

# **Talos Arctica & Glacios Training Documentation Packet**

## Table of Contents

1. Contact Information .....	3
2. Sample Preparation .....	4
a. Vitrobot .....	4
b. Examples of Good and Bad Ice .....	5
3. Sample grid loading (cartridge loading) .....	9
4. Cassette loading (loading cartridge into cassette) & Cab docking & Inventory .....	10
5. Microscope .....	12
a. Optics .....	12
b. Column .....	13
i. Gun .....	13
ii. Spot Size .....	13
iii. Condenser Zoom .....	14
c. Detectors .....	16
d. Thon rings .....	18
e. Information Limit .....	19
f. Parallel Illumination .....	20
6. EPU .....	21
a. Direct Alignments .....	21
i. Beam Tilt Pivot Points .....	21
ii. Beam Shift .....	21
iii. Rotation Center .....	22
iv. Coma-free Alignment .....	22
b. Presets .....	23
c. Calibrate Image Shift .....	24
i. Performing Calibration .....	24
d. Acquire an Atlas .....	26
e. Calibrate Focus and Eucentric Correction .....	27
f. Starting a Session .....	28
i. Define a Session .....	28
ii. Square Selection .....	29
iii. Hole Selection .....	29

## Contact Information

Account Manager:

Phone:

Email:

Global Tech Support:

Phone:

Email:

Field Service Manager:

Phone:

Email:

Field Service Engineer:

Phone:

Email:

Applications Manager: Jason Pierson, PhD

Phone: (301) 204-1795

Email : jason.pierson@thermofisher.com

Applications Engineer:

Phone:

Email:

**Sample Preparation:**

The preparation of samples for cryo-EM requires the sample to be frozen in a thin layer of ice by freezing it in liquid ethane using a Vitrobot. This process is known as vitrification.

Vitrification is the process where water transitions from liquid phase to an amorphous phase without the formation of ice crystals.

• **Vitrobot:**



The Vitrobot was designed to make the vitrification of samples easy and fast for researchers. It allows for setting of optimal blotting times and the pressure with which the blotting is done. It has filter pads that converge onto the grid from both sides with the user desired force and time.

The Vitrobot offers fully automated vitrification, fast and easy. It performs the cryo-fixation process at constant physical and mechanical conditions like temperature, relative humidity, blotting conditions, and freezing velocity. This ensures high quality cryo-fixation results and a high sample preparation throughput prior to cryo-TEM observation. The Vitrobot's controlled environmental technology prevents cooling and concentration of artifacts that are inevitable in other freezing protocols.

**Some Important Notes:**

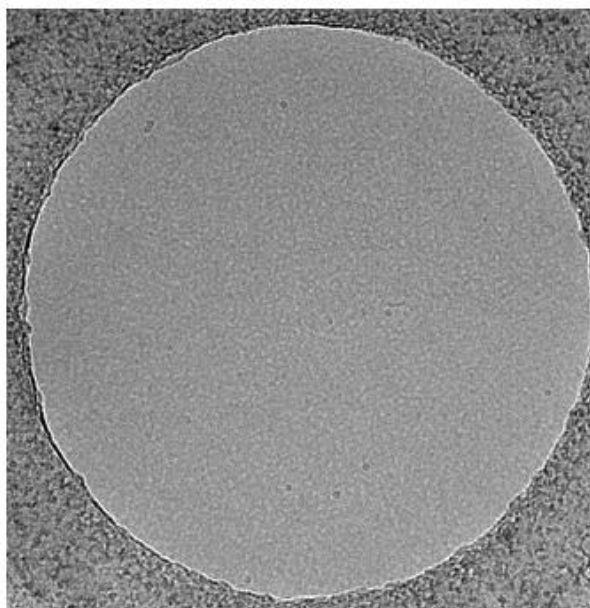
1. Be sure to set the Vitrobot temperature to 4°C and humidity to 100% prior to sample freezing.
  - This may take some time, use this time to set up all tweezers, sample, liquid ethane, etc. Place blotting pads into the Vitrobot while the atmosphere is being established; this will ensure that samples are blotted equally during freezing.
    - **Note:** this is a **user preference**, some users prefer to have the blotting papers not saturated when starting up the Vitrobot.
2. Set your desired blot time and hold times.
3. Cycle the vitrobot a few times without sample grid to make sure it works well and the blot force is correct.

**Sample Freezing Notes:**

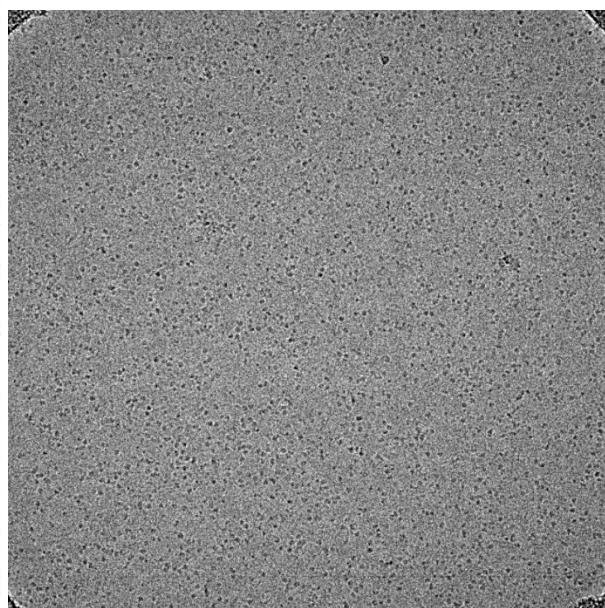
- Ice must be thick enough to allow for particle location within the TEM specimen (holes), but thin enough so that the particles do not pile up in the projection images.
- Depending on the molecular weight and viscosity of the sample, the concentration should be optimized by trial and error.
- A concentration of approximately 10 mg/ml is a good starting point (reduce concentration for particles larger than 100nm, and vice versa).
- Where the sample cannot be diluted, the blotting parameters will determine the final sample thickness.

**Examples of Good and Bad Ice:**

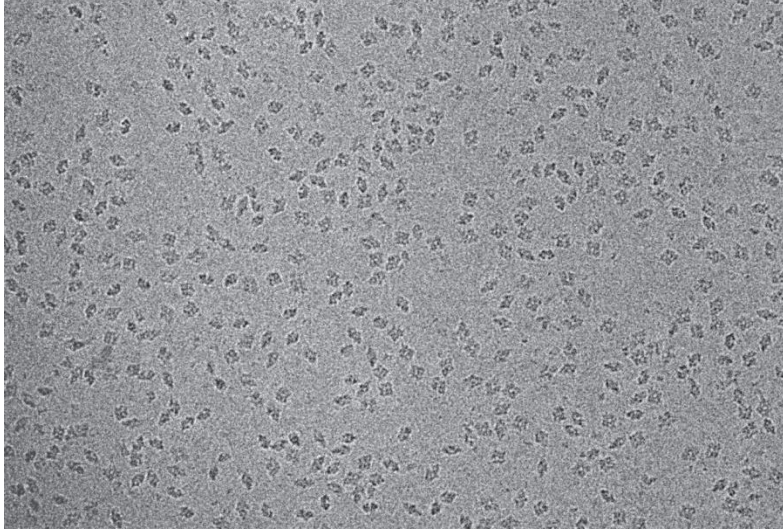
The GOOD:



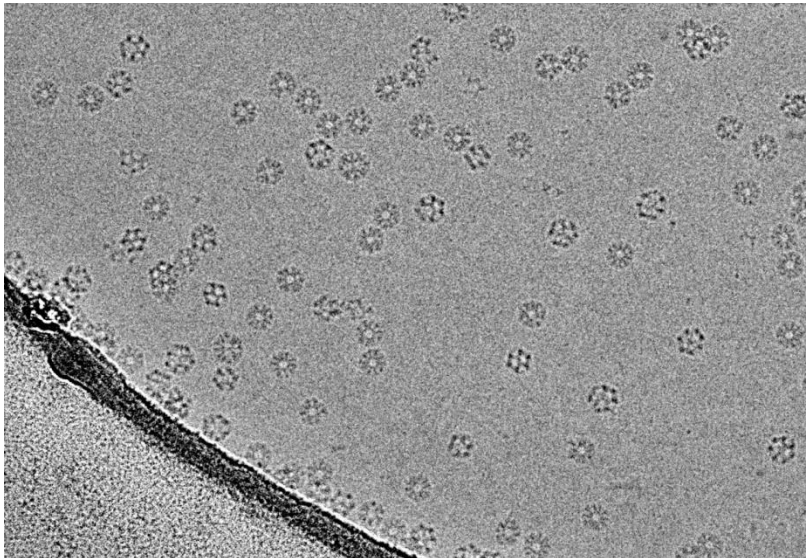
*Example of perfect ice, no sample*



*64kDa sample, well frozen*

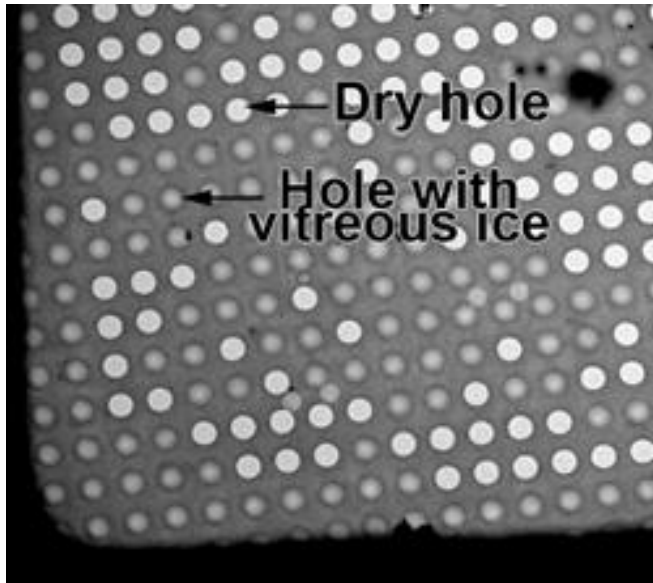


*450kDa sample, well frozen*

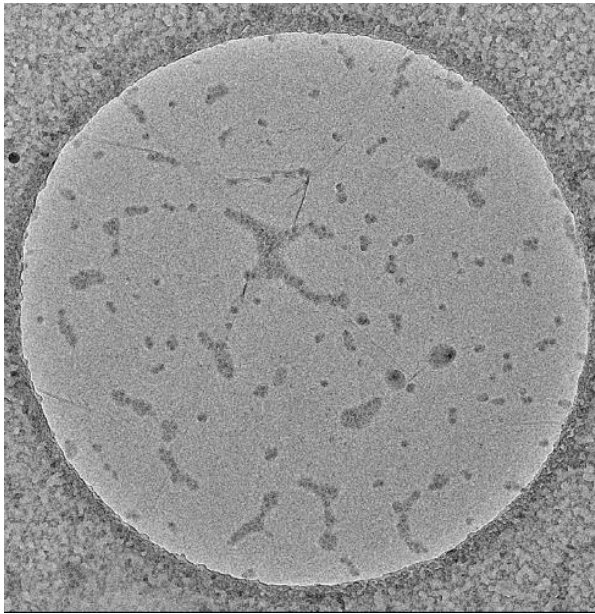


*1.6MDa sample, well frozen*

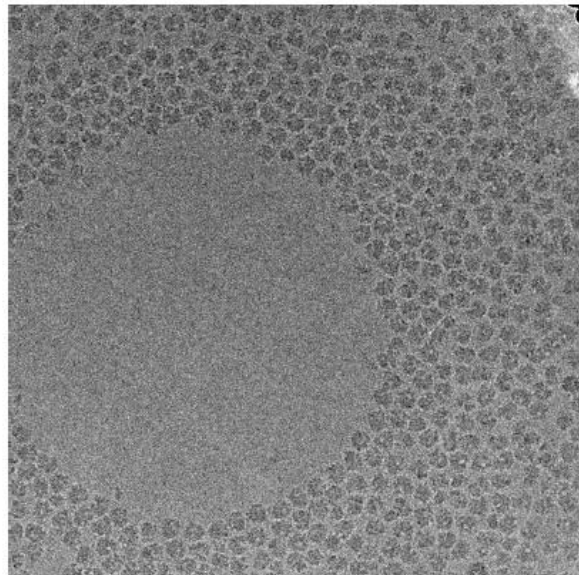
The BAD:



