safer to re-acquire gain when collecting data at different magnifications.

- See EPU>Preparation tab>Presets. Select Grid Square and click Preview.
- Confirm that the position is in a carbon tear in the Grid.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click Set.
- See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
- Press R1 to insert the fluorescent screen.
- Confirm that the beam covers the entire region of the green circle.

- See Microscope Software Launcher>Tools>Camera and detector.
- Select **Falcon4i Reference Image Manager**.

- See the window and **select PostCounting Gain file since it is in Counting mode.**
- Press R1 to retract the fluorescent screen** and then click Measure Dose button.
- If the magnification is set to x215K, the bar should be in the green range.
 - (*It should be ~5.1 e/px/s) (*It is known that 1-12.5 e/px/s is appropriate for Falcon4i.)
 - (*If value is too low, check if fluorescent screen, Beam Blank, Camera Insert are correct.)
- When the bar comes to the green range, click Acquire (takes about 20 minutes).

- When finished, confirm that the date of PostCounting Gain file has been updated.
- See EPU>Preparation tab>Presets. Select Data Acquisition and click Preview.
- Confirm that the image is flat.
- (.gain file is copied in OffloadData/ImagesForProcessing/EF-Falcon/300kV/
when the data collection is started in EPU)

- Close the window of Falcon4i Reference Image Manager, when gain reference is finished.

11. Centering images between different magnifications (daily)

- See EPU>Preparation tab>Presets. Select Hole/Eucentric and find a contamination image.
 - Right-click on the vicinity of a contamination and select **Move stage here** to move it.
 - See EPU>Auto Functions tab. Click **Auto-eucentric by stage tilt**.
 - See Presets. Select Hole/Eucentric and click **Start**.
 - See EPU>Preparation tab>Presets. Select Hole/Eucentric and click **Preview**.
 - Right-click on the corner of the contamination and select **Move stage here**.

 - See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
 - Press **R1** to insert the fluorescent screen. (*Change C2=150, if the field of view is narrow.)
 - Move the corner of the contamination to the center of the green circle with joystick.

 - See EPU>Preparation tab. Click **Calibrate Image Shifts** and click **Start Calibration**.
 - See x215k image. Click **Proceed**. (*Do not double-click here to move. It will be displaced)
 - See x6500 image, and double-click where it matches the center of x215k image. **Proceed**.
 - See x580 image, and double-click where it matches the center of x6500 image. **Proceed**.
 - See x100 image, and double-click where it matches the center of x580 image. **Proceed**.
 - The procedure is finished when you see "finished successfully"

 - See EPU>Preparation tab. Click **Acquisition and Optics Settings**.
 - See EPU>Preparation tab>Presets. Select Data Acquisition and click **Set**.
- (*Note for KEK staff: If we want to save FEG registers or Preparation file, save them here.)
- If screening session is to be started, the following operations should be performed.
 - See EPU>Preparation tab>Presets>Data Acquisition.
 - Change mag to x130k and click **Set**.
 - See TUI>Autoloader tab>Apertures. Confirm that **C2=50, Obj=none**.
 - See EPU>Preparation tab. Click **Get** x4 from Data Acquisition to Zero loss.**
 - See EPU>Preparation tab>Presets. Select Data Acquisition again.
 - Change Presets to Atlas and click **Set**.

V. Others

1. Settings for EPU Preparation

An example of EPU Preparation settings is shown below for reference.

Condition #1: Counting mode, 130k with phase plate (*Mag was changed from x215k)

	Camera	Binning	Readout	Exp. time, etc.	Probe	Mag	Defocus	Spotsize	Ill. area
Atlas	Falcon4i	2	Full	Linear,No,No 1s	Micro	x100	-1000	5	1413um C2:50
Grid Sq	Falcon4i	2	Full	Linear,No,No 1s	Micro	x580	-200	5	272.55um C2:50
Hole/ Eucentric	Falcon4i	2	Full	Linear,No,No 1.00s	Micro	x6,500	-50	5	25.00um C2:50
Data Acq	Falcon4i	1	Full	EER,No,No ~50 e⁻/Å² ~3.1s, (1 fr)	Nano	x130k (0.96Å)	-1	5	0.8um C2:50 Slit:10eV
Autofocus	Falcon4i	2	Full	Linear,No,No 0.50s	Nano	x130k	--	5	0.8um C2:50
Drift Mes	Falcon4i	2	Full	Linear,No,No 1.00s	Nano	x130k	-2	5	0.8um C2:50

