Setting up SerialEM for single particle acquisition v0.2

(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 from View to 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, also have 100 counts)
- 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..., ask about..., NOT use View params. Select filename "**atlas1.st**".
- Montage control panel → Start. At the end click Yes to save the montage as a map. File → Close.
- Navigator → tick Autosave nav. file. Navigator → Save as "**nav1.nav**"

Tips:

- If the montage looks sloppy, 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 magnification (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)	0.5s uProbe!	4, full	3600x	7	100um	-25um	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 not too far from 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. stigmation.

- 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 <= 0.02e/A^2. Also, set defocus relative to R, e.g. -5um, 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 press “Set”.
  - Turn back on “Move stage for big mouse shifts”.
- 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). 1e/px/s is ~ 3.35 e/A^2.
- Mean_Counts = 0.5 (divide by 2 for 16-bit camera) * Dose (e/A^2) * Conversion_Rate (counts per electron=30 by default) * Pixel_Size^2 (at the sample level=1.21A^2 at 59000x). So, for the Record we will have 0.5*33.5*30*1.21~600 counts. More info

Grid squares acquisition.

- Load Atlas (LM). 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 a point in the center of all other interesting squares.
- Find some dirt in the first square. Add point in LM map, go to it. Take V, find the same spot, 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 10-15% overlap, total about 40-50 tiles. Tick “using View”, select filename “**squares-grid1.st**”.
- 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 10%. If montaging fails, you can try higher overlap, e.g., 25%. 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 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:

- Add a polygon item to exclude bad areas.
- Add 5 points to define holes geometry:

  XOOOO

  OOOOO

  OOOOO

  XOOOO

  XXOOX
- Select any of 5 points, Navigator → Montaging & Grids → Add grid points, type Enter, No (to move by zigzag). Add flag “A” to acquire all 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 params 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).
- To select multiple holes/items, switch on Edit mode and use Crtl+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. Also it is possible to modify correlation parameters:

Use Filter Set 2 command (Camera - Correlation Filter 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.

Notes / TODO (do not read this :)

calibrate astigmtism/coma in SerialEM if possible, calibrate dose, gain ref, coma and astigmatism

300x montage is not very good, displacement max 90px - because of grid bars, while LM montage is ok

if the first hole is at the square edge, realign to item (tile center) fails (due to grid bars?)

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

Post-actions command (Camera menu)

This command toggles a mode in which various time-consuming actions are initiated immediately after camera exposure ends, at the beginning of the image readout. These actions can be magnification changes, image shift, and stage tilting.