## Demonstration: SerialEM saving data

**PREREQUISITES:**

- Data recorded

### You managed to record images

From the menu File > Save A will save the latest recorded image.
In the settings dialog box that follows, choose:

- TIFF
- No compression
- Unsigned integers



# CryoTEM

Introduction

Version 1 – May 2024

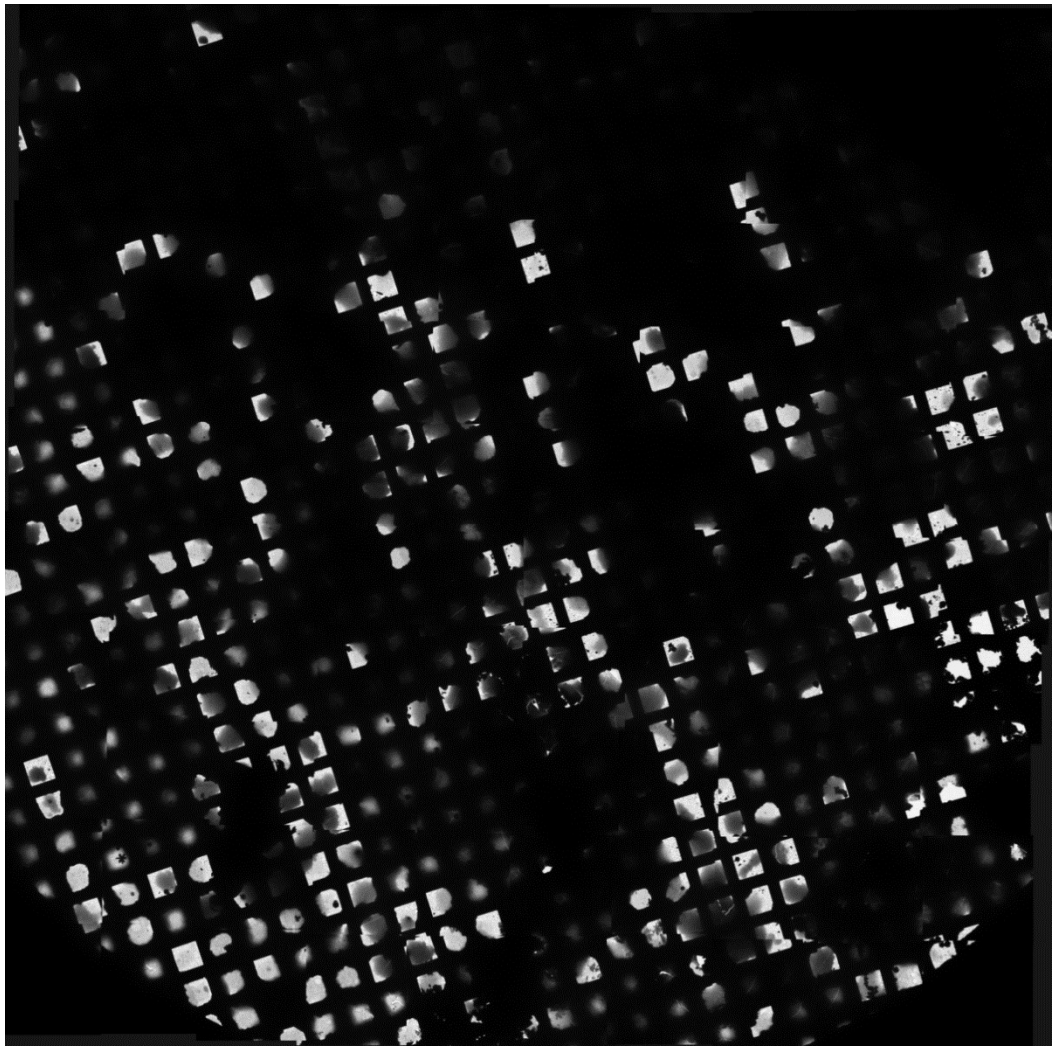
**Part V: Imaging**

starts at: grid inserted in TEM, ready to image
ends with: recorded image


LEICA EM GP

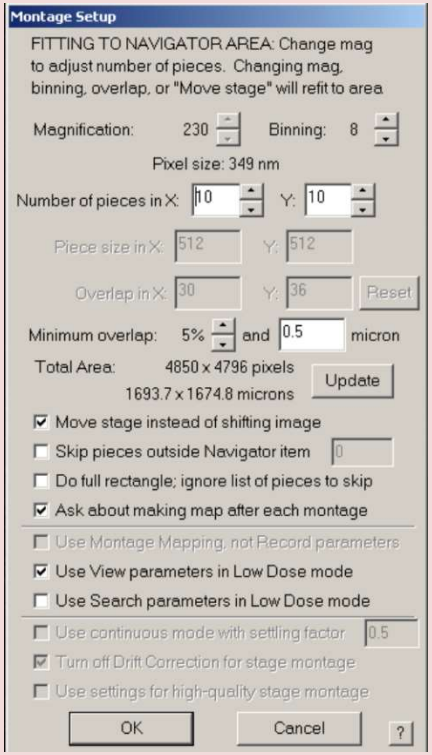






**Experiment: record a full grid map**

Start the full montage: menu Navigator > Montaging & Grids > Setup Full Montage




**Settings**

Number of pieces: 10 X 10

Minimum overlap: 5%

Tick:

- Move stage instead of shifting image
