

Leginon single particle data collection

Updated at 8/1/2019

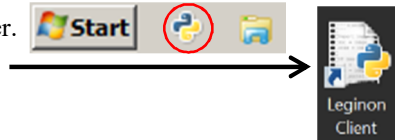
This document is not a full version of Leginon tutorial, but a protocol of how to smoothly set up a single particle data collection on Titan Krios with K3 camera at FSU BSIR. To avoid of confusion and make it easier to follow, most of the details are not mentioned, but only that relevance is addressed.



1 Create a session

1.1 Start Leginon clients

- Hermes is the Leginon host Linux computer.
- Start Leginon client on the task bar on Titan computer.
- Start Leginon client on the Desktop of K3 computer.



1.2 Switch to your account

- Open a new Linux terminal on Hermes computer, or use an existing one.
- Type `su your_user_name` to switch to your account.
- The username on Hermes is the same as your RCC account, but needs to be initialized by me.

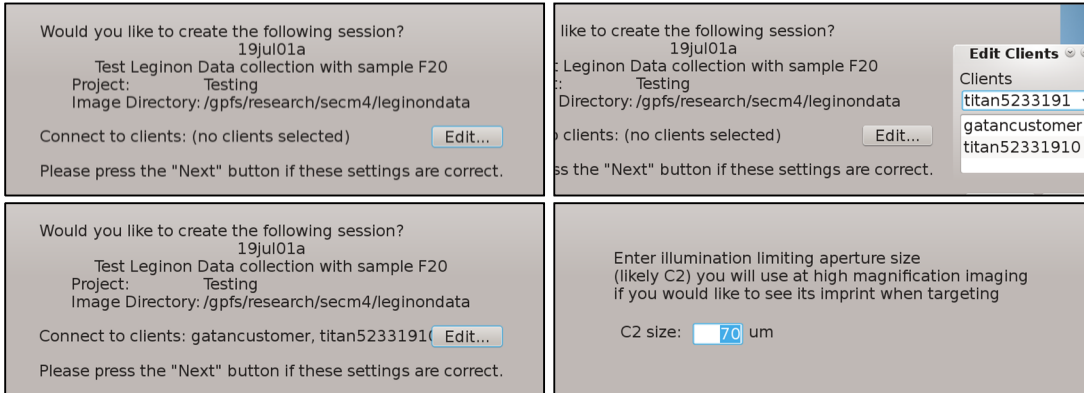
1.3 Launch Leginon

- Type `module load myamidev` on the Linux terminal.
- Type `start-leginon.py` on the Linux terminal.
- Make sure you have a `leginon.cfg` file existing in your home directory (`ls ~/leginon.cfg`), which stores (i) your full user name for Leginon login, and (ii) the location on GPFS to save your Leginon data. The `leginon.cfg` file is automatically created when your account on Hermes is created. Do check and edit the `cfg` file if it is your first time to use Leginon on Hermes.

1.4 Start Leginon GUI to create a new session

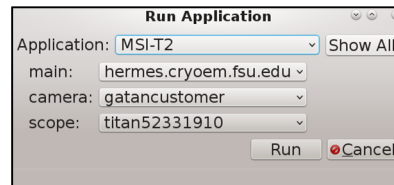
- Create a new session
 - Name: automatically generated
 - Holder: unknown (default)
 - Description: write down a short description of the session, for example the sample info
- Project: choose an existing project, or ask me to create it for you if it is a new project
- Image Directory: it should come out as what you have in `leginon.cfg`, but you can change it here
- Connect to clients: Edit to add titan5233191 (Titan) and gatancustomer (K3) to the list
- C2 size 70 μ : you can give any number here. It is just a note for Leginon database. We use 70 μ m C2 condenser aperture for K3, and 50 μ m for DE64 counting.
- Leginon GUI will pop up.

<p>Leginon Setup</p> <p>Decide whether you would like to create a new session or use an existing session.</p> <p>Session Type</p> <ul style="list-style-type: none"><input checked="" type="radio"/> Create a new session<input type="radio"/> Return to an existing session <p>Click the "Next" button to continue.</p> <p>Click the "Back" button to return to the previous screen.</p> <p>Click the "Cancel" button to exit the entire setup wizard if you want to continue.</p>	<p>Please confirm the session name and enter an optional description. You may change the suggested session name if needed.</p> <p>Name: <input type="text" value="19jul01a"/></p> <p>Choose holder from list or enter new one: <input type="text" value="Unknown Holder"/></p> <p>Description: <input type="text" value="Test Leginon Data collection with sample F20"/></p> <p>Then press the "Next" button to continue.</p>
<p>Select the project this session will be associated with.</p> <p>Project: <input type="text" value="Testing"/></p> <p>Then press the "Next" button to continue.</p>	<p>Select the directory where images from this session will be stored. (A subdirectory named after the session will be created for you)</p> <p>Image Directory: <input type="text" value="arch/secm4/leginondata"/> <input type="button" value="Browse..."/></p> <p>Then press the "Next" button to continue.</p>



1.5 Choose Legikon application

- Legikon GUI Menu -> Application
 Application: MSI-T2 (single particle)
 Main: hermes.cryoem.fsu.edu
 Camera: gatancustomer
 Scope: titan52331910
- If some of the options are not available it probably suggests that the Legikon client is not on.

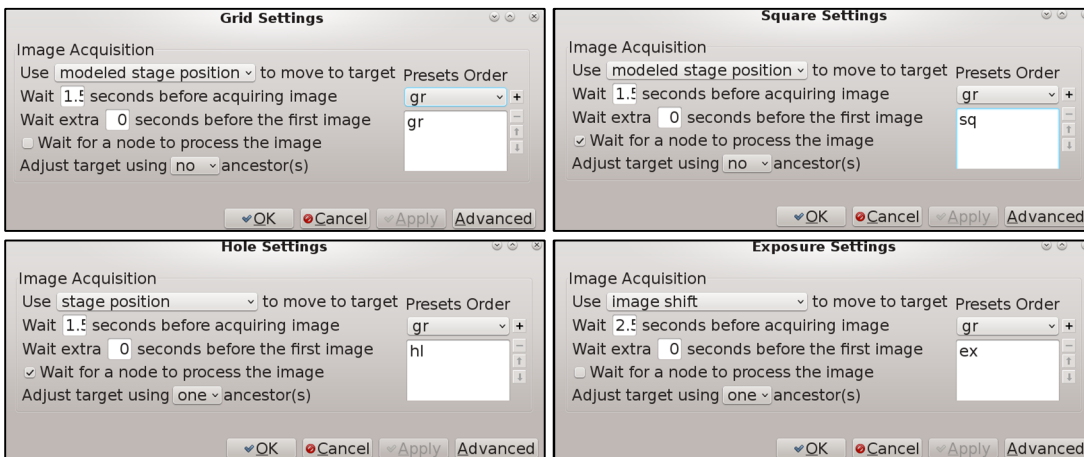


2 Legikon setup

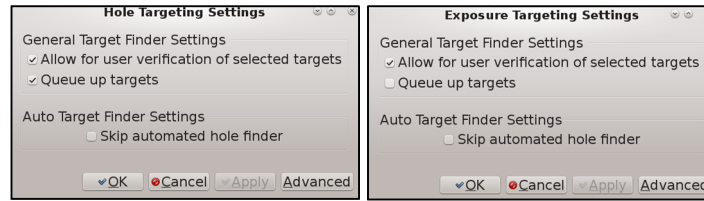
Everything in the left column on Legikon GUI is called 'node' in this document, for example the top two are Presets_manager node and Beam_Tilt_image node.


2.1 Pre-setup

- Grid node -> Settings: Use 'modeled stage position', set 'gr' as it's only preset on the list.
- Square node -> Settings: Use 'modeled stage position', set 'sq' as it's only preset on the list.
- Hole node -> Settings: Use 'stage position', set 'hl' as it's only preset on the list.
- Exposure node -> Settings: Use 'image position', set 'ex' or 'ec' as it's only preset on the list.
- The reason of using 'modeled stage position' for 'gr' and 'sq', but not 'stage position', is because K3 camera has an 180° rotation between LM and SA, and only 'modeled stage position' takes this rotation in account.