*Empty vs ice-filled holes*



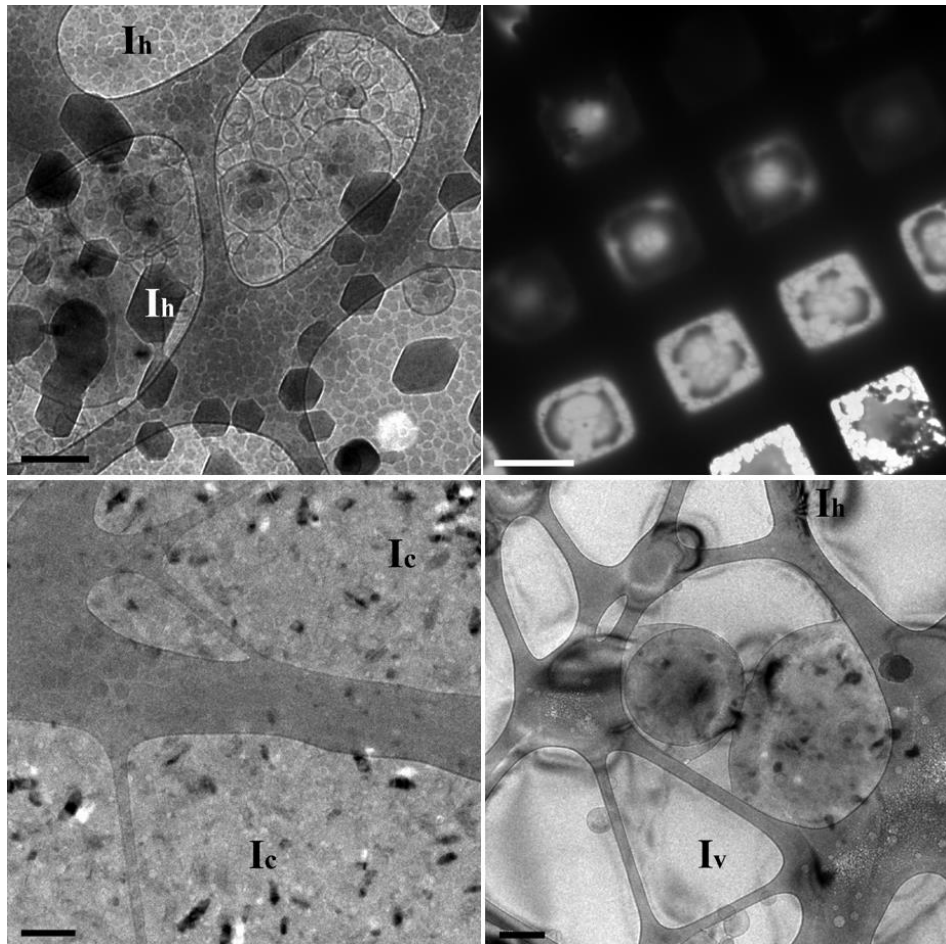
*Freeze-dried damage*



Ribosomes, Chris Russo

*Overblotted*

The UGLY:



**Examples of common artifacts in thin-film cryo-TEM samples**

**A: Small hexagonal** ice crystals (“leopard skin; black Ih): the sample has been warmed up above devitrification temperature or was poorly frozen.

**Large hexagonal** ice crystals (white Ih). Very stable under the beam.

**B: Unevenly distributed and frozen sample:** poor hydrophilicity of the carbon film.

**C: Cubic** ice crystals (Ic) covering the entire grid: insufficient cooling rate; thin film was too thick.

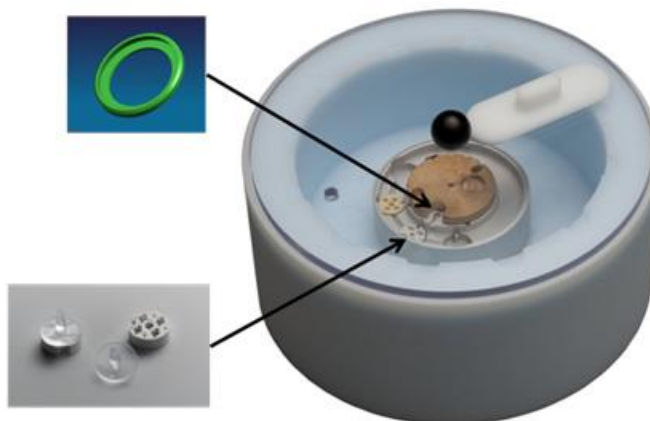
**D: Thin layer of hexagonal** ice (Ih) covering the surface of the grid: ice contamination of freezing or transfer vessels. The underlying sample contains **vitreous** ice (Iv).

Scale bars: B = 50  $\mu\text{m}$ ; A, C and D = 0.2  $\mu\text{m}$ .



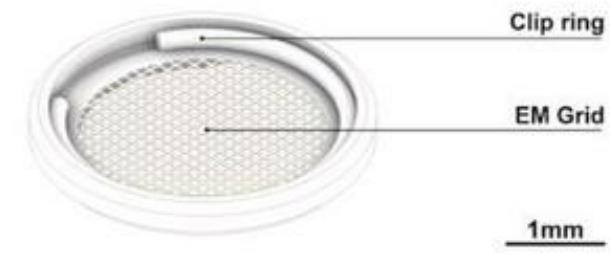
**Sample Grid Loading & Clipping (cartridge loading):**

1. Cool the AutoGrid assembly workstation and place the required parts in the corresponding places inside the workstation (C-clip ring, grid box that holds the frozen grid, and empty AutoGrid Container).



2. Place one of the 3 mm frozen sample grids into the C-clip ring, **carbon side down**.

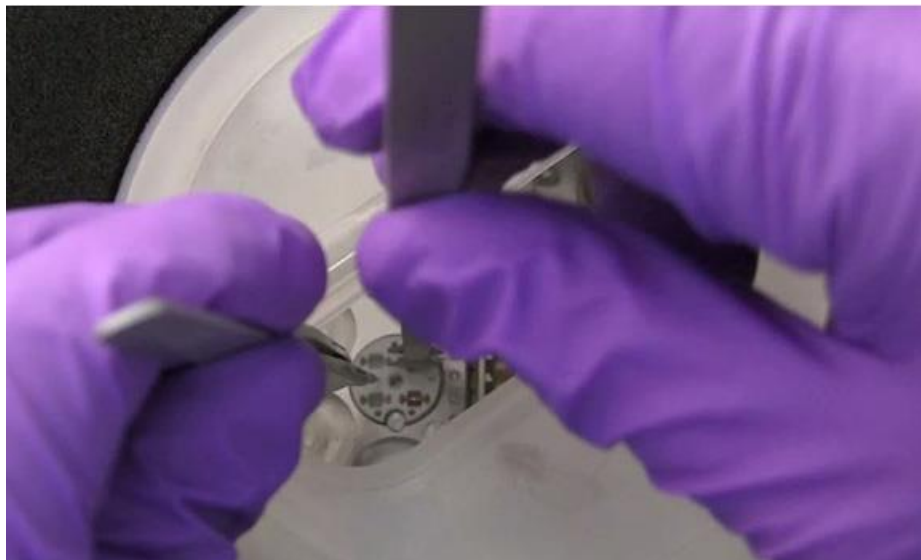
Place a C-clip into the C-clip insertion tool, tilt the C-clip to be at ~45 degree angle inside of the tool, put the tool with the tip against a flat surface and press down on the plunger to ensure that the C-clip is flat against the edge of the tool. This will ensure that the C-clip will not be bent while clipping. Cool the tip under liquid nitrogen. Clip the grid into the cartridge as instructed during training session. What a correctly clipped grid looks like is shown below.



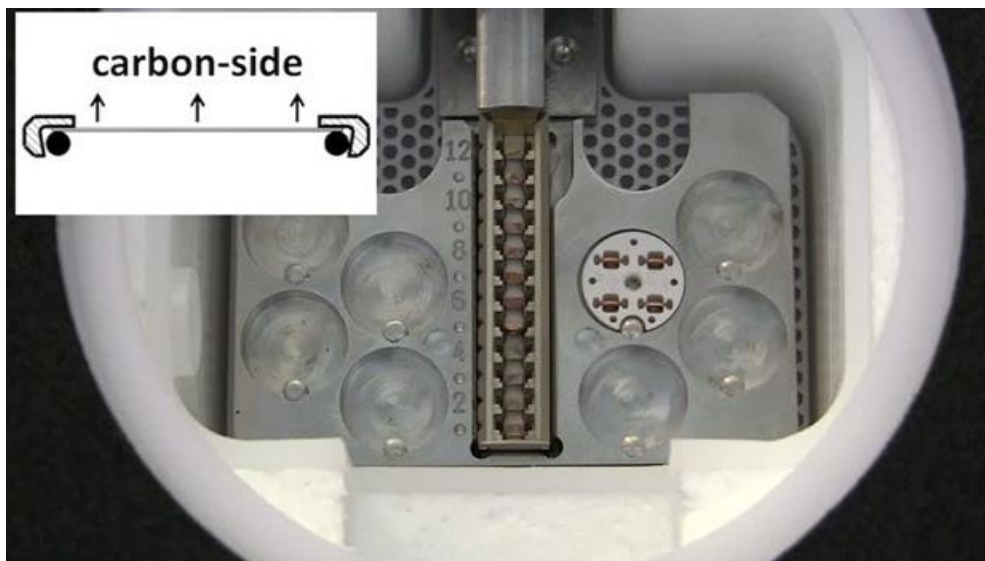
3. Place the correctly clipped samples (cartridges) into the AutoGrid storage containers.