- Ask about making map after montage
- Depending on your preparation, Tick “use View…” or “Use Search…”

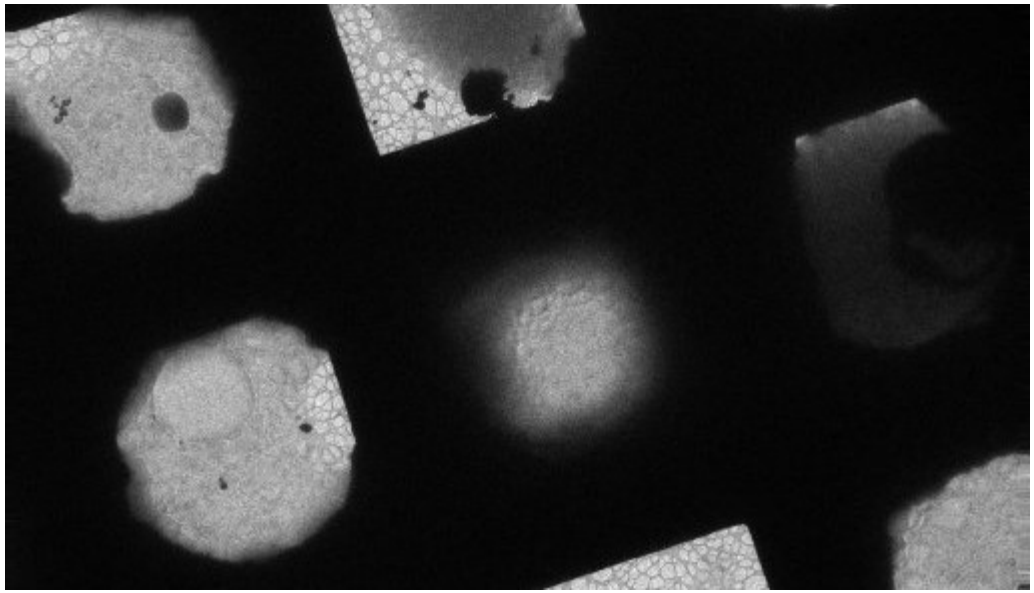
Click OK. In the Montage control panel, click Start

After the recording is finished, you will be asked to Save the montage as a map. Click yes.

The Navigator will get a new entry. Use the moving around as detailed before (page 19). To call the map, click “load map”







PART V: Imaging

**Demonstration: SerialEM creating a full grid map for moving around**

**PREREQUISITES:**

- Properly setup microscope

Make a full map of the grid, use it to move around

An overview map of a grid takes about 15 minutes to record, is not perfect but can be useful to find good quadrants (or empty quadrants for alignment!).