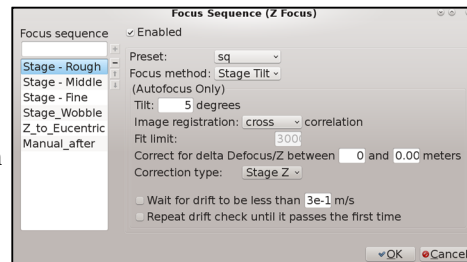
- Hole_Targeting node -> Settings: check 'Allow for user verification of selected targets' and 'Queue up targets', uncheck 'Skip automated hole finder'.
- Exposure_Targeting node -> Settings: check 'Allow for user verification of selected targets', uncheck 'Queue up targets' and 'Skip automated hole finder'.



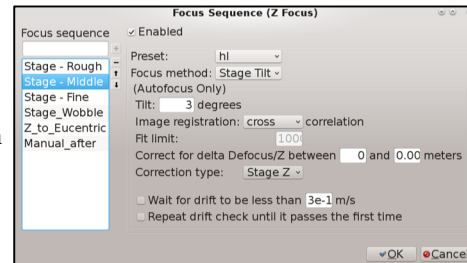
- Z_Focus node -> Focus Sequence : enable Stage-Rough and Stage-Middle, disable all others.
- Z_focus is to adjust the grid z to encentric height by tilting stage (encentric height is the optimal height where microscope is aligned). It will be automatically executed for a new a square during data collection. It can also be done at current position by clicking the 'simulate' icon.



- Stage_Rough: Preset: sq
Focus method: Stage Tilt
Tilt: 5 degree
Image registration cross correlation
Between 0 and 0.0004 meters
Correction type: Stage Z
Uncheck 'wait for drift'

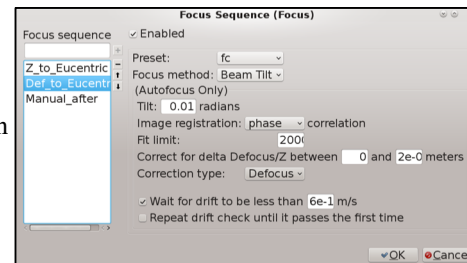


- Stage_Middle: Preset: hl
Focus method: Stage Tilt
Tilt: 3 degree
Image registration cross correlation
Between 0 and 0.0001 meters
Correction type: Stage Z
Uncheck 'wait for drift'





- Focus node -> Focus Sequence: enable Def_to_Eucentric only, disable all others.
- Focus is to slightly change the Obj. lens current to adapt to the requested defocus value. Differing from Z_focus of tilting stage, Focus here is to tilt the beam to find the in-focus condition (defocus 0) and then apply the requested defocus.

- Def_to_Eucentric: Preset: fc
Focus method: Beam Tilt
Tilt: 0.01 radians
Image registration phase correlation
Fit limit: 2000
Between 0 and 2e-05 meters
Correction type: Defocus

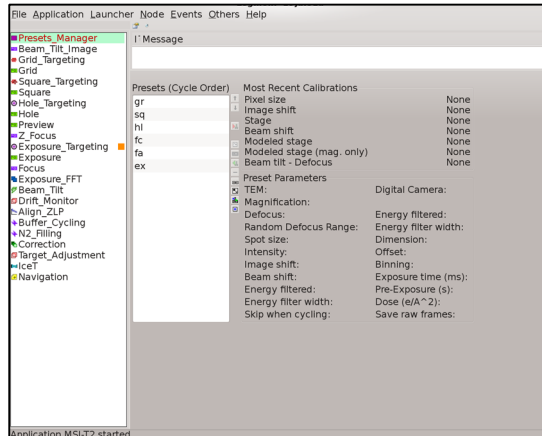
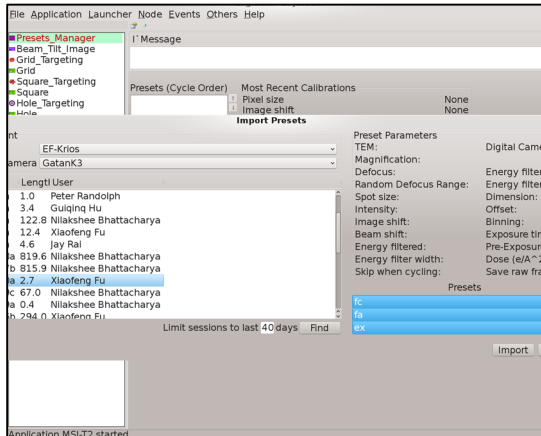
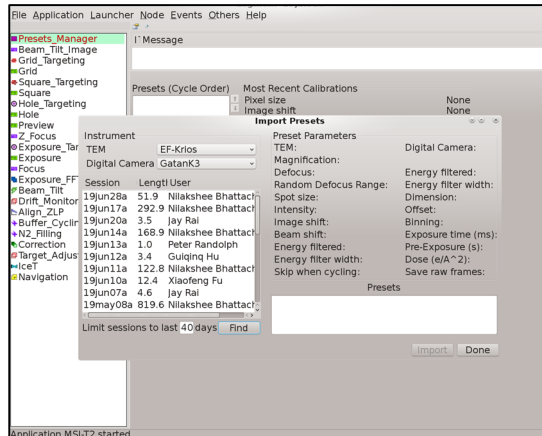
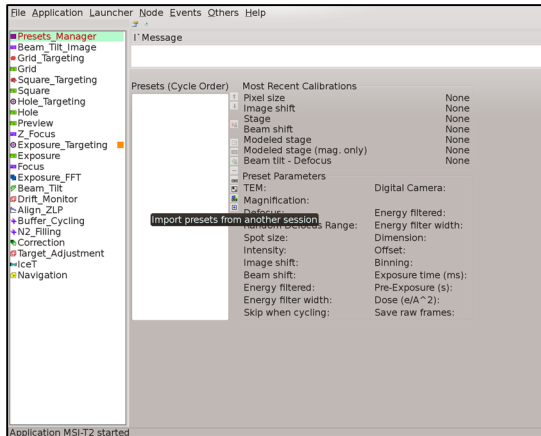


- All above settings only need to be done once.

2.2 Load presets

- Presets_manager node -> Import presets from another session  -> Instrument TEM: EF-Krios; Digital camera: GatanK3 -> Find someone's presets in the past xx days on the left panel -> select all presets from the right panel, usually including gr, sq, hl, fc, fa, ex (or ec) -> Import.
- Edit presets as the example below. 

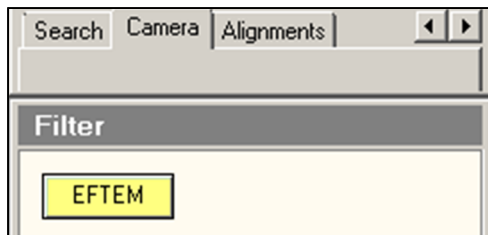
Preset	Mag	Defocus	SP	Intensity	EF	Camera	Exposure	Purpose
gr	135	0	8	0.00096	0 eV	1440X1023 bin8	1001 ms	atlas
sq	275	0	8	0.00038	0 eV	1008X1008 bin8	1001 ms	Square
hl	4800	-8e-05	8	1.322e-05	40 eV	1008X1008 bin8	1001 ms	Hole
fc	81000	-2e-06	7	1.1411e-06	20 eV	1008x1008 bin8	1001 ms	focusing
fa	81000	-2e-06	7	1.1411e-06	20 eV	1008x1008 bin8	1001 ms	Melting ice
ex	81000	-2e-06	7	1.1411e-06	20 eV	5760X4092 bin2	4875 ms	Data



2.3 Check if Hermes communicates with Titan and camera

- Cycle presets: select one preset and send to microscope. You should see mag changing on EM GUI

If you use K3 camera make sure EFTEM is on before cycling presets.