**Cassette loading (loading cartridge into cassette):**

- Make sure to have 1-2 4L dewars of fresh LN<sub>2</sub>.
- Prepare all tools needed.
- Cool down the transfer station and place frozen cartridge box into the transfer station.
- Use the Autogrid tweezers to transfer the cartridge into the cassette.



- When inserting the cartridge into the cassette be sure to have all cartridges in the same orientation, **C-clip facing down towards the bottom of the cassette** as shown in the figure below.

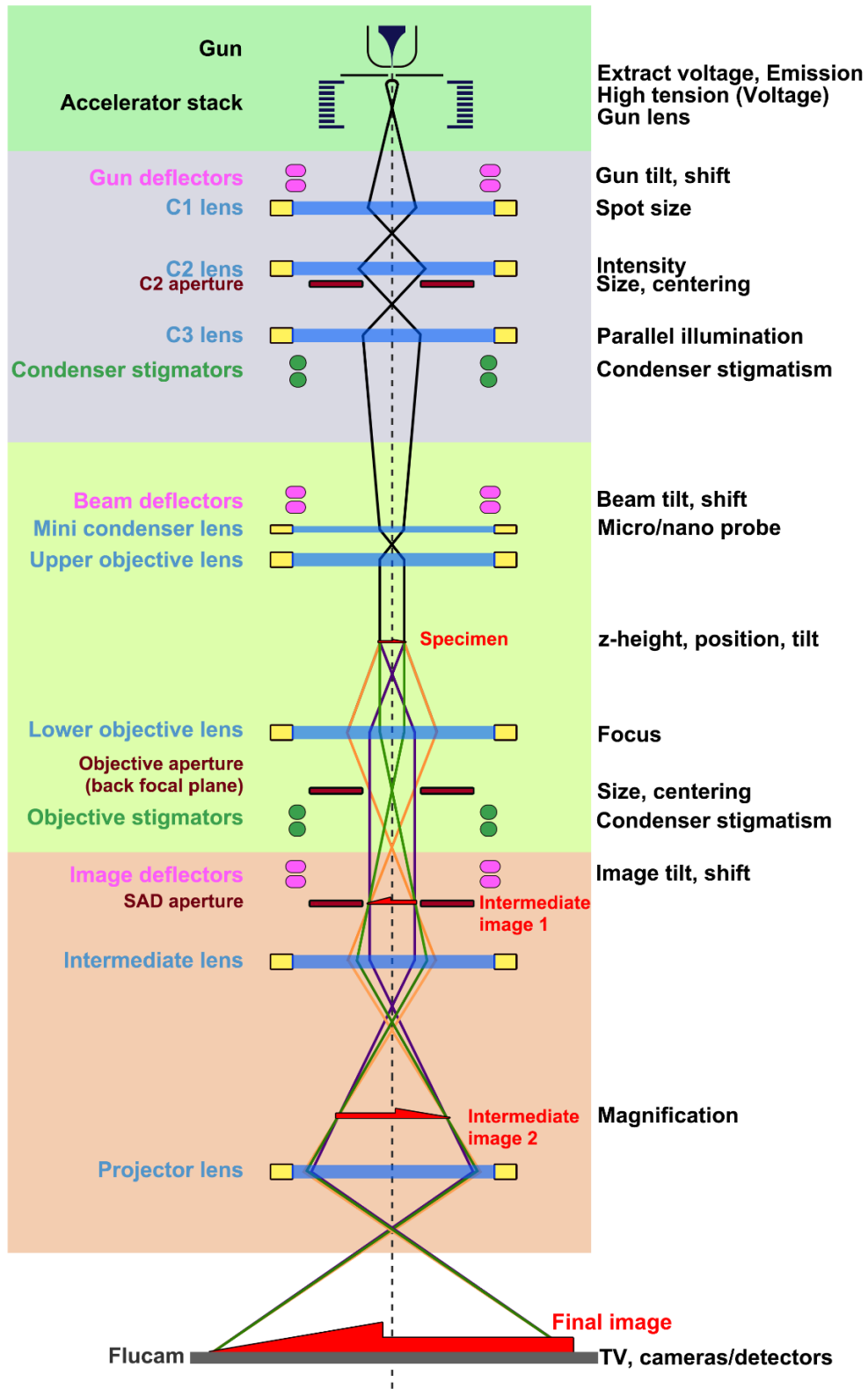


- Once all cartridges are loaded into the cassette, transfer the cassette into the LN2 nanocab attached to the transfer station.

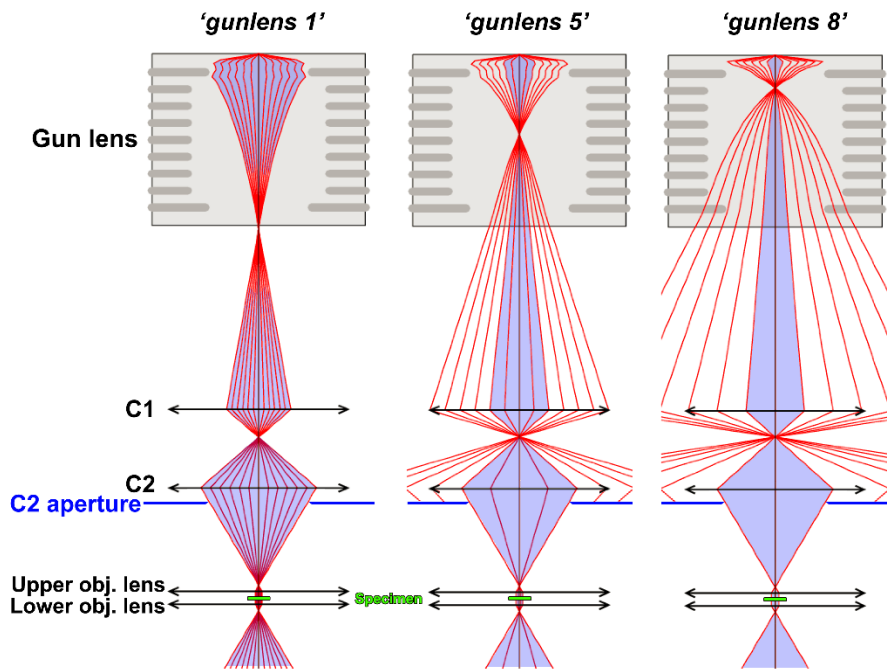


- Take the nanocab into the microscope room and dock the cab, making sure the small pin on the top of the nanocab is set properly by gently pulling up on it. This sometimes gets stuck and the autoloader may fail detecting the cassette if it is not in the correct position.
- When docking is complete, remove the empty nanocab, wait until the temperature ready (~170 °C), and then start the inventory of your samples.
- While the inventory is running clean up the sample transfer area and disassemble the transfer station and set it to dry.