Condition #2,3-165k: Counting mode, 165k (*Mag was changed from x215k)

	Camera	Binning	Readout	Exp. time, etc.	Probe	Mag	Defocus	Spotsize	Ill. area
Data Acq	Falcon4i	1	Full	EER,No,No ~50 e ⁻ /Å ² ~3.1s, (1 fr)	Nano	x165k (0.75Å)	-1	5	0.8um C2:50 Slit:10eV
Autofocus	Falcon4i	2	Full	Linear,No,No 0.50s	Nano	x165k	--	5	0.8um C2:50
Drift Mes	Falcon4i	2	Full	Linear,No,No 1.00s	Nano	x165k	-2	5	0.8um C2:50
Zero Loss	Falcon4i	4	Full	Linear,No,No 0.1s	Nano	x165k	--	5	0.8um C2:50 Slit:10eV

Condition #2,3-215k: Counting mode, 215k (*D:/Preparation/EC_215k_230117.sxml)

	Camera	Binning	Readout	Exp. time, etc.	Probe	Mag	Defocus	Spotsize	Ill. area
Data Acq	Falcon4i	1	Full	EER,No,No ~50 e ⁻ /Å ² ~3.1s, (1 fr)	Nano	x215k (0.58Å)	-1	5	0.8um C2:50 Slit:10eV
Autofocus	Falcon4i	2	Full	Linear,No,No 0.50s	Nano	x215k	--	5	0.8um C2:50
Drift Mes	Falcon4i	2	Full	Linear,No,No 1.00s	Nano	x215k	-2	5	0.8um C2:50
Zero Loss	Falcon4i	4	Full	Linear,No,No 0.1s	Nano	x215k	--	5	0.8um C2:50 Slit:10eV

Condition #2,3-270k: Counting mode, 270k (*Mag was changed from x215k)

	Camera	Binning	Readout	Exp. time, etc.	Probe	Mag	Defocus	Spotsize	Ill. area
Data Acq	Falcon4i	1	Full	EER,No,No ~50 e ⁻ /Å ² ~3.1s, (1 fr)	Nano	x270k (0.46Å)	-1	5	0.8um C2:50 Slit:10eV
Autofocus	Falcon4i	2	Full	Linear,No,No 0.50s	Nano	x270k	--	5	0.8um C2:50
Drift Mes	Falcon4i	2	Full	Linear,No,No 1.00s	Nano	x270k	-2	5	0.8um C2:50
Zero Loss	Falcon4i	4	Full	Linear,No,No 0.1s	Nano	x270k	--	5	0.8um C2:50 Slit:10eV

2. Acetone treatment of grids (if necessary)

*Do this work on a stainless-steel desk. If you do this on an ordinary desk, the acetone may melt the surface of the desk and the petri dish may stick to the desk.

- Place filter paper in a glass petri dish.
 - Carefully lay out about 25 grids on the filter paper (be careful of static electricity).
 - Pour acetone slowly so that the grid is soaked.
 - Stays at room temperature (overnight) .
 - Absorb as much acetone as possible with Kimwipes, etc.
 - Move the grid over the new filter paper with tweezers.
 - Cover with a glass petri dish and leave to dry for at least 1 hour.
 - Grid is stored in a grid box (white rectangular) .
- (*Be careful with static electricity. Be careful not to bend the grid with the grid box lid.)

3. Refilling of liquid nitrogen for grid preparation (if necessary)

- In the morning, pumped out from 120L liquid nitrogen tank in 2 x 5L Siebel bottles.