- Take an image with Navigation node: select navigation node -> send gr to microscope -> take an image



2.4 Preset alignment

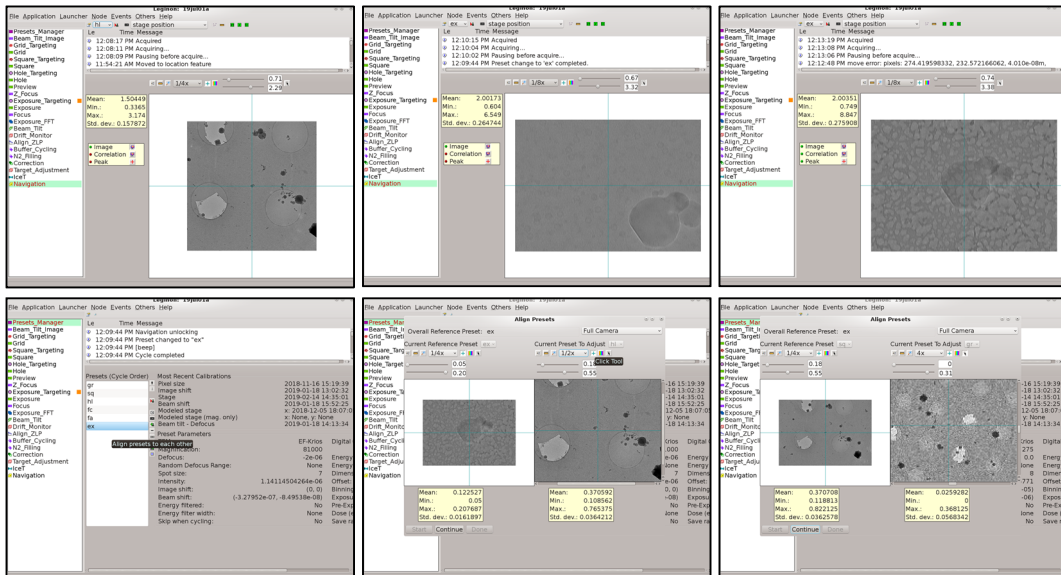
- The goal of preset alignment is to ensure they image at the same object by applying image shift for each individual preset.
- Check preset ex settings to make sure its image shift is zero, otherwise go EM GUI -> Tune -> Image Settings -> Reset shift, or simply fill 0,0 to image shift for preset ex setting. The reason is that any image shift will induce beam tilt which should be avoided of for data collection beam.





- Center beams for all presets. Use multiple X&Y button (beam tilt) to make the beam center on the green circle on Flu camera. Usually you only need to center those SA mode beam, including hl, fa, fc, and ex, but not touch the LM mode beam including gr and sq since they are pretty stable for long time. -> remember to update the beam shift in each preset once you make changes.



- Go to 'feature' square and move to an ice contamination or junk on carbon with proper size
- Center the object at ex preset with stage shift
- Go ex preset -> align presets to each other  -> you will be doing alignment between ex and hl, hl and sq, and sq and gr. Notice there is a 180° rotation between hl and sq for K3 camera. Use the Click Tool to center the same feature for each preset. 
- At the end every preset except for ex will have a new image shifts which is automatically calculated and filled in the preset settings. Preset ex should have 0 image shift because it is the reference that all others align to.









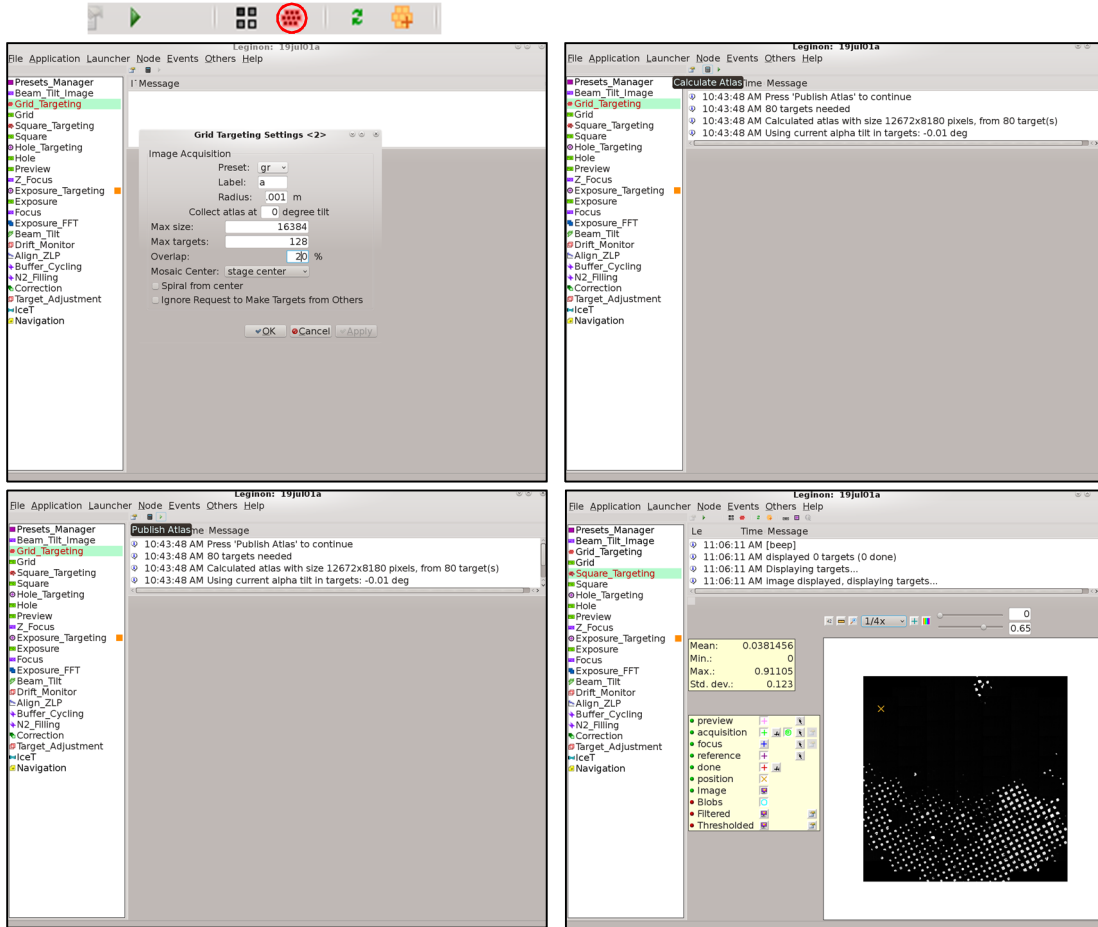
2.5 Collect atlas (overview map of the whole grid)

- Select Grid_Targeting node
 - Settings: Preset: gr  
- Label: a (you can give any character)
- Radius: 0.001m; Collect atlas at 0 degree tilt (only concerns this if you do tilted data collection)
- Max size: 16384 (default); Max targets: 128 (default)












Overlap: 20% (more overlap will be slower due to taking more images, but the atlas stitching will be better) Mosaic Center: Stage center



Uncheck 'spiral from center' and 'ignore request to make targets from others'

