

Setting up SerialEM for single particle acquisition v0.3

(Quantifoil grids)

Preparation (FEI)

Align Titan in Nanoprobe 59kx (=105kx in EFTEM), spot 7:

- Spot size-dependent Gun shift
- Beam tilt pp and beam shift in u/nanoProbe. Beam shift is important when switching between View and Record.
- Also align Rotation center in both modes
- Center C2, C3 (Condenser center TEM)
- Align Cs
- Insert obj. aperture (if used) and center it

Preparation (Gatan)

- Find a place without specimen.
- Check in Linear mode Search view (in DM) to have ~2000 counts/px/s at 105kx, spot 3, nProbe EFTEM.
- Calibrate ZLP in DM4 interface: center ZLP then Tune filter (Full tune). Resulting ZLP Offset should be 0 eV.
- Prepare Gain references for linear (30s) and counting/super-res (9 min) modes at spot sizes 3/7, respectively. Also update HW dark reference (7 min).

SerialEM setup and calibrations (not full list...)

Start SerialEM, switch on EFTEM if it went off.

Calibrations required:

- Autofocus 30um range in nanoProbe (you must be in focus at the start and have enough signal)
- Image shift, Stage shift
- Standard Focus set for LM (275x) and SA (both 3600x and 105kx)
- Image & Stage shift -> Mag IS Offsets for all mags from 105kx till 170x.
- Tick box Adjust Image Shift between Mags in Image Alignment and Focus panel.
- Check File → Continuous save to autosave log file
- Make sure you have scale bar by pressing “Scale Bars” button in Image Display Controls panel. Also Autozoom option is useful.

Atlas acquisition (LM map)

- LD off. Set Record: 275x spot 7, 0.3s, bin 2, slit 40eV, EFTEM, uProbe. No obj. aperture. Record is about 100 counts ~ 5-10 e/px/s at camera (spread beam almost to max). This is 1.27mm beam size.
- At the grid center, run Tasks → Euc. rough. Press Euc. focus. Set LM standard focus here.
- Navigator → Open. Navigator → Montaging & Grids → Setup Full Montage. It will give 9x8 pieces at 275x. Set options: move stage instead of image shift, NOT use View params. Select filename “**atlas1.st**”.
- Montage control panel → Start. At the end do File → Close.
- Navigator → tick Autosave nav. file. Navigator → Save as “**nav1.nav**”

Tips:

- If the montage looks misaligned, untick “Treat as very sloppy montage”, select acquired map and click Load.
- Navigator window: check “Rotate when load”. This will rotate LM map during loading when you are at higher magnifications (e.g., SA range).

Low Dose setup

- LD on. Setup Camera params for each mode (all EFTEM, nanoProbe):

Area	Exp	Bin	Mag	Spot	Beam	Defocus	Slit	Mode
View (V)	0.01s uProbe!	4, full	2250x	7	45um	-20um	40eV	Linear
Focus (F) / Trial (T)	2s	2, half + 20%	105kx	7	525nm	-	20eV	Linear
Record (R)	7s, 0.25s/frame, dark subtracted	3*, full	105kx	7	700nm	0	20eV	SR
Preview (P)	0.06s, continuous	4, full	105kx	7	700nm	0	20eV	Linear

*(only applied for display, SerialEM always saves full resolution frames)

- Go to safe area with no ice / good carbon with holes (Add point and Go to XY in LM map). Tick box “Keep T/F identical”. Prepare View mode and do Tasks → Eucentric Both. Also check if you are in focus.
- Setup R and P now. Make sure image shift is zero in R (center beam with Direct alignments and press “Reset Image Shifts”). Spread the beam as table indicates, to get 2.5e/px/s for SR mode or 10e/px/s for C mode (see *Tips*). Set Processing to “Dark Subtracted”. Copy settings to T and F using a button. Also, setup folder for frame acquisition and associated parameters (save files as TIFF with LZW compression; no rotation/flip of images (attention!! the hand will be changed!!); packing SR as 4-bit).
- You can set up P to continuous mode and use Process → Live FFT to correct astigmatism. In R mode check rotation center, coma-free alignment and obj. stigmatism.
- Finish V setup: make smaller beam, tick “Set add. beam shift”, center it on FluCam with

trackball. Untick the box to save shifts. View dose must be $\leq 0.02 \text{ e}/\text{A}^2$. Also, set defocus relative to R, e.g. -20um, in “Defocus offset for View”. If V and R positions are not aligned (image shift is needed^{*}):