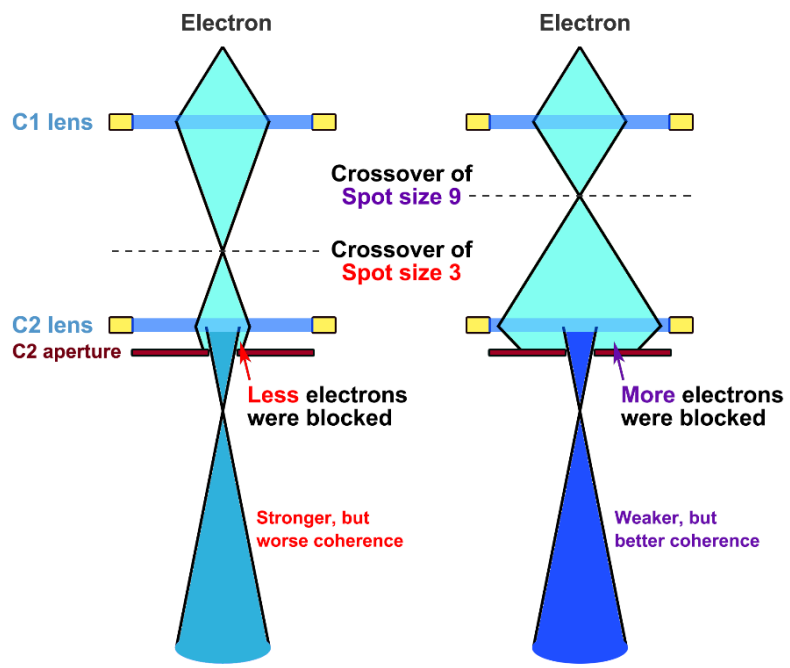
Microscope:  
• Optics



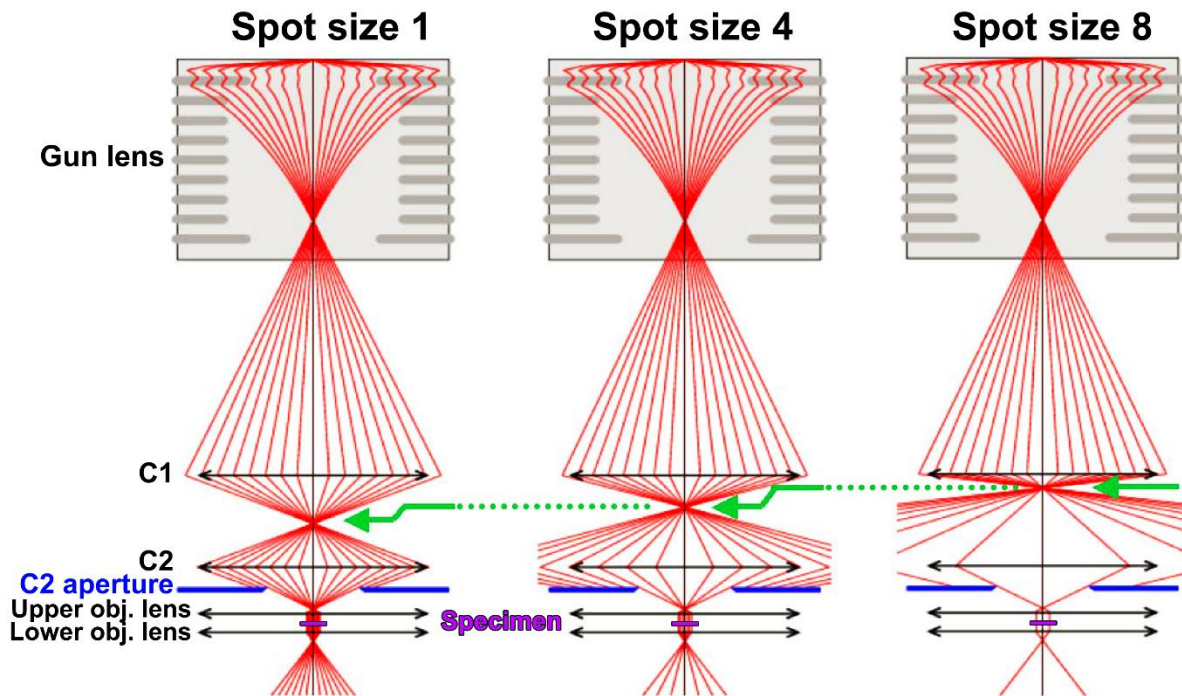
- Column:
  - Gun



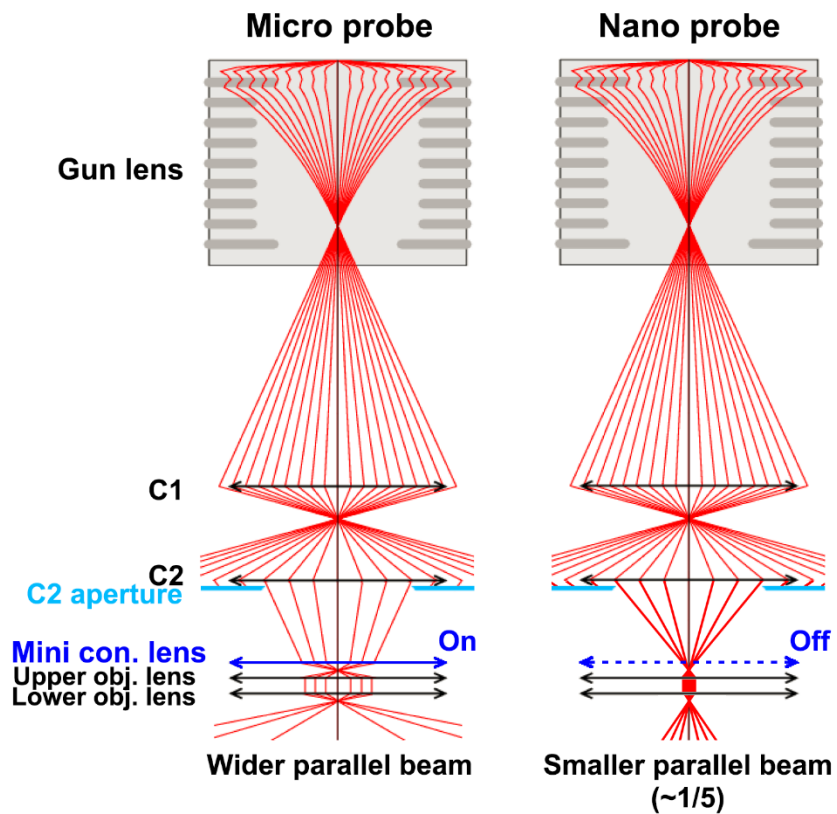
**Condenser Lens  
Spot Size**



C1-C2 zoom

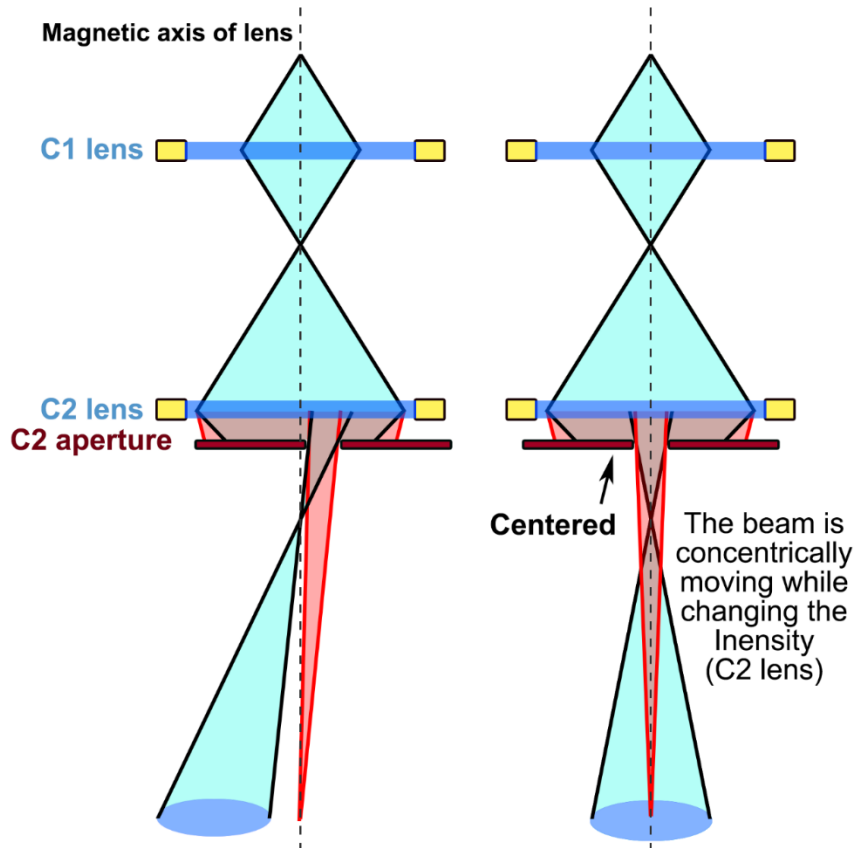


Probe



**Notes:**

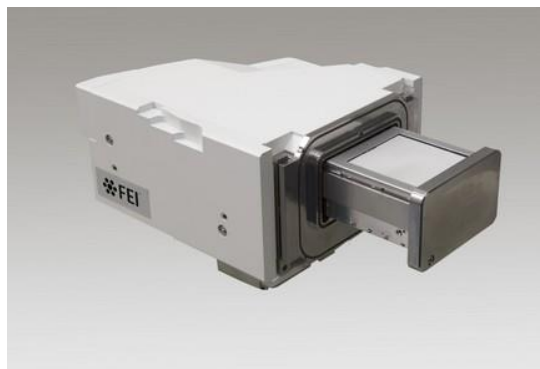
- In a 2 condenser system there is a value (%) of the C2 lens where parallel illumination is achieved. This value is different for every spot size so it is important to check to be sure that the system is in parallel illumination prior to high-resolution data collection.
- It is important to have the microscope column well aligned before data collection. A poor alignment will affect the quality of the data. One such alignment is the centering of the C2 Aperture, via the Direct Alignments menu in the TEM User Interface.



## Detectors

### 1. Ceta

- All-in-one camera functionality for the highest performance and throughput.
- The Ceta 16M camera is the first camera to deliver high speed readout for simultaneous dynamic imaging and large field of view. This unique combination follows the natural workflow in TEM imaging: from fast navigation to find the area of interest-to easy optimization of the image quality via optical adjustments-to the final result: a 4k x 4k image unrivaled in quality and detail.
- Key Benefits:
  - a. **Consistently clear images, from mesoscopic to atomic scale:** Largest field of view combined with high speed readout delivers clear images quickly, even when moving from mesoscopic to atomic scales.
  - b. **Optimum performance at any high tension (20 - 300kV):** High sensitivity, robust fiber optic-coupled scintillator combines with large 14µm pixel size to deliver the best quality images regardless of high tension selection.
  - c. **Movie acquisition for dynamic studies:** 16-bit dynamic range movie recording enables high quality 4k x 4k movies at 1 fps or 512 x 512 movies at 25fps.
  - d. **Optimized settings for any material or application:** Select low dose imaging for beam sensitive materials, or high dose for diffraction applications, with more than 16 bit dynamic range using fast frame-adding.



### 2. Falcon 3

- The Falcon 3EC fully integrates into a fast, automated workflow on the Thermo Scientific™ Titan Krios, and Talos Arctica, and Glacios. Not only has the detector sensitivity been improved, but so has the handling of data produced by the detector.
- Key Benefits:
  - a. The Falcon 3EC features electron counting capabilities that enable the highest sensitivity (DQE). Electron counting can significantly counteract the effects of electron beam-induced motion, which can degrade resolution considerably. The Falcon 3EC also boasts improvements to its data handling capabilities. Its next-generation imaging pipeline allows for high data transfer rates and on-the-fly drift correction. Ultimately, these innovations result in sub-4Å resolution images.
  - b. Based on Falcon II feedback, the Falcon 3EC features additional improvements, such as a non-invasive dose protector, ability to image in LM (low-magnification) mode, and lightning-fast imaging.
  - c. The Falcon 3EC detector is fully integrated, making it considerably easier to use. The large, 14µm pixels ensure the highest signal and lowest noise. In addition, the Falcon 3EC's next-generation imaging pipeline and data storage solution are specially optimized for multiple frame recording.



### 3. Gatan K2

- The K2 Summit<sup>®</sup> electron counting direct detection camera is simply the highest performance detector available for cryo-electron microscopy. The unmatched frame rate of this direct detector allows electron counting modes to improve both contrast and resolution for large and small molecules alike.
- Only the K2 Summit can produce a DQE as high as 80%.
- First to identify ground-breaking structural discoveries using cryo-EM.
- K2<sup>®</sup> XP sensor provides the highest performance sensor for both resolution and contrast.
- Superior detective quantum efficiency (DQE) means better science.
  - a. Image samples below 500 kDa at high resolution.
  - b. Reliably resolve differences with heterogeneous samples.
  - c. Higher quality cryo-tomography.
- Super-resolution mode means 4x larger area in one image.

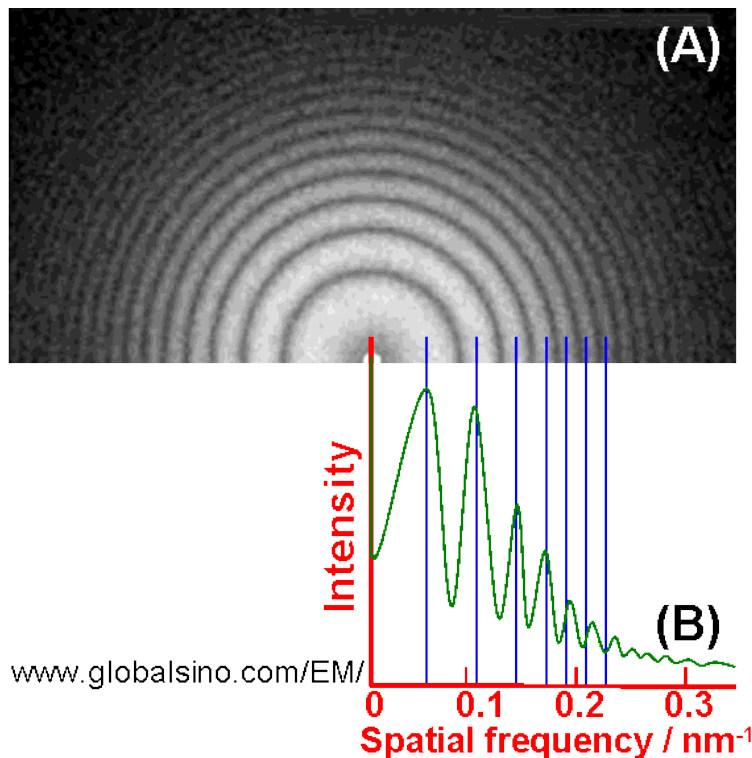


### 4. Gatan K3

- Powerful inline signal processing will raise the DQE beyond that of the K2 camera.
- Optional inline, GPU-based motion correction avoids the need to save terabytes of raw frames.
- 24 megapixels (5,760 x 4,092) field of view – 1.6 times the size of the K2 camera.
- 1,500 full frames per second – 3.75 times the speed of the K2 camera.