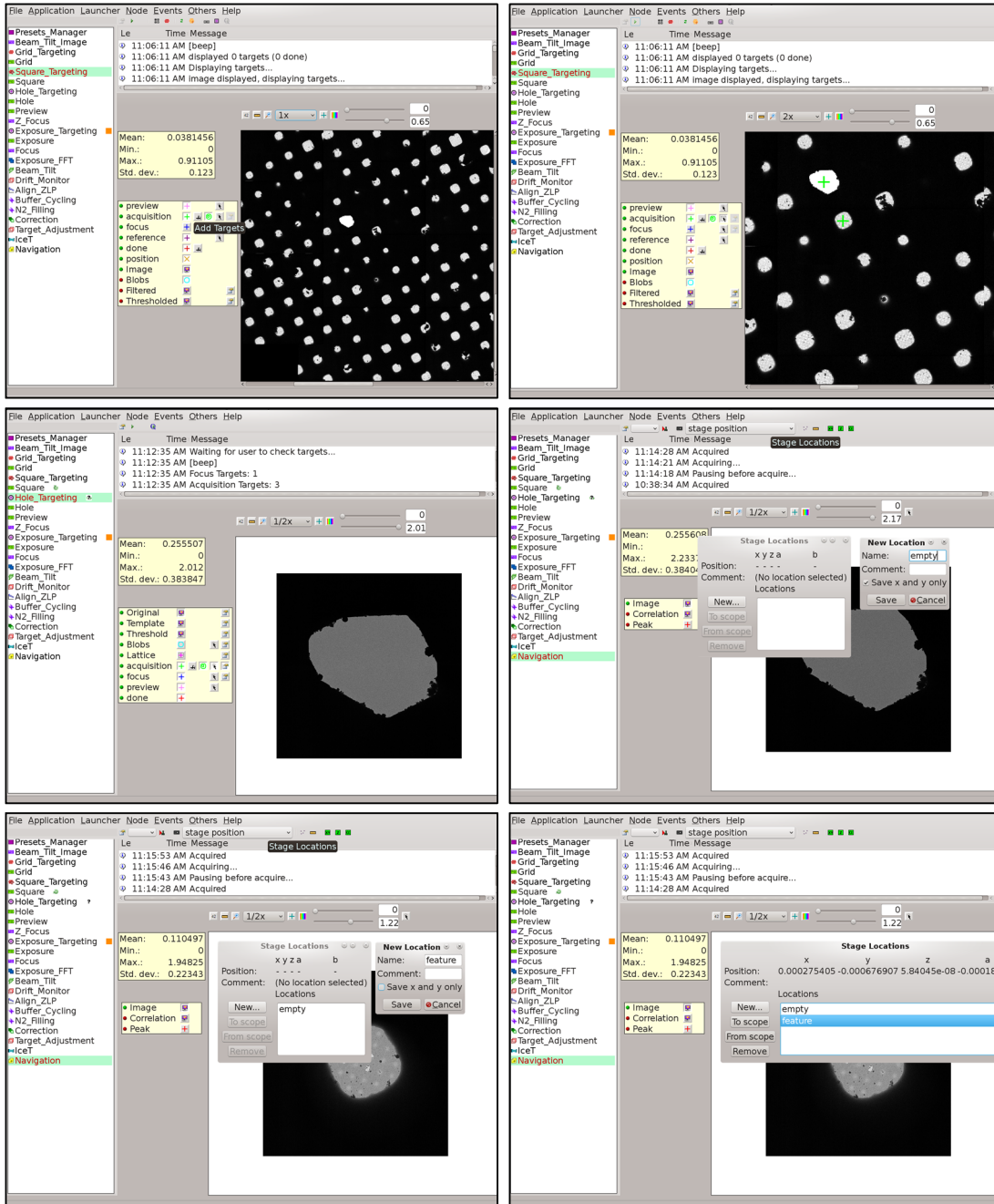
- Calculate Atlas (calculate how many tile images needed to complete the atlas map)   
- Publish Atlas   
- Each sq image will be published at Grid node simultaneously when the image is recorded, and the stitched atlas will be published at Square_Targeting node.
- If the atlas image is too compressed (default is 512), change it to 2048 at Square_Targeting node:



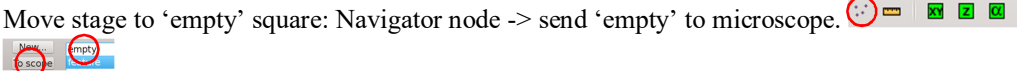
2.6 Pick up two squares: one 'empty' and one 'feature'

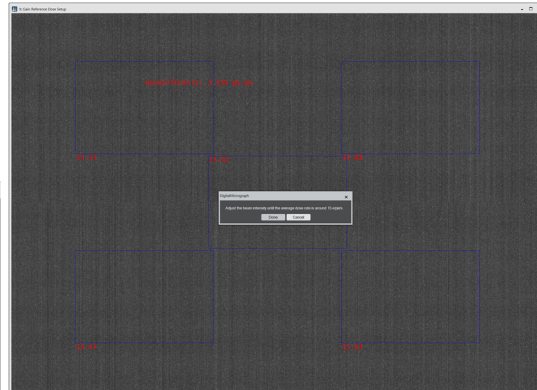
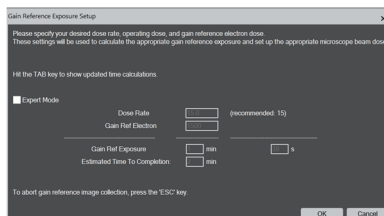
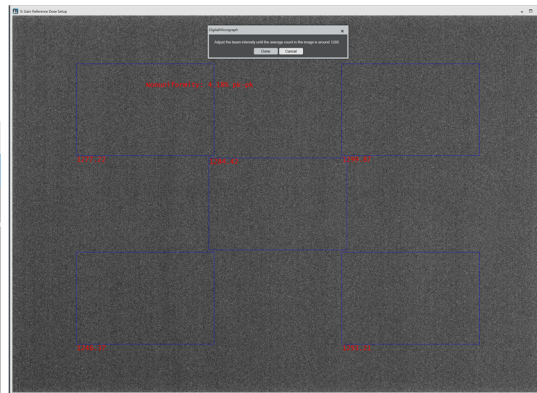
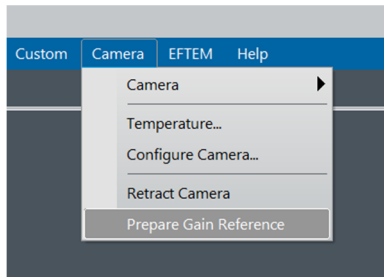
- The 'empty' square means a broken empty square for camera gain preparation, dose estimation, or phase plate charging, and so on.
- The 'feature' square means a square with normal looking ice and carbon for the purpose of objective lens astigmatism and coma-free correction, and with some proper size ice contamination or junks for preset alignment.
- Square_Targeting node -> acquisition Add Targets      -> select one broken empty square. Right click again is to delete the selected target -> submit queue 
- Hole_targeting node -> acquisition settings      -> clear targets. Some targets might have been automatically picked (green cross and box) with your previous Hole_targeting setting; we do not need any target but only the square coordinate at this moment.

- Save the coordinates for the 'empty' square: After the broken empty square image is published on the Hole_Targeting node, select Navigation node -> Stage Location -> New -> Name: 'empty' -> save. 
- Do the same operation to save the coordinate for a 'feature' square. Save the 'feature' square coordinate and name it as 'feature' (uncheck 'save x and y only' to have z saved)
- Later, simulate Z_focus to update the Z height for the 'feature' square at Hole node -> 



3 Camera gain preparation

- Move stage to 'empty' square: Navigator node -> send 'empty' to microscope. 
- Send ex preset to microscope and take an image to ensure the image is blank.
- DigitalMicrography Menu -> Camera -> Prepare Gain Reference
- It doesn't matter which mag or spot size you choose for K3 gain preparation. Here we use Mag81K, spot size 3 for Linear Mode and 7 for Counting Mode.
- Collect Gain Reference for Linear mode (Mag81k, spot size3).
- Adjust Intensity button (on the left EM control panel) to reach counts ~1280 (five red values on the image).
- Insert the Flu camera (R1 on the EM right control panel) to make sure the beam is bigger than the camera (green circle on the Flu camera indicates the size of K3 camera) and centered, otherwise center beam by multiple X&Y button on EM control panel.
- Retract the Flu camera (R1 again) to start taking gain
- Collect Gain Reference for Count Mode (Mag81K, spot size 7)
- Keep all parameter in popup windows 'Gain Reference Exposure setup' as default
- Adjust Intensity button to reach counts ~15 (five red values on the image).
- Insert the Flu camera (R1 on the EM right control panel) to make sure the beam is bigger than the camera (green circle) and centered, otherwise center beam by multiple X and Y button on EM control panel.
- Retract the Flu camera (R1 again) to start taking gain
- Check if gain is properly done.
- Go to Legikon GUI -> Select Navigation node -> send ex to microscope and take an image -> make sure image is clear, no any visible lines or features present. Zoom in to see the whole image to ensure no beam edge or fringes on the image corner as well.