**Experiment: prepare for a full grid map**

In Low dose control, change the column parameter set **Search or View** to these settings (tick continuous update, select Vie.):

- Magnification: LM 230 X
- Spotsize: 3-5
- C2: 100%

Untick continuous update.
In camera & script, adjust to have the
View parameter set:

- Exposure time: 0.2s

Finally: remove the objective aperture (out)

**Note: The stage movement depends on physical cogwheels. Do not expect a precision better than 0.5 µm**





**Demonstration: Assessing the quality of the ice**

PREREQUISITES:

- Grid is inserted, shutter retracted, column valves opened
- Magnification 390X, C2=100%, Spot size = 2

Good quadrants vs bad quadrants

Find your way in the grid and spot good quadrants

**Experiment: Landing**

Look onto the fluorescent screen. Make sure:

- the camera is retracted
- the fluorescent screen is down (R1)
- the column valves are open

**Assess your stage**

Two possibilities: either you see

- Nothing (darkness)
- something green (see below)

- Darkness

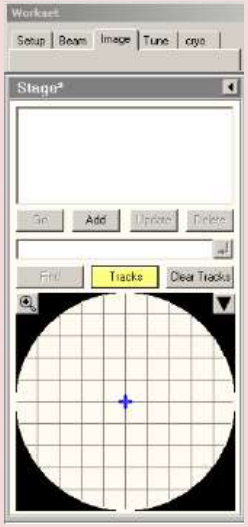
Assess all settings:

| | | | |
|---|---|---|---|
| Magnification | M 390X | SAED | Out |
| Camera | out (green LED on) | Shutter | retracted |
| Intensity | 100% | Column valves | Opened |
| obj aperture | Out | | |

If all correct: **you have too thick ice → search for better ice**

**Experiment: Search for good ice / quadrants**

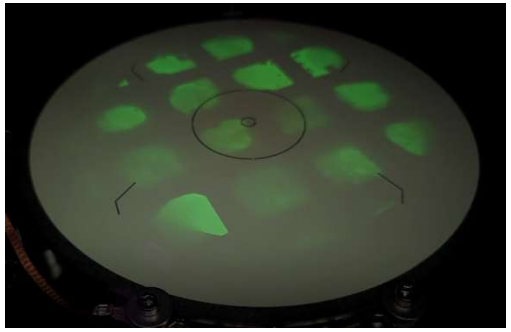
**Prepare your stage**
- Open the Image tab of your graphical user interface.
- In Stage$^2$, activate the “tracks”.




**Scout**
- double LMB near the top left corner: the stage will move there
- Meanwhile, lookm in the camera compartment of the TEM
- Scout for patches that are green (=electron transparent)

**Repeat**
- Meander through the entire grid (top left to bottom right)
- When good regions are found, click Add (=saved in the list)

- You have found “something green”



**Congratulations!**

**Experiment: alignments**

Run:
- A eucentric height + eucentric focus (max 6000X)
- Objective aperture alignment (by diffraction). Use Obj Ap position 2