**Thon Rings:**



(A) Power spectrum of typical bright-field image of amorphous carbon film presenting concentric Thon rings taken in TEM.

(B) Radial intensity of the power spectra.

Thon rings emerge in FFT as part of the contrast transfer function of the objective lens when observing an amorphous area under a relatively high defocus (around -2  $\mu\text{m}$ ). They are used to prove the microscope resolution per camera pixel at middle magnifications (around 100 kx).

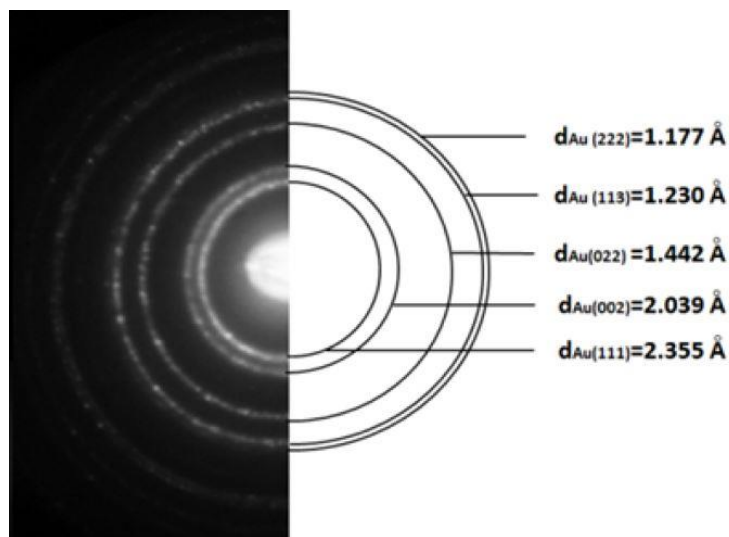
The spacing and the shape of the rings depend on the electron–optical parameters that describe the image, principally the amount of defocus and astigmatism. Thon rings from amorphous carbon are routinely used to adjust astigmatism and set the defocus of a microscope (Henderson et, al. 2015).

**Procedure to check Thon Rings:**

- Use Platinum Iridium grid.
- Select the 100  $\mu\text{m}$  C2 aperture and center it.
- Retract objective aperture.
- Make sure eucentric height is adjusted accurately.
- Use spot size 2 or 3 and condense beam so it is slightly larger than the detector.
- Go to magnification  $\sim 200$  kx and defocus to  $\sim 2$   $\mu\text{m}$ .
- Correct for any condenser or objective astigmatism.
- Take a 1 sec exposure with either TIA or AutoCTF.
  1. In TIA:

- After acquiring a 1 sec exposure, click FFT (on left side) to generate FFT of Ptlr image.
  - Click FFT and click Rot Avg (on left side) to generate plot of Thon rings from FFT image.
2. In AutoCTF:
- After acquiring a 1 sec. exposure, click 1d plot tab on right side of image.
- Can repeat with defocus of  $\sim -1 \mu\text{m}$ .

**Information Limit:**



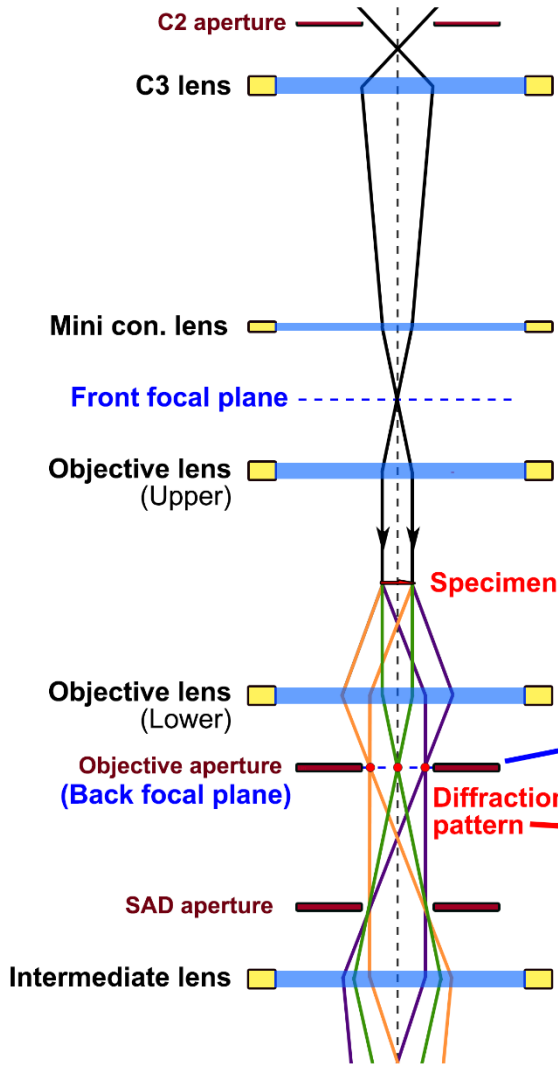
**Procedure to check Information Limit:**

- Use Gold cross-grating grid.
- Select the  $100 \mu\text{m}$  C2 aperture and center it.
- Retract objective aperture.
- Make sure eucentric height is adjusted accurately.
- Use spot size 2 or 3 and condense beam so it is slightly larger than the detector.
- Go to magnification  $\sim 200 \text{ kx}$  and defocus to  $\sim 1 \mu\text{m}$ .
- Correct for any condenser or objective astigmatism.
- Take a 1 sec exposure with TIA.
- After acquiring a 1 sec exposure, click FFT (on left side) to generate FFT of image.

**Helpful hints:**

- Find an area heavy with gold (this will give nice diffraction rings).
- Concentrate the beam to get rings to appear crisp.
- If rings are barely visible, try adding exposure time and/or defocus.

Parallel Illumination:

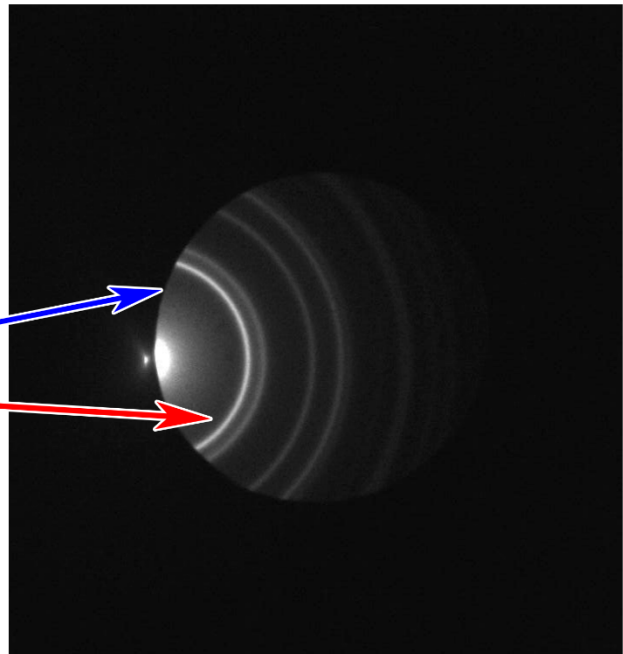


**Parallel illumination:**

The **last condenser crossover** is at the **front focal plane** of the upper objective lens. THEN the **diffraction pattern** is focused at the **back focal plane** of lower objective lens (where the objective aperture is).

Therefore, focus the **objective aperture** and the **diffraction pattern simultaneously**, parallel illumination can be achieved.

**Procedure:** At *diffraction mode*, sharpen the **edge of the objective aperture** with the **Focus knob**, shapen the **diffraction pattern** (e.g. gold of cross grating grid) with the **Intensity knob**.



**EPU:**

The microscope must meet the following conditions:

- The column must be properly aligned.
- HT is stable.
- The imaging detector is at constant temperature.
- Gain and dark correction images should be available and well averaged.
- An alternate shutter should be chosen for CCD exposures or the use of both shutters should be enabled in case a pre-exposure is desired. (See the CCD control panel in the microscope UI.)
- Enough time should be allowed for settling after any actions that may have introduced exceptionally strong drift (cooling, inserting holder).
- Alignments, FEG registers, and necessary calibrations should form a consistent set. It is best to always use the same set for EPU.
- Calibrations must have been done for LM and SA ranges (Magnification Calibrations control panel in microscope UI).
- For non-FEG instruments, the gun saturation (heating) must be optimized and the emission chosen. Gun settings may need to be optimized via the direct alignments gun-tilt and gun shift.
- Apertures should be centered correctly.
- Focus calibration must have been done (currently a task that is performed in EPU).
- Direct alignments should be checked/adjusted in the modes that are used for EPU (LM range and SA range nanoprobe/ Microprobe).

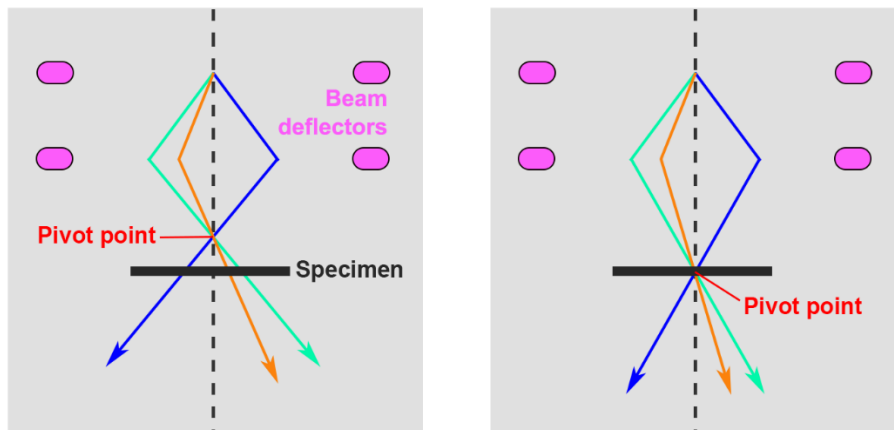
**Direct Alignments**

- These can be found on the bottom right section of the TEM UI when logged in as **Supervisor**. Click on the drop-down menu and select Direct Alignments.