4 Determine Exposure beam for data collection

4.1 Rough setup beam for data collection

- Navigation node -> send 'empty' square to microscope.
- Send ex to microscopy
- Change to the Mag for your data collection. Usually your target resolution determines the pixel size, the pixel size determines the Mag. (Three common Mag on K3: 64K 1.414 Å; 81K 1.12 Å; 105K 0.87Å)
- Switch spot size to 6 or 7 as a starting spot size
- Adjust Intensity button to ensure the beam diameter slightly bigger than the green circle on Flu Camera
- Slightly adjust condense astigmatator if the beam is not circular (EM GUI -> Tune -> Stigmator -> Condenser).
- Update all above adjustment to your ex preset

4.2 Fine determination of beam

- Here is an example of total dose $60\text{e}/\text{Å}^2$ for Mag 81K with pixel = 1.12 Å.
- Presets_Manager node -> ex ->  Acquire dose image for the selected preset
- Determine dose rate: Dose rate (beam intensity) is the key of using any direct electron camera. Too high dose rate will cause electron coincidence loss (electron comes to the camera too fast that the camera does not have time to read out each individual electron and may read two fast electrons as one). Too low dose rate will cause super long total exposure time for one image. The example below is $3.9\text{e}/\text{p}/\text{s} * 4 \sim 16\text{e}/\text{p}/\text{s}$ (the pixel in Legikon is based on super resolution mode, here is converted to counting mode by multiplied by 4; the recommend dose rate range for K3 is between 15~40 e/p/s). The best dose rate we use is between 15~20. Change spot size or Intensity then acquire dose image to measure dose rate again until it reaches this range. Update all above adjustment (Mag, spot size, beam diameter, and beam shift) to your ex preset

Use the measured dose $60.21\text{ e}/\text{Å}^2$ for this preset?

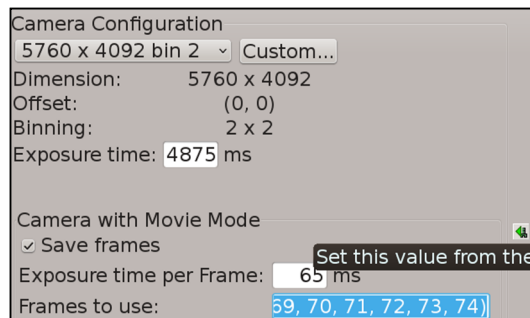
(Dose rate on camera pixel: 3.9 electrons/second)

(Dose on camera pixel per frame: 0.2 electrons)

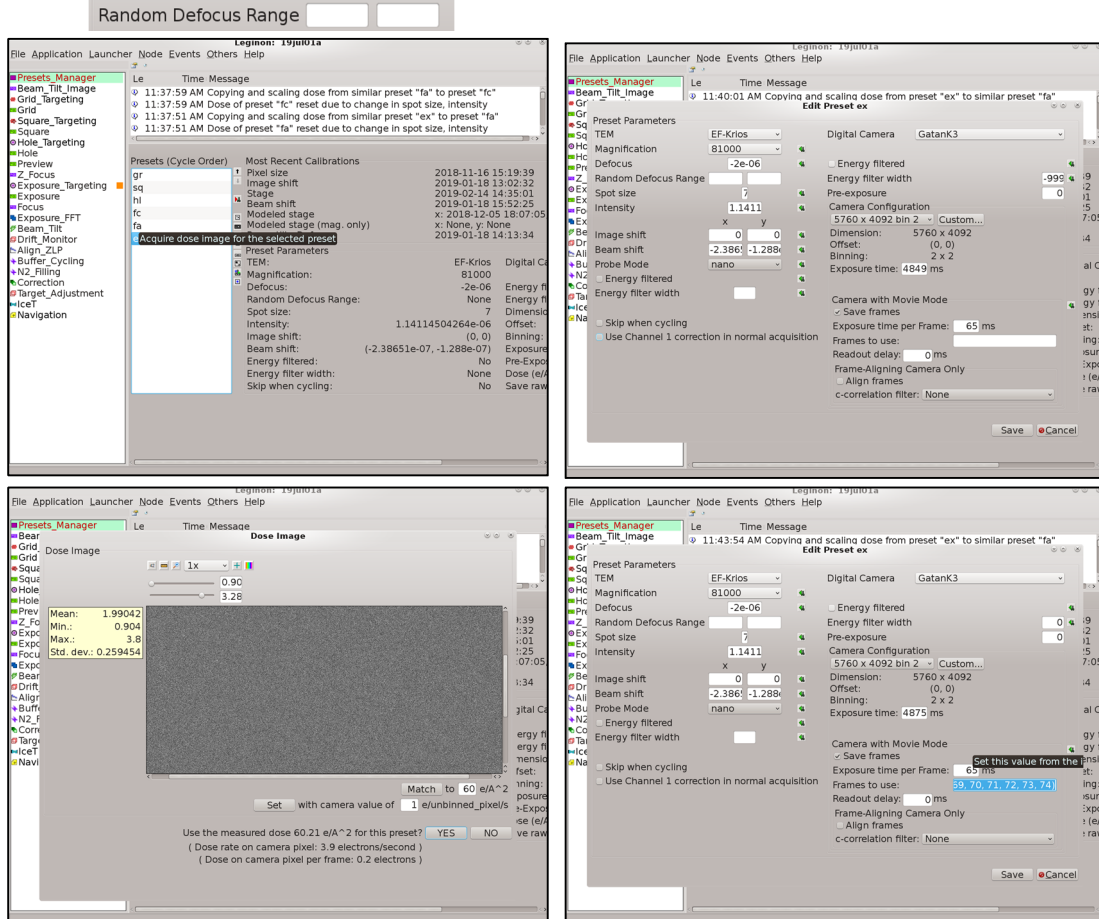
$3.9 * 4 \sim 16\text{ e}/\text{pixel}/\text{second}$

$0.25 * 4 = 1\text{ e}/\text{frame}/\text{pixel}$; 0.2 here indeed is 0.25

- Determine exposure time: match dose to $60\text{e}/\text{Å}^2$, Just click the Match button then the exposure time of ex preset will be automatically updated. **Match to 60 e/Å²**
- Determine frame number: $60\text{e}/\text{Å}^2 = 60\text{e}/(1\text{ pixel}/1.12)^2 = 75\text{e}/\text{pixel}^2$, which means that every frame will have 1e/frame/pixel if asking for 75 frames. 1.12 here is the pixel size for mag 81k.
- Calculate Exposure time per frame=Total exposure time/frame number=4875/75=65ms

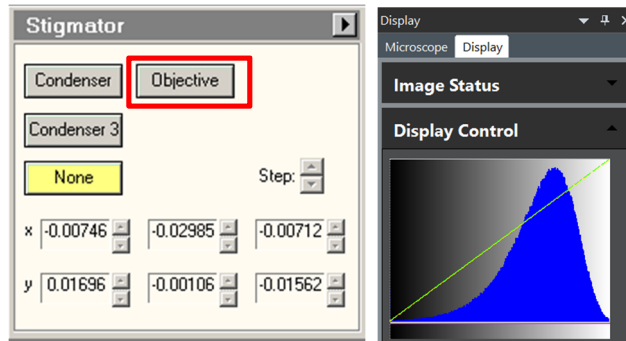


- Fill the calculated Exposure time per frame to ex preset **Exposure time per Frame:** . Acquire dose image again, and then the frame number will be automatically calculated and filled in (0, 1,, 74).
- Have 'save frame' checked to save frames
- Have defocus range setup for ex preset at 'Random Defocus Range', usually 0.6~3.0 μm defocus