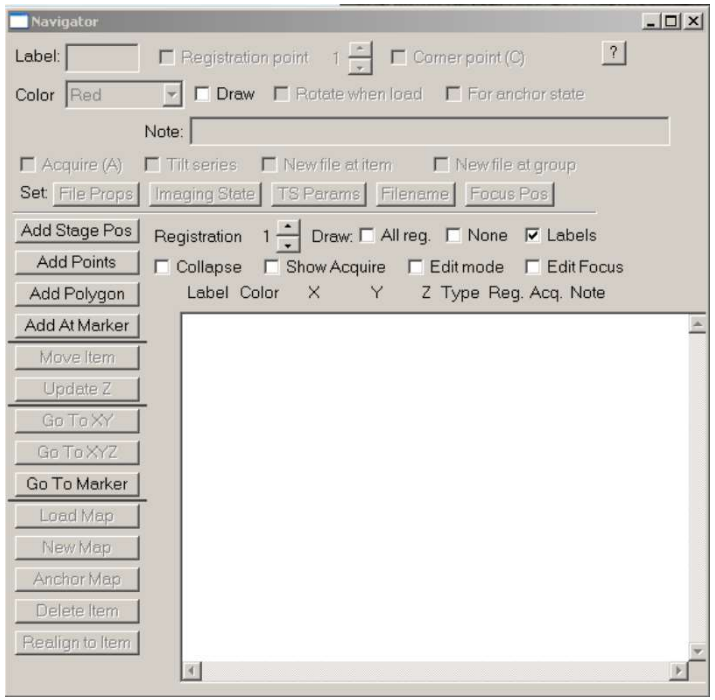


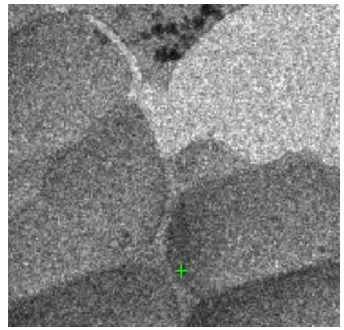
**Demonstration: SerialEM moving around**

**PREREQUISITES:**
- Properly setup microscope

Continuous imaging is not possible due to the dose

Open the **navigator**: Menu navigator > Open
A new window opens. Place it on the right screen.



**Experiment: Using the navigator to move around**

- Make an image (e.g. using the View parameter set, ca. 4800X).
- LMB somewhere on the view image. This leaves a small green cross on the image
- Press “Go To Marker” in the navigator. The stage will move to this position.

**Experiment: defocus**

**1. Set the defocus target**

Menu Calibration > set Target.

What target (defocus) you want?

- < zero (you want defocus, not overfocus)
- something between -1 and -10 µm
- the higher the magnification, the less defocus
- the more defocus, the more contrast, but the lower the resolution

As an example, here a target of -4 µm will be used

**2. Measure the defocus and manually set the focus**

Menu Calibration > Measure defocus.

the system will use the focus camera parameter set and the focus column parameter set

The **log window** will output the results, e.g.

```
Measured defocus = 1.06 microns drift = 0.00 nm/sec
```

In the example (Target = 4 µm), the setting is 5 µm off (1 - -4 = 5).

- On the right panel, press R2.

This reset the relative defocus to 0


- Using the focus knob, change the focus until the "Def" entry in the Microscope panel reads -5 um.

**2. Autofocus**

Menu Calibration > Measure defocus.

- The system will measure the defocus, and adjust the focus setting to match the target.

```
Measured defocus = 1.06 microns changed by 5.06 to target…
```

**Note: autofocus is not very precise. Repeat and check if needed**

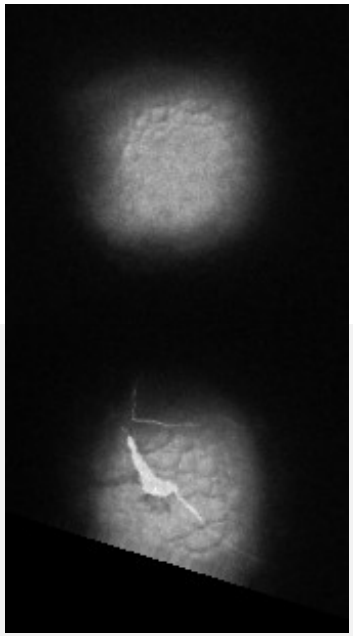




**Experiment: Coarse ice thickness assessment**

- Now you see something green, insert the objective aperture
- do a coarse objective aperture alignment[1]
- Focus on entire quadrants: check if the ice is still transparent for the beam:
  - The green quadrant becomes black→ the ice is too thick for imaging
  - The green quadrant remains green (albeit a bit darker) → ice is not too thick