- Image Alignment & Focus → uncheck “Move stage for big mouse shifts”
- Take R, center feature with Shift+right mouse. Usually you may burn a hole in ice.
- Take V, shift feature with right mouse button, press “Set”. A new V will be taken automatically. Check if the feature is centered. Shift it again if not and then press “Set”.
- Turn back on “Move stage for big mouse shifts”.

** After this procedure, you should re-do “Shift to marker” (between Atlas and GridSquare mag)*

- To set up F/T area while in V, click radio button. Adjust Focus area position along the tilt axis with mouse. Select “Rotate inter-area axis” to rotate the axis between R and F/T.
- Switch to R, then T/F and check if beam shift is necessary, if so, set it as for V.

Tips:

- Use checkbox “Continuously update...” to modify area settings on-the-fly.
- You might setup Search area with settings from Atlas (LM mag) to use it for fast grid navigation with screen down.
- Always cycle through the modes in the same order, V → F → R → V → F → R ..., to prevent hysteresis in image and beam shift coils.
- For a SR exposure, the frames format is 4-bit unsigned int, means all the pixel value in a frame is within the range 0-15. Therefore, we have to set our imaging condition accordingly. For example, if the beam is at dose rate of 10 electron per physical pixel per second, and if we use 1.5 seconds or more as frame time, then we could reach the limit of 15. In this case, the pixel value will overflow, and we lose information. Although this is almost unlikely to be the real condition we ever to use, we should be aware of such limitation.
- For K2 Counted and Super-res image, the dose rate is small, usually around the range of 10e/unbinned px/s (8 counts/unbinned px/s). $1 \text{ e}/\text{px}/\text{s}$ is $\sim 3.35 \text{ e}/\text{A}^2$.
- $\text{Mean_Counts} = 0.5$ (divide by 2 for 16-bit camera) * Dose (e/A^2) * Conversion_Rate (counts per electron=30 by default) * Pixel_Size² (at the sample level= 1.21 A^2 at 59000x). So, for the Record we will have $0.5 * (33.5 * 7 \text{ s}) * 30 * 1.21 \sim 4200$ counts. [More info](#)

Grid squares acquisition.

- Load Atlas (LM map). Click on a square’s center, Go to marker. Get eucentric and in focus.
- Add polygon for this square (it should be a bit smaller than the square size). Add points in the center of all other interesting squares.
- Find some dirt in the first square. Add a point in LM map, go to it. Take V, find the same spot (move only with stage if necessary), left click on it, select Navigator → Shift to marker, Yes. This will adjust the coordinates of all items in order to align LM montage and V.
- Select the polygon item, Navigator → Montaging & Grids → Setup Polygon Montage. Set 15% / 5um overlap, total about 20 tiles. Tick “using View”, select filename “**squares-grid1.st**”. Untick “Treat as very sloppy montage” option.

- Add “Acquire” flag for all square points in Navigator and for the first polygon.
- Run Navigator → Acquire at points, check “Euc. rough” and “Acquire montage map”. At the end close montage file.

Tips:

- Percentage of overlap between tiles should be at least 15% and about 5um. Also, it helps to switch off "Treat as very sloppy montage" and reload the maps.

Selecting foil holes

First, you need to create a template of a single centered hole: take V with only the center of camera frame (can increase by 10-20%) to be sure that your image includes only a single hole (with neighboring half-holes) with no strong features around it. Save image to file “**hole.st**” in the folder where you put navigator file and map files.