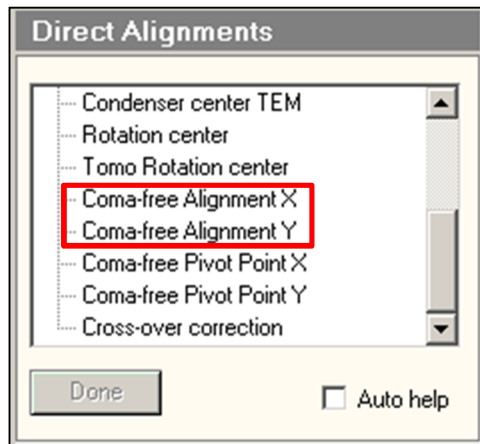


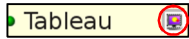

5 Objective astigmatism and coma-free

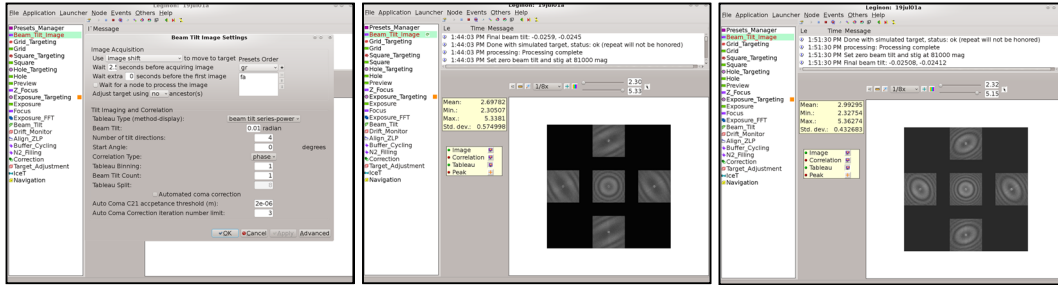
- Move stage to 'feature' square -> make sure it is at the right Z height, otherwise click 'simulate' at Z_Focus node to have Z eccentric -> slightly move stage X&Y to image fully on carbon,
- Send ex to microscope
- DigitalMicrograph -> view (with 0.1~0.15s exposure time) -> Menu Process -> live FFT
- EM GUI -> Tune -> Stigmator -> Objective -> multiple X and Y, adjust to have Thon ring circular at defocused condition (defocus ~1 μm), then change focus towards in-focus as much as you can until the Thon ring is most spreading but still visible (defocus 0.1~0.2 μm) to further adjust objective Stigmator. You might need to adjust the display to make Thon ring contrast sharper. Adjust Thon ring image contrast at DigitalMicrograph -> left panel 'Display' -> Display control. Finally acquire a one-second-exposure image to make sure there is no obj. stigmatism. Remember to turn off Objective Stigmator after you finish.



- Come back to 1~2 μ m defocus and go EM GUI Alignment -> Direct alignment -> coma-free alignment X and Y.
- Correcting coma-free alignment is to make sure there is no beam tilt for the exposure beam. Once coma-free alignment X is selected, an additional beam tilt is added on +X and -X periodically. User would see two identical distorted Thon rings flashing on Live FFT if the coma-free is good, meaning your beam tilt is zero. Otherwise, adjust multiple X button to have the two distorted Thon ring most close to each other, then do the same adjustment for coma-free alignment Y, and back and forth. You may still not be able to have them perfectly identical to each other since the beam tilt you changed will affect the Obj. astigmatism. Go back to do Obj. astigmatism, and come back to do coma-free again. Repeat this operation multiple times to have them perfect. The Thon ring has to be perfectly circular at the end.

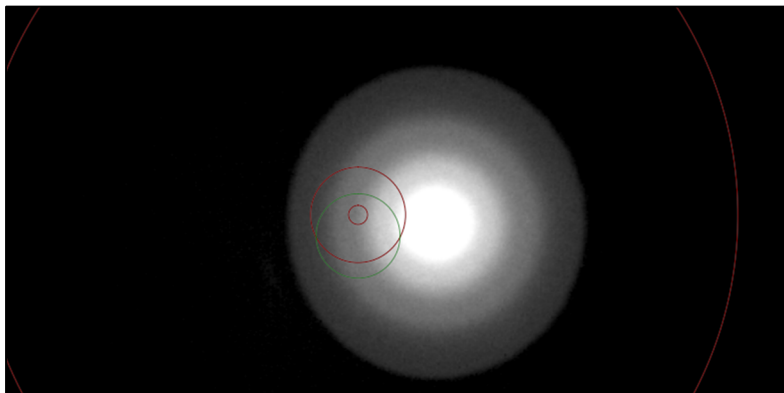
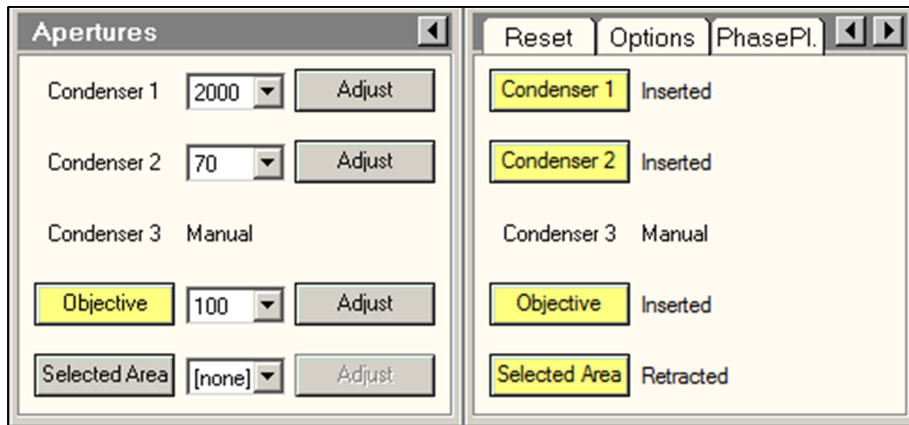


- Alternative way to do coma-free in Legikon as below.
 - Select Beam_Tilt_image node -> settings: Use image shift to move to target Presets Order; fa; Beam tilt 0.01 radian; Number of tilt directions: 4; Start Angle: 0; Correlation Type: phase; Tableau binning: 1; Beam Tilt Count: 1;
 - Preset fa has to be chosen to use a box size of camera, meaning equal size on x and y, otherwise the FFT will be distorted on the tableau map.
 - Simulate -> It will take five image at beam tilt of 0, -x, +x, -y and +y, separately.
 - Click Tableau. It will show those five FFT images at the tableau map 
 - Two tableau maps below were taken at the same good coma-free but with different defocus (-1.0 μ m vs. -1.5 μ m)
 - If the +-x or +-y is not identical to each other, click  on the tableau map a little off from the center, meaning to apply a little beam tilt on that direction, and then a new tableau will be taken to show the effect. Repeat this multiple times to reach a good coma-free. -> sent the coma-free to microscope.



6 Insert Objective aperture

- Send 'feature' square to microscope, and slightly move onto carbon area. Make sure it is on the right Z height.
- Presets_Manager node -> send ex preset to microscope
- EM GUI -> Camera -> turn off EFTEM. The reason is that Obj. aperture edge is invisible in EFTEM mode.
- Have the Flu camera inserted.
- Push Diffraction button on the right EM control panel to enter diffraction mode. You will see diffraction rings. Scroll the middle mouse to adjust the contrast on the Flu image. It is not a problem if the rings are not centered on the green circle.
- EM GUI -> Aperture -> click Objective button to insert it in and choose 100 for 100 μm aperture.
- Activate 'Adjust' button and use multiple X&Y on EM control panel to adjust the position of Objective aperture to make sure it is centered on the diffraction rings. Do not forget to deactivate the 'Adjust' button after you finish.





- EM GUI -> Camera -> turn on EFTEM
- Quite Diffraction mode.
- Presets_Manager node -> send preset ex and hl to microscope separately to ensure beam is not blocked by Objective aperture, suggesting the aperture is well centered.