Only save quadrants with good ice in your list. With the remaining quadrants, do a ice quality assessment:



**Perfect quadrant:**

- Thin enough in the center
- With a smooth falloff towards the gridbar
- Few/no black artefacts (crystalline ice)

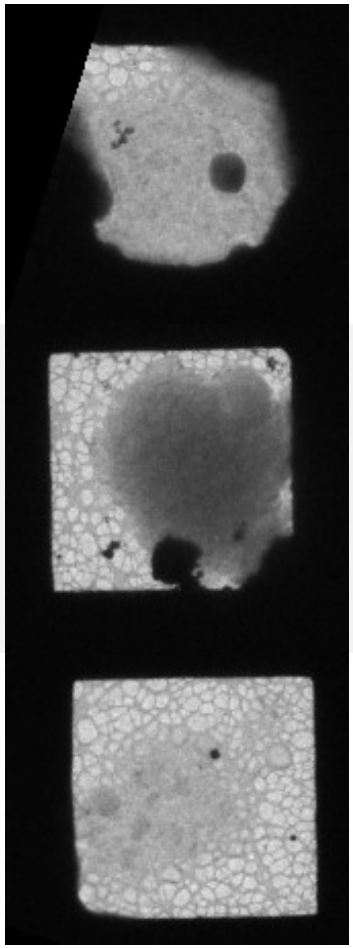
Good images very well possible

**Almost perfect quadrant**

- Same as above
- Some cracks in the vitreous water, but enough good parts around it

Good images very well possible

[1] Just use the knobs on the objective aperture mechanism to get the inserted aperture roughly centered at 390X. Better: do a proper alignment at 6000X, as described in the TEM booklet



**Medium quality quadrant**

- Thin enough near the center
- The falloff towards the gridbar is steep, not smooth (→ probably too thin ice = not a lot of material)
- Large crystalline ice artefacts (dark, round) obstruct parts of the quadrant

Good images very may be possible

**Medium quality quadrant**

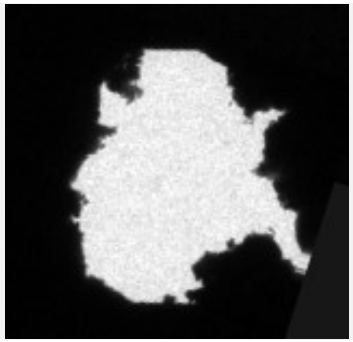
- No falloff towards the gridbar → probably no ice / too long blotted. Risk of finding no material
- Large crystalline ice artefacts (dark, round) obstruct parts of the quadrant
- Dark gray cloud obscures most of the quadrant

Low chances of good images

**low quality quadrant**

- No falloff towards the gridbar → probably no ice / too long blotted. Risk of finding no material

Low chances of good images



**Broken quadrant**

- Lacey, ice and sample are absent

Low chances of good images, BUT:
**important spot** for alignments (e.g. beam centering)