For each grid square map do the following:

- Get eucentric (it seems better to do it yourself for each grid square)
- Add a polygon item to include only good areas. Make sure it is close to bad holes to avoid excluding good ones nearby.
- Add 5 points to define rectangular pattern inside the created polygon:

XOOOO

OOOOO

OOOOO

XOOOO

XXOOX

- (optional) To add a line, add a group of 3 points like this: XXOOOOX
- Navigator → Montaging & Grids → Set group radius = 50um since we want to have 1 group per grid square and the first point in the square center. Tick Navigator → Montaging & Grids → Divide points by groups
- Select any of 5 points, Navigator → Montaging & Grids → Add grid points, enter polygon number, set “Acquire” for newly created points.
- Switch to V before starting any macro. This will ensure that RealignToNavItem does not switch between modes all the time.
- Load LD-Group and MyFuncs macros, modify parameters if required.
- File -> Open Old -> hole.st
- Run Navigator → Acquire at points, check Run macro LD-Group and press GO.

Tips:

- Check the macro on 1-2 holes first.
- Once you have added a grid with a 5-point pattern like this, you can add the same grid in a new square/polygon by simply adding a single point in the new area (it must be the point in the main corner). Remember: it's easier

to add all holes of a square and then remove bad ones – this way they will all be in the same group.

- To select multiple holes/items, switch on Edit mode and use Ctrl+left mouse to select multiple points in the image. Middle button click will add a point to the group to which the current point belongs. You can delete selected items all at once with Delete item button.
- If you need to stop+kill the running macro, press STOP and “End macro” and “STOP” again.

Troubleshooting

Q1: Autofocus fails.

A1: First, make sure it is calibrated (i.e. no errors are shown after calibration is over). If Autofocus fails, you can increase binning / intensity in F or adjust low-pass filter. Set program properties to apply a stronger high-frequency filter to the images. This filter can be controlled by entries in the file SerialEMproperties.txt: 'FocusFilterRadius2' for the cutoff frequency (default 0.25/pixel) and 'FocusFilterSigma2' for the Gaussian rolloff of the filter to zero (default 0.05/pixel). Smaller values are used to apply more filtering.

Q2: Montage of a grid square is very bad.

A2: First, make sure you are at eucentric height and in focus, on the square center. Second, correct the shift between Atlas mag and Square mag using Shift to marker option. Another possibility is to tweak “Treat as very sloppy montage” option. Make sure you have set enough overlap for the pieces of a montage. Also, it is possible to modify image correlation parameters (SerialEM beta version):

Use Filter Set 2 command (Camera – Set minimum counts submenu)

The Correlation Filter submenu has a set of commands for controlling the filter used when correlating montage overlap zones. There are two sets of filter parameters available. Each set has default values, but the defaults can be changed by adding entries for MontageFilterR1R2S1S2 or MontFilterSet2R1R2S1S2 to the SerialEM properties file. The only difference between the defaults is in the low frequency filtering applied when 'Treat as very sloppy montage' is checked in the Montage Control panel. Filter Set 1 has a strong low-frequency filter, which has been found to be important when there are strong, high-contrast features like grid bars in the overlaps zone. However, this amount of low-frequency filtering can be detrimental when aligning low-dose montages without many features beside the regularly spaced holes. Filter Set 2 eliminates the extra low frequency filter and may work better for such specimens.

Use this command to switch to Filter Set 2 unconditionally. If you change filters and want to see the effect on alignment of an existing montage, you need to turn on the “Redo Corr when Read” option so that correlations are recomputed when the montage is read in.

Q3: When switching between V and R, defocus changes keep accumulating.

A3: It happens if micro and nanoprobe modes are not well aligned. SerialEM reimposes a number of settings that were present when it was last in that mode ([More info](#)). To equalize focus between the two modes while in Low Dose mode, turn off Tasks → Special options → “Adjust Focus on Probe Mode Change”, turn on “Continuous update of Mag and Beam”, and autofocus in each mode. Use the microscope interface to change modes. Similarly, to center the beam independently in the two modes, set up Trial parameters for a condensed beam that can be centered, turn on 'Continuous update of Mag and Beam', and change between the modes with a script or in the microscope interface.

Notes / TODO

Add usage of Imaging state dialog in Navigator

Calibrate astigmatism/coma and dose in SerialEM if possible

Check Post-actions command in Camera menu

For 7s exposure: 70s per hole (1 img), 80s per hole (1 img with realign center), 4.5min per 4 img=1hole