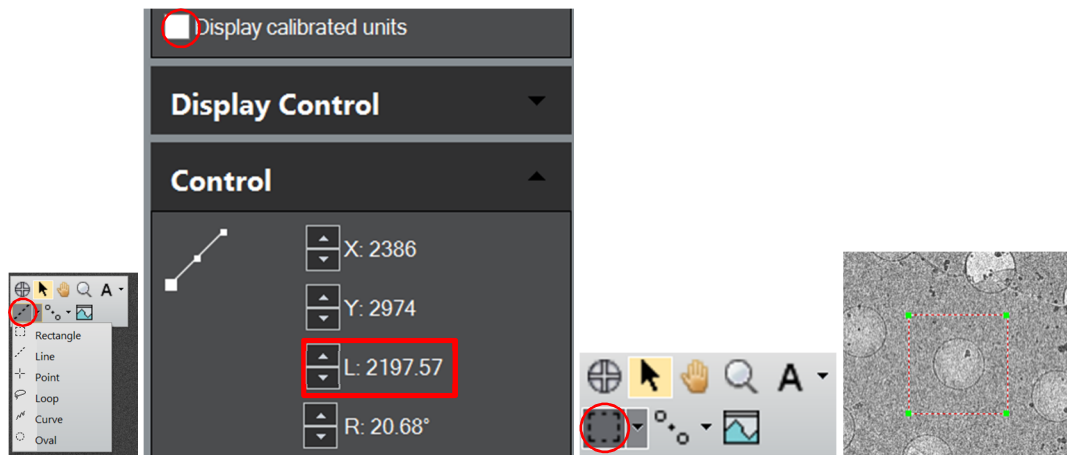
7 Square targeting

- Square_Targeting nodes -> acquisition Add targets -> select those squares with proper ice thickness and without cracks or big ice contaminations. -> submit queue.

8 Hole targeting

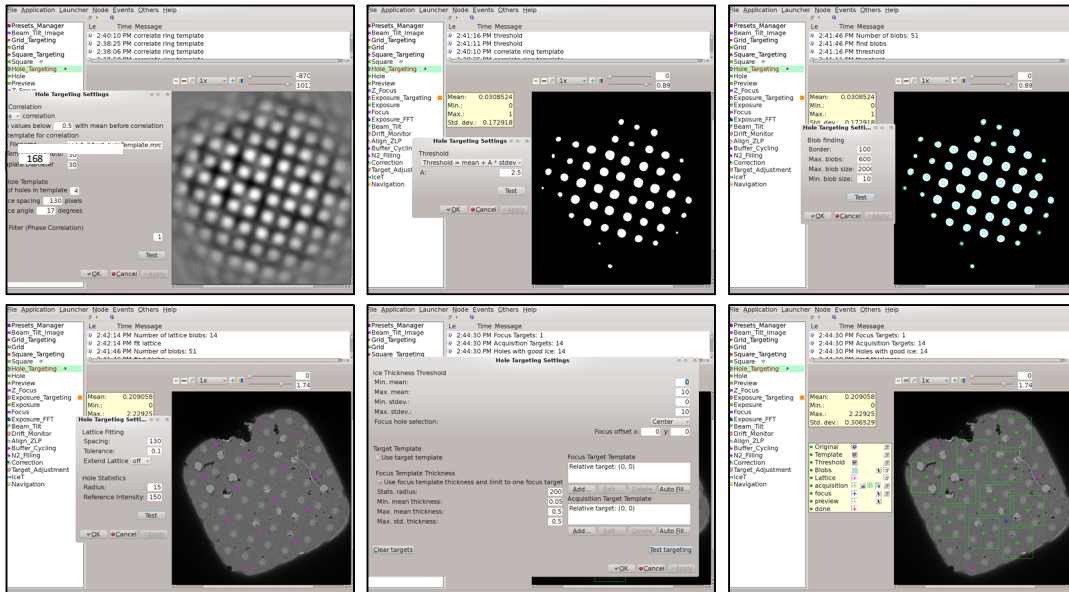
- Go to Hole_Targeting node, you should see the first gr image showing on the GUI windows
- Template setting: (below is an example to target on the center of each four holes)
 - Use 'cross' correlation
 - Fill image values below '0.3': this value should be bigger than the counts measured on the grid bar (activate 42 then the cursor will show counts , and smaller than inside the square, for example carbon or ice.
 - Uncheck 'invert template for correlation'
 - Template Filename: Leave it blank to use the default Leginon template
 - Original Template Diameter: 168
 - Final Template Diameter: measure the diameter of the hole. 
 - Number of holes in template: 4
 - Hole lattice spacing xx pixels: measure the spacing between two neighboring centers of four holes, practically you always put a little bigger value than measured
 - Hole lattice angle xx degrees: measure the angle between two neighboring centers of four holes
 - Click Test button
 - Ideally you would see strong sharp white peaks on the center of each four holes, try to adjust the 'Final Template Diameter' or 'Hole lattice spacing' to make the peak white and strong. If you see the peaks are at the right position but is black, it means your 'Final Template Diameter' is far away from what it should be.
 - The Leginon hole template works very well with well-defined hole grids, for example gold grid, but for thick ice or deformed grid, an alternative is to use the real hole from the current grid
 - Send sq preset to microscope
 - Capture a image through DigitalMicrograph with the same exposure time as you set up in Leginon for sq preset, and with count mode
 - On the image, right click to select line tool to measure the hole diameter (pixel unit), then select rectangular tool and Alt + left to drag out a hole.
 - Ctrl+C and then Ctrl+Alt+V to create the hole image, save the image with a name 'hole_template.mrc' at X:\LatitudeData\
 - Go to Hermes terminal to copy the file to Hermes at your home directory:


```
rsync /k3/k3_data/LatitudeData/hole_template.mrc ~/
```
 - Set the 'Template File' as /home/your_user_name/hole_template.mrc
 - Set the 'Original Template Diameter' with the measured diameter in DigitalMicrograph



➤ If neither of above methods works for you, then you have to manually do hole targeting.

- Threshold setting
 - Threshold: $\text{Threshold} = \text{mean} + A * \text{stdev} *$
 - A: xx. Adjust this value so you can see separated sharp peaks
 - Test
- Blobs setting
 - Border: xx. How many xx pixels close to edge should be excluded for blob finding
 - Max. blobs: estimate how many blobs should be present, always more than it should be
 - Max. Blob size: always give a very very big number
 - Min. Blob size: threshold to exclude those too small noise peak
 - The blob finding doesn't have to be perfect; those bad ones could be excluded at the next step 'Lattice'.
- Lattice setting
 - Spacing: the same value as the 'hole lattice spacing' in previous template setting
 - Tolerance: increase this value to select more if your grid or the hole geometry is deformed.
 - Extend Lattice: off. Turning it on will extend the lattice over the whole image based on the blobs geometry it found
 - Radius: the radius around the lattice spot (pink) for statistical analysis.
 - Reference Intensity: the count measured from an empty hole. Using default should work.
- Acquisition setting
 - Ice Thickness Threshold: Min. mean: 0; Max. mean: 10; Min. stdev: 0; Max. stdev: 10. Here since we select all possible lattice targets, so we give a big range as above to cover them all.
 - Focus hole selection: center
 - Focus offset x: 0; y: 0
 - Uncheck 'use target template'
 - Focus Target Template, Add one spot with $x=0$, and $y=0$, it should show 'Relative target (0, 0)' in the list.
 - Acquisition target Template, add one spot with $x=0$ and $y=0$, it should show 'Relative target (0, 0)' in the list.
 - Test targeting
- At the end of targeting you should see green crosses for acquisition and one blue cross for Z_Focus, otherwise go back to double check.