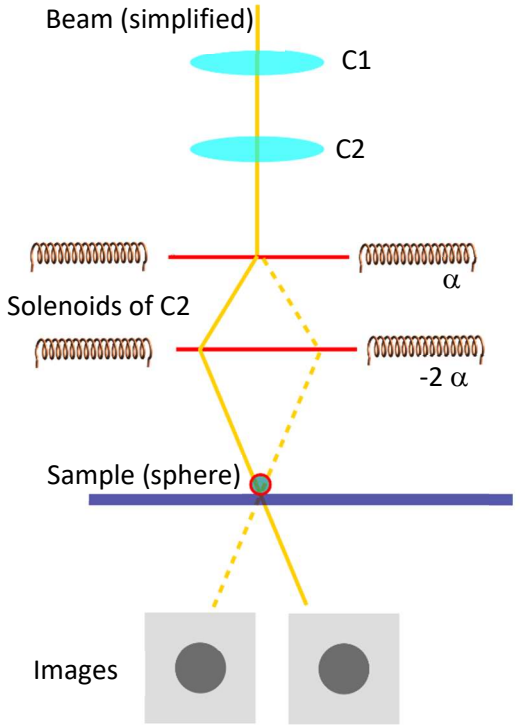
## Demonstration: SerialEM focus

**PREREQUISITES:**

- Properly setup microscope

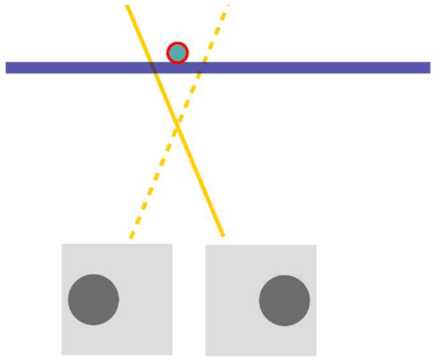
### Focusing your object with minimal dose

Focusing is needed, but in cryoTEM the dose plays a role, too. Learn to focus at minimal electron dose.




The defocus is measured by tilting the beam by the C2 solenoids (2 solenoids, both in X and Y).

Two images are made ($\alpha/-2\alpha$ and $-\alpha/2\alpha$). The difference between the images is a function of the defocus (If the images are the same, then the object is exactly in focus)



## Demonstration: SerialEM Dose calibration

**PREREQUISITES:**

- Properly setup microscope

### Electron dose is essential in cryoTEM

The electron dose calibration is for a particular spot size but short-term (1 day by default)[2].

**Experiment: calibration of the dose**

**1. Setup buffer A**

- Move to an broken/empty quadrant (or a hole in the ice)


- take an image (record) of the unblanked, opened beam with at least 0.2 seconds exp time, within a range where intensity is within the directly calibrated range[3] (Spot is blue).

**2. Calibrate the dose**

- With the right image in buffer A, find the menu Calibration > Electron Dose[4]

[2] Requirement: beam intensity must be calibrated and SerialEMproperties.txt contains an entry for CountsPerElectron for the camera.

[3] In the compartment "Microscope", the background color of "SPOT" must is blue (=calibrated), light cyan (not calibrated, but extrapolated), orange (completely outside the calibration range), or magenta if there is no calibration on the current side of crossover.

[4] The calibration will be most accurate if the spot size is normalized: Go to Normalizations in the Microscope User Interface, select TEM Spotsize, and check Spotsize. Then change spot size to get a normalized beam, take a picture, and calibrate the dose with it.





## Demonstration: Using SERIAL EM

**PREREQUISITES:**

- A Stage[2] list filled with at least 1 position of a good quadrant (not too thick ice)

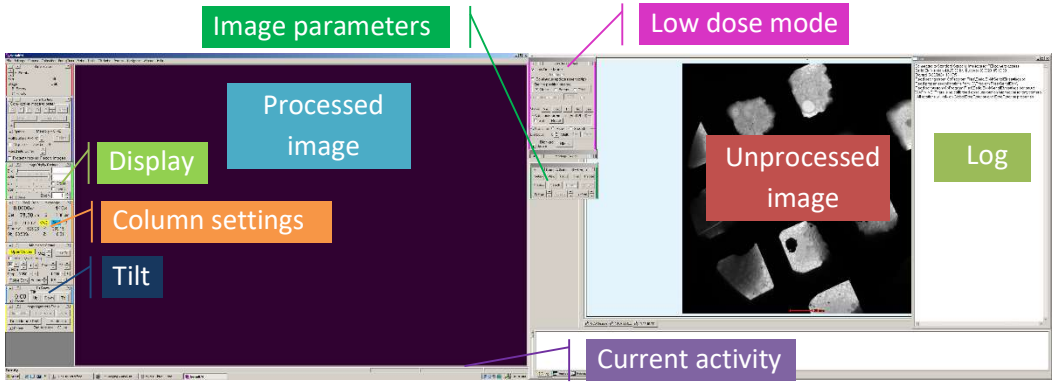
### You have good ice in the TEM

SerialEM is a software that takes care of the many settings of the TEM column and the camera, allowing you to concentrate on the imaging.