○ **Beam Tilt Pivot Points**

1. These should be checked at the accurate eucentric height and focus. When the beam is tilted, the beam (spot) should not move (important for auto-focus/stigmator).
2. Enter direct alignment, focus spot. Is spot movement minimized?

**Beam tilt pivot point alignment:** the beam tilt wobble should give no beam shift, so only one spot is visible in the image. i.e. the tilt pivot point coincides with the specimen. (*Focus dependent!! Must be done at eucentric height and in focus.*)

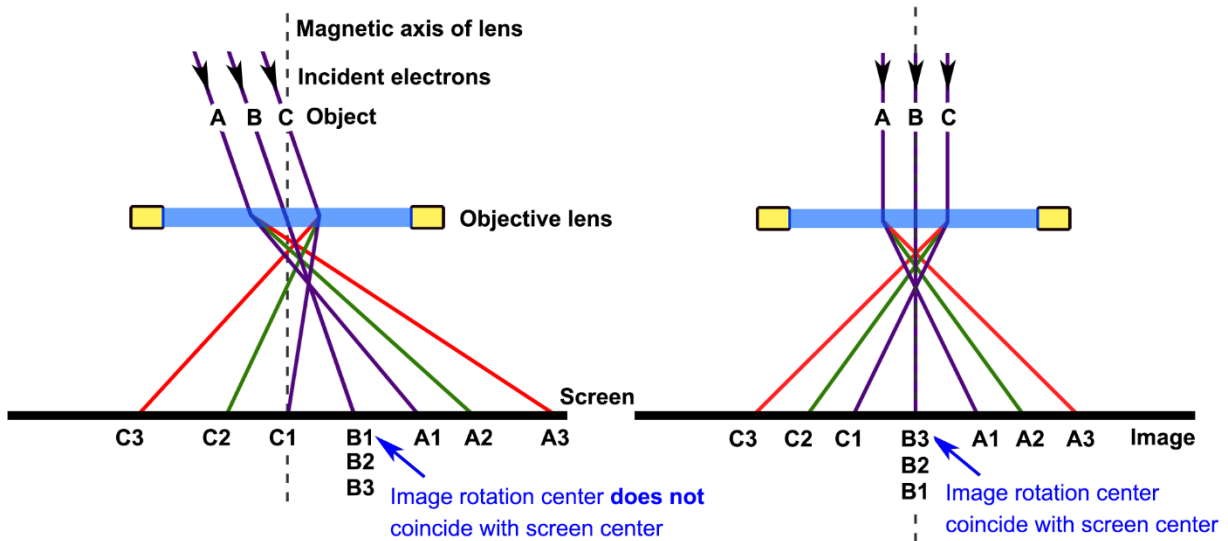


○ **Beam Shift**

1. To center beam when no user beam shift is applied.
2. Perform at highest magnification that will be used in EPU.
3. Use MF-X and MF-Y to center the beam once it is focused into a point on the screen.

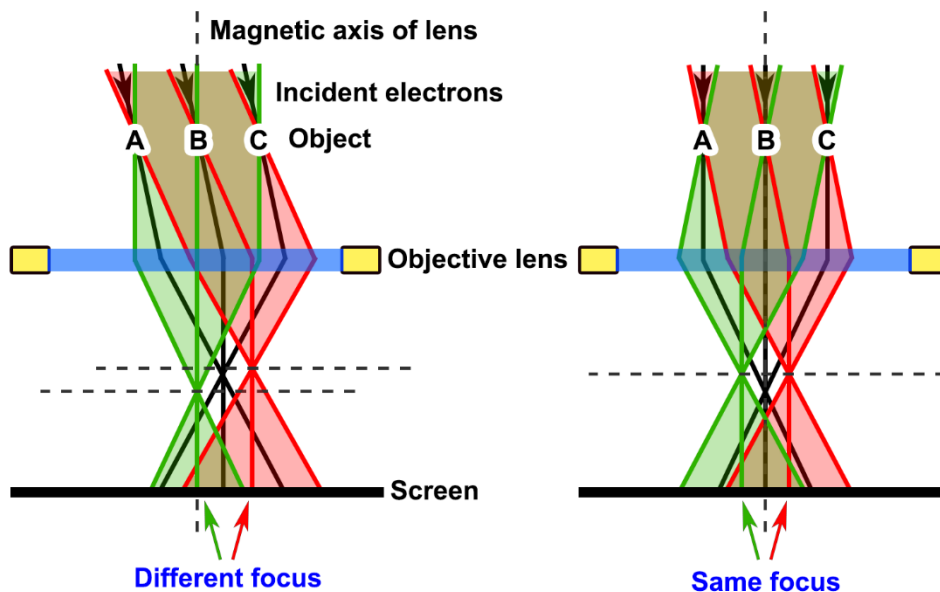
○ **Rotation Center**

1. When focusing, the central image features should stay on the optical axis, not move.
2. Perform at highest magnification that will be used in EPU.



○ **Coma-free Alignment**

1. For recording high resolution images, it is important that the illumination is coma-free. This procedure can only be done properly with the help of a thin carbon film and a CCD camera running with live FFT at the same time. Contrast and FFT should not change when the beam is tilted.



### Presets

- **Atlas Preset**
  - An atlas will be recorded to give an overview of the specimen and to find grid squares suitable for automatic data recording. Such a grid square should fulfill the following conditions:
    1. Thin specimen area, electron translucent.
    2. No broken carbon film.
    3. Ice-filled holes.
  
- **Grid Square Preset**
  - The purpose of the grid square setup is to be able to find or define suitable target areas for data acquisition.
  - Typical magnification is LM 400–600 X, illuminated area 150–200µm, but numbers depend on the type of grid and the microscope configuration.
  - A parallel beam is advantageous, as a convergent beam may lead to an effective change of magnification when out of focus.
  
- **Hole/Eucentric Height Preset**
  - This preset is used for:
    1. Performing automatic adjustment of eucentric height during automated run.
    2. Acquiring low dose images of target areas for inspection.
    3. Quantifoil support grids:
      - a. Image, find and center holes (the positions of which were defined at the grid square level).
      - b. Define a template of acquisition positions and a focus position. This template will be applied to every hole during data acquisition.
      - c. Use a magnification here with at least one hole completely visible in the image. However, the algorithm for finding holes is more reliable if other holes are also visible (at least half visible).
  
- **Data Acquisition Preset**
  - Particular care must be taken to set the illumination conditions for the actual data acquisition. Adjust the required resolution and dose on the sample by selecting magnification, C2-aperture, beam diameter, spot size, TEM mode (Microprobe/Nanoprobe). The illumination should be parallel.
    1. The illuminated area should be selected to fulfill specific needs. For example:
    2. A small illuminated area prevents double-beam exposure when there are closely spaced target areas.
    3. A larger illuminated area is good when there are widely spaced targets and it is important for the beam to also hit some carbon.
  
- **Autofocus Preset**
  - To avoid problems due to slight misalignments, set the illumination conditions identical to the data acquisition conditions (spot size, TEM imaging mode, and intensity or beam diameter). It can be advantageous to set the beam diameter a little bit wider because the Autofocus routine will tilt the beam, which will lead to a small shift of the illuminated area when not already close to focus.
  
- **Drift Measurement Preset**
  - To have EPU pause and wait until drift is within a threshold set by the user, a setup is defined that allows EPU to record images for drift measurement at a meaningful magnification, illumination size, and CCD setting.
  - This preset is used for:
    1. Drift measurements during data acquisition (applicable only to Quantifoil type specimens).

2. Drift measurements that are started as standalone functions (**Auto Functions** tab). This is useful to determine the typical settling time that the application must wait after moving the stage to the next target for data acquisition.
  - Select a CCD setting that will result in a fast readout in order to avoid overhead in the drift measurement. Using only half the CCD frame helps in this case. In addition, binning will increase the readout speed, but FEI recommends staying at binning 1 because the accuracy is needed.
  - If the drift threshold is set very low, it may be necessary to increase the magnification such that it takes less time until a small drift is detectable as a shift in the image.

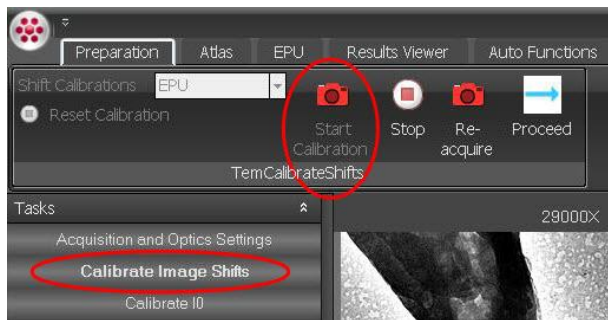
### **Calibrate Image Shifts**

- The calibration determines corrective image shifts between the defined optical settings (i.e., the presets) used in EPU. Usually, a good lens series alignment should ensure that switching magnification will not shift the image of the feature.
- EPU needs an especially precise alignment and certain factors induce deviations from a perfect alignment:
  - The alignment is done on a camera or flu screen that can have a different center than the camera used for EPU.
  - For the transition between SA and LM modes, a shift is easily induced by readjustment of the LM rotation center.
  - When aligning the microscope, the normalization of the lenses may have been handled differently (especially on Tecnai and Talos systems).
  - An inaccuracy in rotation center alignment will induce small shifts when EPU presets use defocus.
- **Performing Calibration:**
  - Search for a feature that will be recognizable both in LM mode and at high magnification. Center this feature at Data Acquisition magnification.
  - Select **Gridsquare** and click **Preview**.
  - Right-click on a feature in the image, choose **Move stage here**.