4. Replacement of 250L liquid nitrogen tank (biweekly)

- See TUI>Autoloader tab>Temperature Control>Option>Filling tab.
- Click and wait for a while.
- Close all valves of 250L LN₂ tank (green one at the outlet and black one) .
- Disconnect the black hose and plastic tube connected to the electron microscope.
- Replace 250L LN₂ tank.
- Connect the plastic tube and the black hose from the electron microscope.
- Fully open the green and black valves of 250L LN₂ tank.

5. Conditioning of Autoloader (biweekly)

- See TUI>Autoloader tab>Temperature Control>Option>State tab.
 - Set "Go to" in Temperature State to and click .
 - For After Conditioning, select ,
set "At" to the date and time you want to restore (*default is 12 hours later), and click .
- (*Conditioning both is done with planned power outages and long vacation shutdowns.)

6. Shutdown and reboot (before long-term vacation)

· Bring the temperature inside the EM to room temperature (leave the vacuum pulled).

Grid and cassette removal

- , . (*no need to put LN₂ in Nanocab)
- Wait until temperature display returns to green.
- Shutdown EPU.
- See TUI>Setup tab>Filter. Click to turn from yellow to gray.

Conditioning

- See TUI>Autoloader tab>Temperature Control>Option>State tab.
Set "Go to" in Temperature State to and click .
- For After conditioning, select and click .
- Wait until Dewars become 0%.
- Wait until temperature reaches room temperature (takes about 8-10 hours!).

Turning off the cooling of Falcon4i and Ceta (*once per 3-4 months)

- See Microscope Software Launcher>Tools>Camera and detector.
- Select .
- See the window>Basic Operations tab and uncheck .
- Change Cooling from to .
- See Microscope Software Launcher>Tools>Camera and detector.
- Select .
- See the window>Basic Operations tab and uncheck .
- Cooling state automatically changes from to .

Others

- Leave the electron gun and HT turned on.
- Leave the vacuum pulled as well.

· Reboot: Set the temperature inside the EM to liquid nitrogen temperature.

- If necessary, see TUI>Autoloader tab>Autoloader>Option, click .
- See TUI>Autoloader tab>Temperature Control>Option>State tab.
See Temperature State Go to, select , and click .
- See Temperature State Go to, select ,
- input the date and time 12 hours later, and click .
- (Wait ~12 hours until finished.)

Turning on the cooling of Falcon4i and Ceta

- See Microscope Software Launcher>Tools>Camera and detector.
- Select .
- See the window>Basic Operations tab and check .
- Cooling state automatically changes from to (takes 10-20 min) .
- See Microscope Software Launcher>Tools>Camera and detector.
- Select .
- See the window>Basic Operations tab and check .
- Cooling state automatically changes from to (takes 10-20 min).

Import alignment files

- (* Wait for the Column's Vacuum to drop below 34 log. Ideally, 1 log is good.)
- See TUI>Autoloader>Apertures>Option>Enable tab.
 - Confirm that and are yellow.
 - See TUI>Setup tab>Vacuum. Click button.
 - Press to insert the fluorescent screen and confirm that the beam can be seen.
 - See TUI>Align tab>Alignments>Option>File tab.
 - Select **300** and move everything in to , and click .
 - See TUI>Align tab>FEG registers.
 - Select **300kV-nP_EFTEM_kek** from the list and click .

*After this, we can use the EM by doing the alignment according to the procedure in Chapter IV (p.15-24).

7. Shutdown and reboot (before planned power outages or breakdown)

In this case, call KEK staff.

8. Lens/aperture configuration of Titan Krios

In Titan Krios, Lens/Apertures are arranged from top to bottom as follows.

Gun (+Gun lens)

C1 Lens (*Spot size)

C1 Aperture (*fixed at 2000 in KEK)

C2 Lens (*Spot size & intensity)

C2 Aperture (*50 or 150 in KEK)

C3 Lens (*intensity)

C3 Aperture (*fixed at 1000 in KEK)

Mini-condenser Lens (*Switching between nano probe and micro probe)

Obj Lens (*stage position)

Obj Aperture (*Back Focal Plane)

SA Aperture (*Almost never used in KEK)

Int. Lens

Pro. Lens