9 Exposure targeting

- The procedure of Exposure_Targeting is very similar to the Hole_Targeting, but we will do more control to exclude those 'bad' holes, for example, empty hole, thick ice hole, or crack holes.
- Go to Exposure_Targeting node, you should see a hl image showing on the GUI windows
- Template setting: (below is an example to target four holes)
 - Use 'cross' correlation
 - Fill image values below '1.1': set this value just a little higher than that big ice contamination
 - Uncheck 'invert template for correlation'
 - Template Filename: Leave it blank to use the default Legion template
 - Original Template Diameter: 168
 - Final Template Diameter: measure the diameter of the hole, always put a little bigger value than the measured value.
 - Number of holes in template: 1
 - Hole lattice spacing xx pixels: measure the spacing between two neighboring holes
 - Hole lattice angle xx degrees: measure the angle between two neighboring holes
 - Click Test button
 - Ideally you would see four strong sharp white peaks on the center of each hole, try to adjust the 'Final Template Diameter' or 'Hole lattice spacing' to make the peak white and strong. If you see the peaks are at the right position but are black, it means your 'Final Template Diameter' is far away from what it should be.
- Threshold setting
 - Threshold: $\text{Threshold} = \text{mean} + A * \text{stdev} *$
 - A: xx. Adjust this value so you can see separated sharp peaks
 - Test
- Blobs setting
 - Border: xx. How many xx pixels close to edge should be excluded from blob finding
 - Max. blobs: 4
 - Max. Blob size: always give a very very big number

- Min. Blob size: threshold to exclude those too small noise peak
- Lattice setting
 - Spacing: the same value as the 'hole lattice spacing' in previous template setting
 - Tolerance: increase this value to select more if your grid or the hole geometry is deformed.
 - Extend Lattice off.
 - Radius: the radius around the lattice spot (pink) for statistical analysis.
 - Reference Intensity: the count measured from an empty hole. Using default should work.
- Acquisition setting
 - Ice Thickness Threshold. Put mouse on the four Lattice target (pink) to read the values for 'Mean Thickness' and 'S.D. Thickness' (highlighted in yellow in the figure below).
 - Set min. mean and max. mean range so that it only include those good ice hole, and exclude that empty ice hole or thick ice hole
 - Set min. stdev and max. stdev range so that it only include those good ice hole, and exclude those crack ice holes.
 - Focus hole selection: center
 - Focus offset x: 0; y: 0
 - check 'use target template'
 - Focus Target Template, Measure the distance from the center of one hole to the center of four holes, record the distance and angle. Auto Fill Targets: number of targets: 4; radius (pixel): measured distance; angle offset (degree): measured angle.
 - Focus Template Thickness: Have 'use focus templates thickness and limit to one focus target' checked. Stats. radius: 200; Min. mean thickness: 0; Max mean thickness: 10, Max. std. thickness: 5.
 - Acquisition target Template, add one spot with x=0 and y=0, it should show 'Relative target (0, 0)' in the list.
 - Test targeting. You should see multiple green crosses for acquisition and one blue cross for Focus, otherwise go back to check.
- Monitor a couple of targets to see if your settings capture all good ice holes and exclude all bad ice holes, otherwise adjust the 'Ice thickness Threshold' .
- Uncheck the setting on Exposure node of 'Allow for user verification of selected targets' and let the data collection continue without interruption.
- Remotely check Appion once a while to make sure that the data collection is going well.

File Application Launcher Node Events Others Help

Time Message
 3:01:49 PM correlate ring template
 3:00:46 PM correlate ring template
 3:00:06 PM correlate ring template

Exposure Targeting Settings

Correlation
 s - correlation
 values below 1.1 with mean before correlation
 template for correlation
 Filename
 Template Diameter 16
 Plate Diameter 59

Hole Template
 of holes in template 1
 hole spacing 1196 pixels
 hole angle 7 degrees

Filter (Phase Correlation) 1

Test

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:06:38 PM correlate ring template
 3:06:02 PM correlate ring template
 3:05:41 PM correlate ring template

Exposure Targeting Settings

Correlation
 s - correlation
 values below 1.1 with mean before correlation
 template for correlation
 Filename
 Template Diameter 16
 Plate Diameter 68

Hole Template
 of holes in template 1
 hole spacing 1196 pixels
 hole angle 7 degrees

Filter (Phase Correlation) 1

Test

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:07:11 PM threshold
 3:07:07 PM threshold
 3:07:04 PM threshold

Exposure Targeting Settings

Mean: 0.0810916
 Min.: 0
 Max.: 1
 Std. dev.: 0.272976

Exposure Targeting Settings

Threshold
 Threshold = mean + A * stdev.
 A: 1.4

Test

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:07:42 PM Number of blobs: 4
 3:07:42 PM find blobs
 3:07:11 PM threshold

Exposure Targeting Settings

Mean: 0.0810916
 Min.: 0
 Max.: 1
 Std. dev.: 0.272976

Exposure Targeting Settings

Blob finding
 Border: 100
 Correction
 Max. blobs: 4
 Max. blob size: 0000
 Min. blob size: 10

Test

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:08:32 PM Number of lattice blobs: 4
 3:08:32 PM fit lattice
 3:07:42 PM Number of blobs: 4

Exposure Targeting Settings

Mean: 1.43102
 Min.: 0.3365
 Max.: 3.39525
 Std. dev.: 0.181605

Exposure Targeting Settings

Lattice Fitting
 Spacing: 1194
 Tolerance: 0.1
 Extend Lattice: off

Hole Statistics
 Radius: 150
 Reference Intensity: 1.8

Test

OK Cancel Apply

Exposure targeting Settings

Ice Thickness Threshold
 Min. mean: 0.01
 Max. mean: 5
 Min. stdev.: 0
 Max. stdev.: 0.09

Focus hole selection: Off

Focus offset x: 0 y: 0

Target Template
 - Use target template

Focus Target Template

Focus Template Thickness
 - Use focus template thickness and limit to one focus target

Stats. radius: 200

Acquisition Target Template

Min. mean thickness: 0
 Max. mean thickness: 10
 Max. std. thickness: 5

Relative target: (0, 0)

Test targeting

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:09:22 PM Focus Targets: 1
 3:09:22 PM Acquisition Targets: 3
 3:09:21 PM template point (-563, -161) passed thickness test

Exposure Targeting Settings

Ice Thickness Threshold
 Min. mean: 0.01
 Max. mean: 5
 Min. stdev.: 0
 Max. stdev.: 0.09

Focus hole selection: Off

Focus offset x: 0 y: 0

Target Template
 - Use target template

Focus Target Template

Focus Template Thickness
 - Use focus template thickness and limit to one focus target

Stats. radius: 200

Acquisition Target Template

Min. mean thickness: 0
 Max. mean thickness: 10
 Max. std. thickness: 5

Relative target: (0, 0)

Test targeting

OK Cancel Apply

File Application Launcher Node Events Others Help

Time Message
 3:19:22 PM Focus Targets: 1
 3:19:22 PM Acquisition Targets: 2
 3:19:22 PM stopping template point (619, 619): out of image bounds

Exposure Targeting Settings

Ice Thickness Threshold
 Min. mean: 0.01
 Max. mean: 5
 Min. stdev.: 0.08
 Max. stdev.: 0.02

Focus hole selection: Off

Focus offset x: 0 y: 0

Target Template
 - Use target template

Focus Target Template

Focus Template Thickness
 - Use focus template thickness and limit to one focus target

Stats. radius: 200

Acquisition Target Template

Min. mean thickness: 0
 Max. mean thickness: 10
 Max. std. thickness: 5

Relative target: (0, 0)

Test targeting

OK Cancel Apply

10 Transfer frame data and run motionCor2 and CTF on-the-fly

- Transfer frame from K3 computer to GPFS (necessary)
Frame data are saved on K3 computer, so you have to transfer them out once data collection starts, in order to not saturate the space on K3 computer.
- Run MotionCor2 (optional)
Give Appion the patch $x=5$, $y=7$ for K3 data, which will be converted to $x=7$, $y=5$ in MotionCor2.
- Run CTF estimation (optional)

11 Clean up after data collection is done

- Close Leginon GUI
- Exit your account from Hermes terminal by typing *exit*
- Close column valve on EM GUI
- Remove Obj. aperture at Aperture panel on EM GUI
It is especially important to retract phase plate before turning on Turbo pump if you collect phase plate data.
- Turn turbo pump on at Autoloader panel on EM GUI
- Insert Flu camera
- Unload the grid when Turbo is ready
- Undock the cassette
- Fill in the paper log book and the electronic log book as well.

Appendix: Diagram for single particle