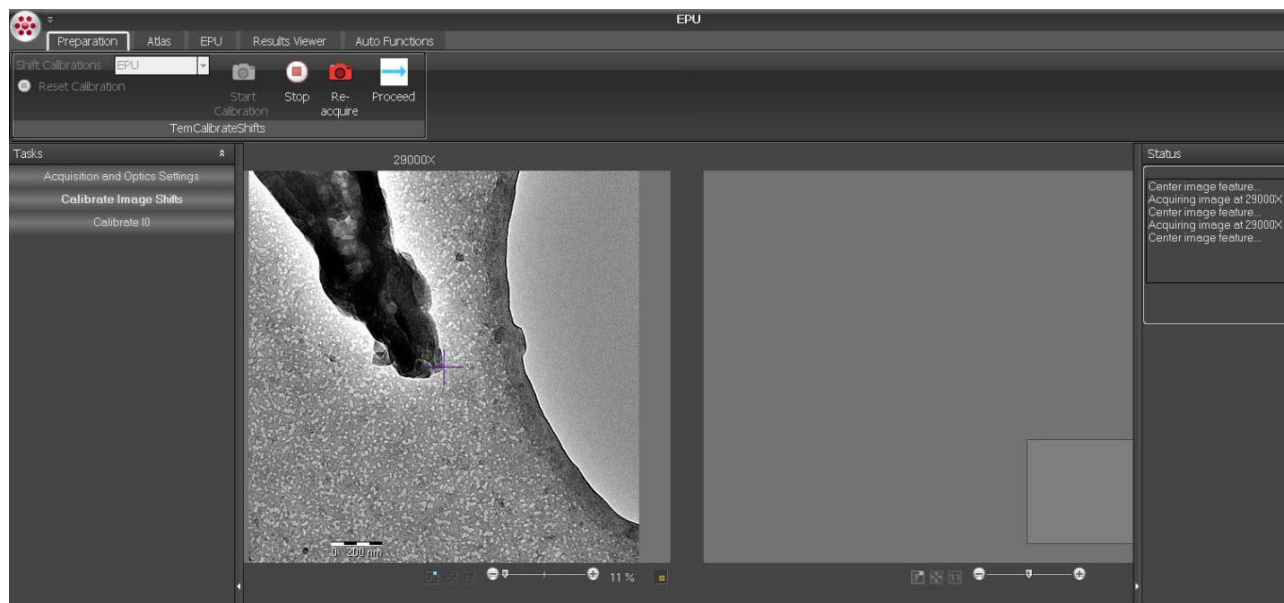


1. Select the “Hole” preset and click **Preview**. The feature is usually visible. If not, use the fluscreen/flucam to search and center it and click **Preview**.
  - a. Right-click on a feature in the image, choose **Move stage here**.
  - b. Select the **Data Acquisition** preset and click **Preview**. The feature should be visible (provided the lens series alignment is acceptable).
- **Calibrate**
  1. Select the **Calibrate Image Shifts** task
  2. Click **Start Calibration**. An image taken with the Data Acquisition preset will be shown on the left.

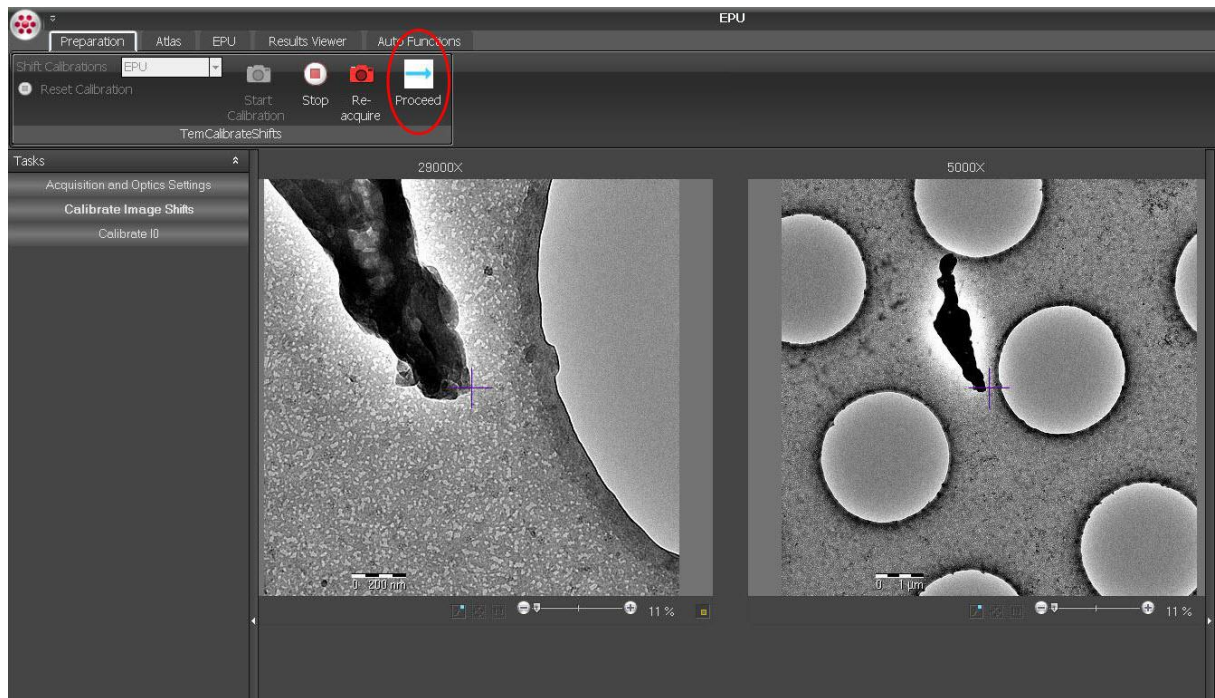




3. Move the hash mark a position on the recognizable feature, and click **Re-acquire**.

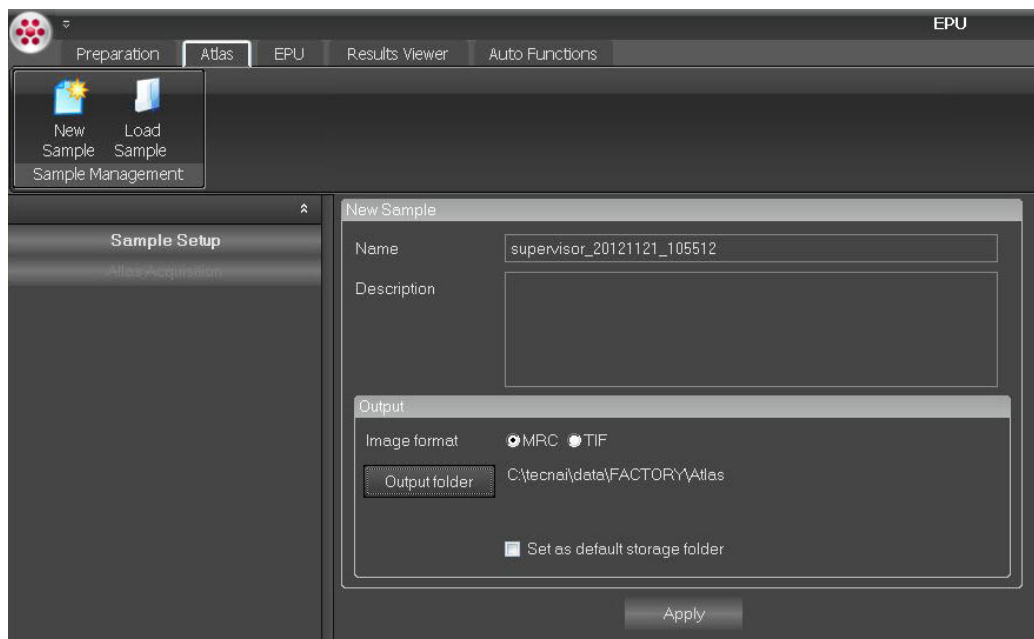


4. If the feature was centered as described above, it should be nicely centered now (within the accuracy of the stage movement). Otherwise, repeat the procedure again for the backlash correction to be consistent.
5. Click Proceed. An image taken with the Hole Acquisition preset will be shown on the right display.
6. On the right display, move the red hash mark to the feature.
7. Click **Proceed**. A new Hole settings image will be shown in the left display, and the feature should be centered now (image shift is used).
8. An image taken with the Grid Square acquisition preset will be shown on the right display. Continue with the calibration until it is completed.



### Acquire an Atlas

- An Atlas is an overview of a sample composed by acquiring low-magnification images at different stage positions (tiled acquisition). The atlas is used for navigation purposes and determining which grid squares to include during an EPU run.
- Start by setting up file name and output folder for your atlas/project, click **Apply** when done.
- Start Atlas acquisition.



### **Calibrate Focus and Eucentric Correction**

- **Focus Calibration**

- The focus calibration is required to make focus measurements work accurately and converge quickly.
- **Preparation:**
  1. Search an area of the specimen with only carbon that is thin (< 20 nm). Avoid thick features.
  2. Bring the specimen to eucentric height.
  3. Select a magnification. The Focus Calibration usually works well at a magnification and imaging conditions, as are typically used for EPU data acquisition.
  4. Correct astigmatism carefully.
  5. Focus the specimen using the weakest contrast. When the specimen is nicely focused, the contrast is minimal.
- **Calibration:**
  1. Select the Auto Functions tab in the EPU ribbon bar.
  2. Select the acquisition preset to use (preferably Autofocus).
  3. Click Focus Calibration.
  4. Click OK on the Manual Focus dialog that displays.
- The calibration now proceeds. Images with tilted beam and cross correlation images are displayed. The encircled bright spot in the cross-correlation image corresponds to the shift between the first two images.