**Experiment: Switch to the Eagle camera**

- The Eagle camera is much more sensitive than the Veleta. Do not use the Veleta for cryoTEM!
- In the GUI, switch to BM-Eagle:
  Image > CCD/TV Camera > Camera
- Start Serial EM from the desktop 





## Demonstration: SERIAL EM – Camera parameters

**PREREQUISITES:**

SerialEM started

### First, assure you get a proper image through the Eagle camera

SerialEM allows 7 different setups for camera parameters. Each of the setups is a combination of different camera variables. It is up to the user how to define them, but a good starting point is provided here: **In Camera & Script > Click Setup.**


| Parameter set | Acquisition | Binning | Area size | Exp time | Use |
|---|---|---|---|---|---|
| Search | Continuous | 8 | Full | 0.05 | Continuous searching at low mag |
| View | Single | 8 | Full | 0.25 | Find quadrants in M mode |
| Focus | Single | 2 | Half | 1 | Beam shifted spot to focus |
| Trial | Single | 8 | Full | 0.5 | Align tilted or shifted images |
| Record | Single | 2 | Full | 2 | Your final acquisition |
| Preview | Single | 4 | Full | 0.25 | Alternative for view or search |
| Mont-Map | Single | | Full | 1 | Used to acquire montage pieces |

Always use Gain normalized Processing and a Drift setting of 0.00

In **Camera & Script** > Click Record.


**Chicken wire**

Is a type of artefact due to incorrect dark references. Click "Force new dark references next time only" in the parameter setup of the affected parameter set.





## Demonstration: SerialEM center beam

**PREREQUISITES:**

- Eucentric height properly set
- Edge of the beam is fairly sharp and appears in the current image

### Center the beam at a minimum dose.

Assure you are on a transparent but not important part of your sample, e.g. a broken quadrant (page 8).

Condense the beam (intensity/C2) until 3 or 4 corners are no longer covered by the beam

Run: tasks > Center beam

The program will analyze the image to find the beam edge then solve for a circle that fits the edge.

It will then move the beam to the center. It will not work if the beam edge is not relatively sharp.

## Demonstration: eucentric height in SerialEM

**PREREQUISITES:**

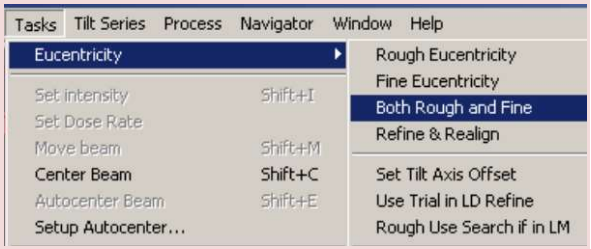
- Eagle camera parameters properly setup
- Column parameters properly setup

### Eucentric height is still important

The eucentric height algorithm is much more powerful than the Tecnai GUI. It will use the **camera View parameter set** in Low Dose mode.

**Experiment: eucentric height**

- Find in the menu Tasks > Eucentricity > Both Rough and Fine




- Assuming you have done the Eucentric height (page 6), this will run Automatically.

The procedure does not make any attempt to keep the starting specimen location in the field of view.





## Demonstration: SERIAL EM – Low dose control

**PREREQUISITES:**

Eagle camera parameters properly setup

### Second, setup the column parameters for cryoTEM

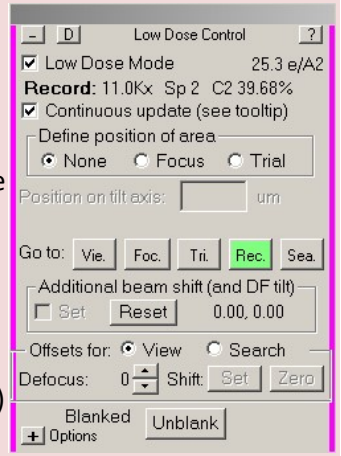
Besides camera parameters, there are also column parameters. These settings include Magnification, Spot size, C2 and Beam shift

- Setup Column parameters

In the pink accented Low Dose controls, you can setup the column parameters.