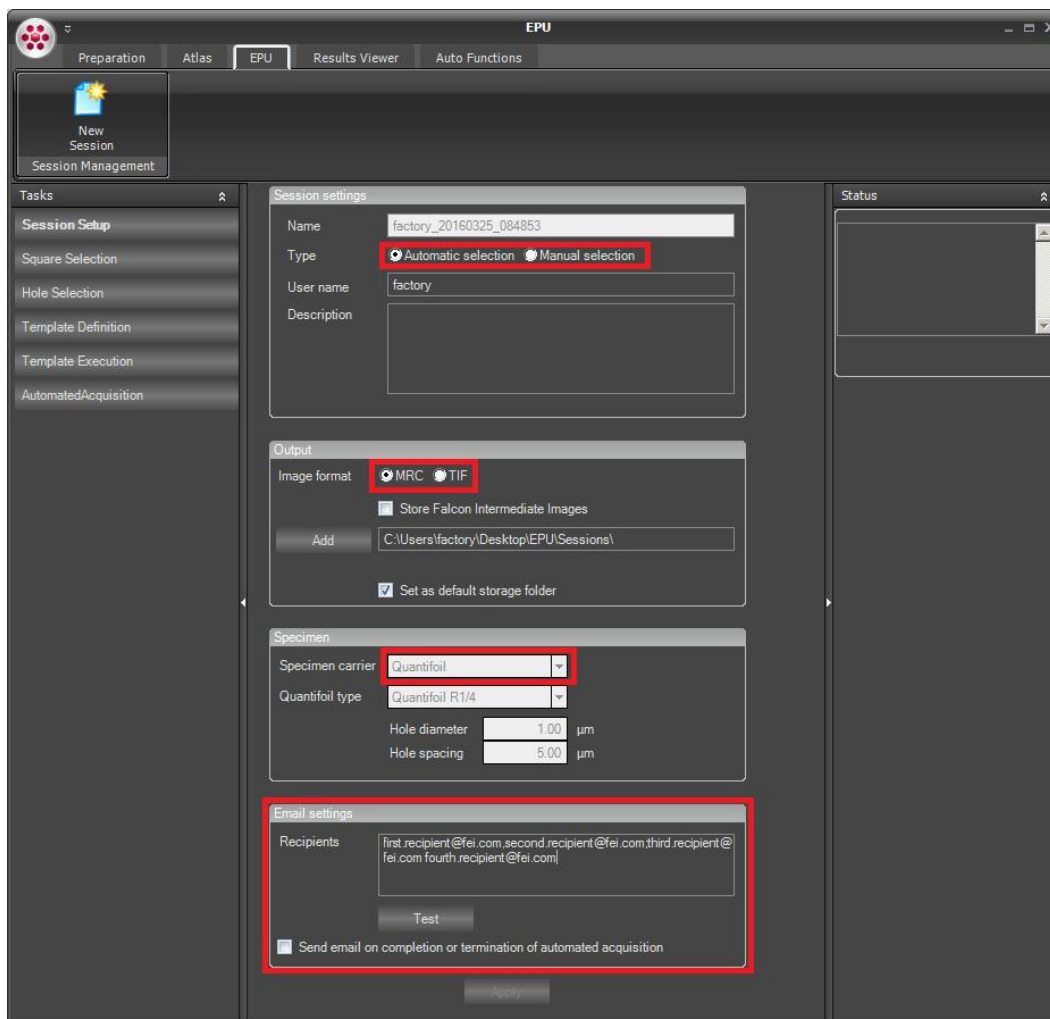


- **Eucentric Correction Calibration**

- The eucentric focus calibration is optional. It is meant to capture the difference between the microscope's eucentric focus preset and the actual focus when the specimen is brought to eucentric height with help of the alpha tilt wobbler. This will be of high importance once the tilted acquisition is incorporated into EPU.
  1. Carefully bring the specimen to eucentric height using the stage wobbler.
  2. Focus the specimen.
  3. Select the foil hole/eucentric height preset.
  4. Click Eucentric Correction Calibration to start the calibration procedure.

### Starting a session

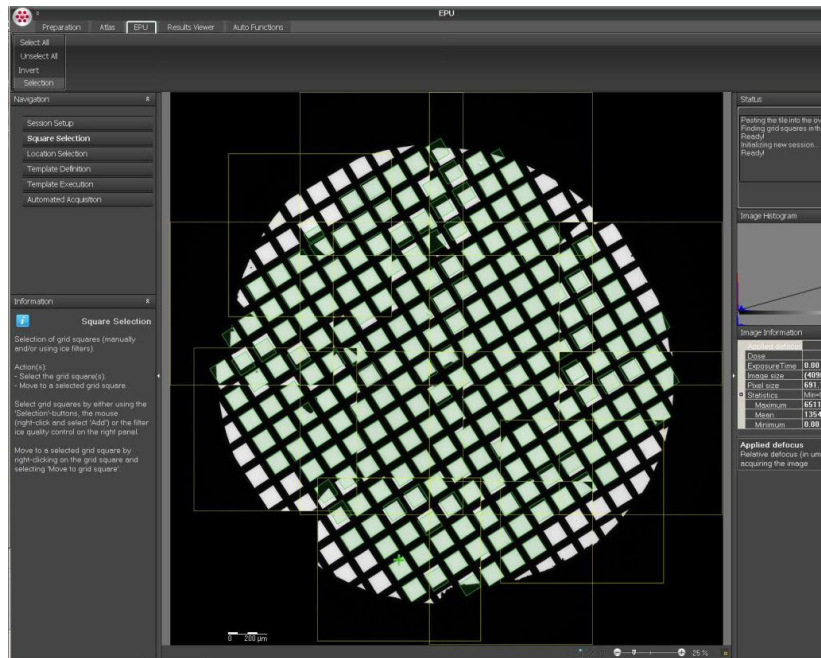
- Switch to the EPU tab. The order of the tasks in the EPU navigation pane indicates the workflow for setting up a data acquisition.



- **Define a Session**
  - Under Session Settings specify **Automatic** or **Manual** if you want to set up a fully automatic run or select suitable target locations manually. It is possible to switch between automatic and manual runs in one session.
  - Under Output, decide where the data is to be stored and in what format (this can also be changed between runs).
  - Under Specimen, enter some properties of the specimen support film to be used in the session. EPU supports two types: lacey carbon and Quantifoil, the latter coming in multiple variations that differ in hole size and spacing.
  - Under Email settings, enter the email addresses separated by commas, semicolons or spaces. Click the check box to enable email notifications to these recipients at the end of a run. Use the 'Test' button to confirm if the required email settings have been configured. These settings can be updated even after a run is started.

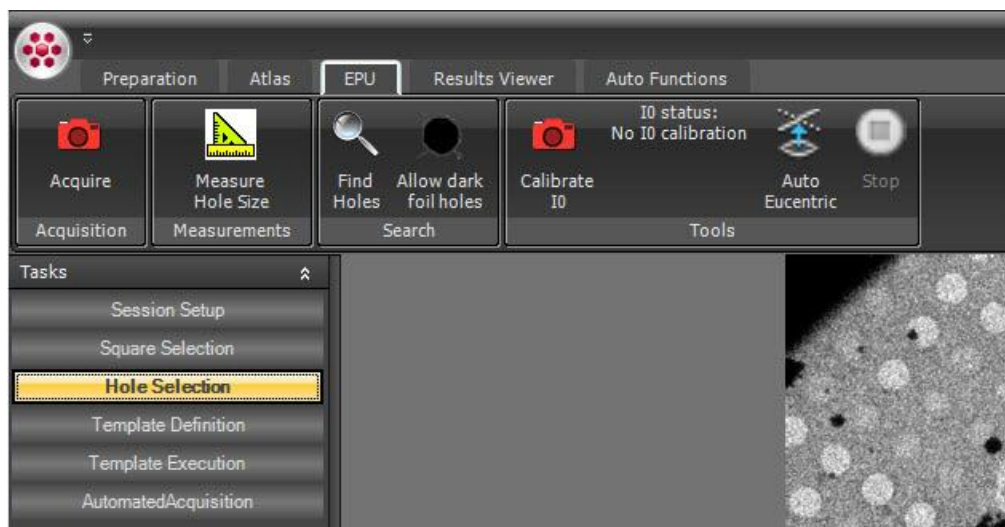
- **Square Selection**

- The EPU session automatically loads the last atlas. The atlas can be exchanged (a new one acquired or an old one loaded), as long as no data has been acquired. Once data runs have taken place, the EPU session continues with this atlas (even if a new one has been acquired) to maintain data consistency.
- The Square Selection view displays the atlas overview image, similar to what is displayed in the Atlas tab. However, all detected grid squares are selected for acquisition by default and therefore outlined in green. Atlas tiles are outlined in yellow.

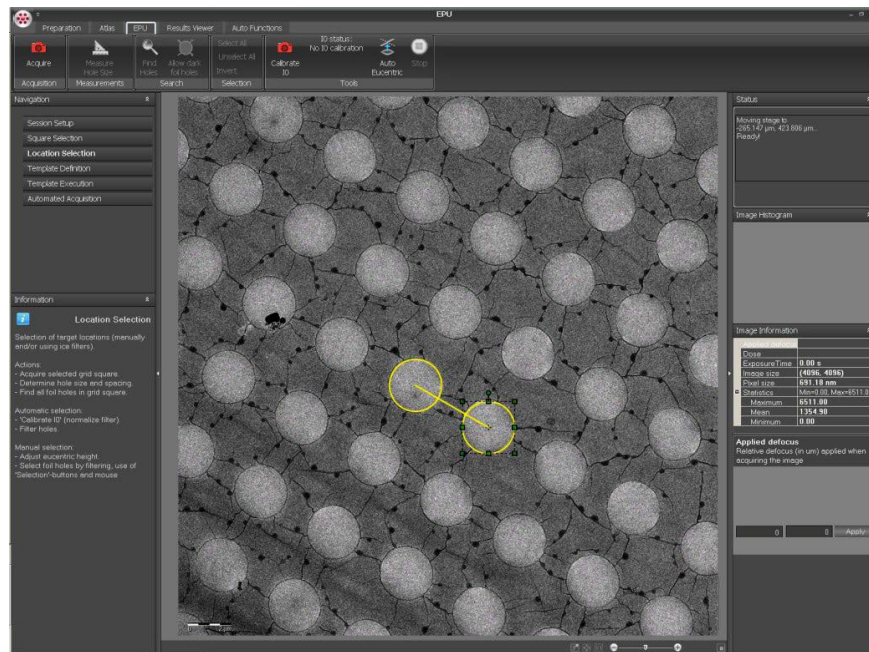


- **Hole Selection (Quantifoil Only)**

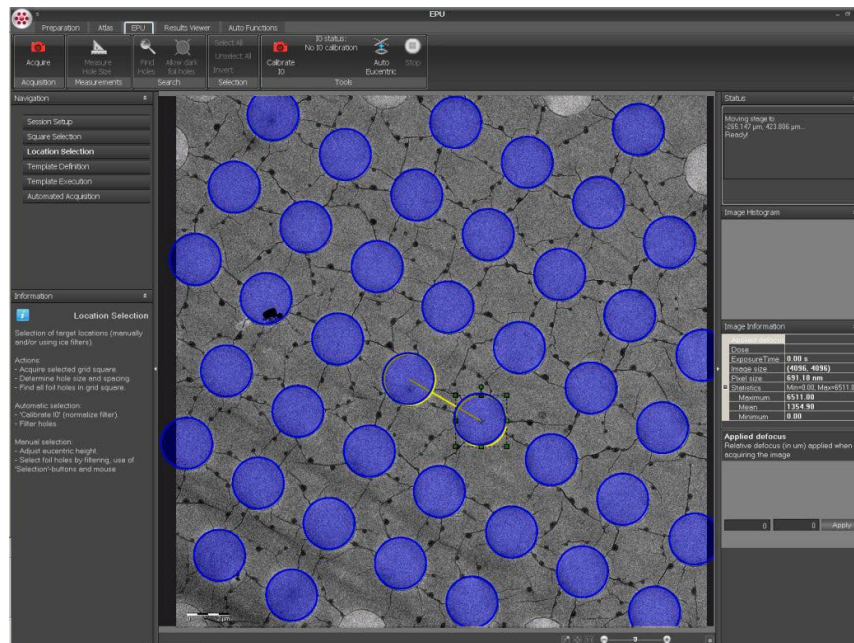
- Navigate to the grid square of your choice in the Square Selection task.
- Click **Hole selection** in the navigation pane on the left.
- Click **Acquire** to obtain an image.
- When the image displays, click **Measure hole size** in the Measurements ribbon bar.



- The yellow circles must be resized and moved to match the hole size and spacing exactly, as shown below. Zoom into the image to position the measure tool more accurately.



- Once set, click on **Find Holes**, and **Remove Holes Close to Grid Bar** to only have holes that are visible inside the grid square.
- Adjust the histogram to be more specific in which holes are selected in the grid square (if necessary)



- Continue with setting up the rest of your session according to EPU manual and instructions provided during training.