**Setting up column parameters**

1. Find the Low Dose control and expand the box.




2. Tick “Low dose mode”.

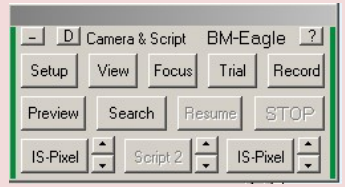
3. Tick “Continuous update”. Any change on the microscope will now affect the settings.

4. In the “go to:” below, select a parameter set (here Rec.)

5. Use the TEM controls (left panel, right panel) to adjust to the required settings (e.g. 11.0Kx, spotsize 2, C2: 39.68%)

6. Untick the Continuous update again.

7. Each time the Column Record parameter set is selected, the microscope will adjust to these settings.




8. Take an image (with the record camera parameter set) by clicking Record in the Camera & Script.

The column parameters are set as needed by the sample and experiment. A starting point can be:

| Column parameter set | Abbr. | Beam shift | Magnification | Spot size | C2 |
|---|---|---|---|---|---|
| View | Vie. | No | < 1.050 X | 2 | 100% |
| Focus | Foc. | Yes | > 11.500 X | 3 -5 | < 45% |
| Trial | Tri. | Yes | 2.900 X – 9.900 X | 2 – 3 | < 50% |
| Record | Rec. | No | > 18 500 X (< 43.000X) | 2-5 | < 45% |
| Search | Sea. | No | Around 4.800 X | 2-3 | < 50% |

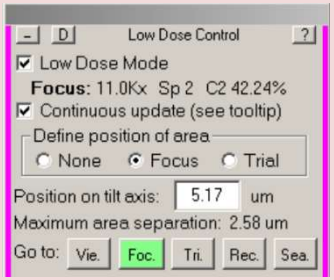
**Despite having the same names, column parameters are not the same as camera parameters! However, they are linked (e.g. View uses Vie.)**

Focus and Record should be as similar as possible (Spot size, C2, magnification), but focus is beam shifted (see below).

- Setup beam shift (focus and trial column parameters only)

The goal is to avoid dose on the region where the image will be acquired. To focus, a beam shift of 4-5 µm must be applied to focus close by but not on that region.

**Experiment: adjust the focus beam shift**




- Record an image using the camera parameter set View.

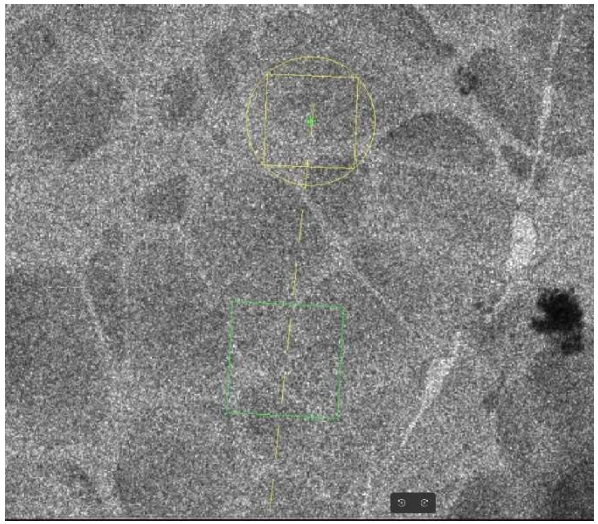
- In the Low Dose control assure “Continuous update” is ticked and that the Focus column parameter is selected.

- In the Position on tilt axis, adjust the value (here: 5.17)

- In the overlay you can see the position for the record (=your final image, green box) and the position of the focus (yellow box). Both boxes lay on the alpha tilt axis, which is calibrated and not necessarily vertical.







Important is that the yellow box is not overlapping with the green box.