

SerialEM Note: Single Particle Data Collection

Adapted from Chen Xu's original file

https://sphinx-emdocs.readthedocs.io/en/latest/singleparticle_seriallem.html#

Edited by Tong Wang (twang1@gc.cuny.edu)

This doc is a working progress. If you have comment and suggestion, please let me know. Thank you! **This is a protocol using stitching MMM, the traditional way.**

Preparation (scope condition, tuning) (staff job)

Before you commit large dataset time, it is always a good idea to check scope condition to make sure everything is good. Calm down and be patient! Here are a few things I usually check.

Microscope TUI

- Make sure “Standardalone Camera” is activated (yellow) in microscope TUI.
Camera>CCD/TV Camera>Shutter>Standalone Camera
- Load cross grating grid to microscope, find one square with broken film and one good square (intact film), record both positions in microscope TUI: *Search>Stage²*.
- Check Gun Lens (3), Extracting Voltage (3750), High Tension (300kV), FEG Emission (~221uA) are correct values. microscope TUI: *Setup>FEG Control*.
- Stare for a few seconds at the focused beam at the highest SA mag (360kx), to see if the beam has good shape and there is no shaking or jumping.

Gatan GMS

- Move stage to square with broken film. For K3 camera, perform full gain refs for both linear and counting modes in GMS interface (~10000x, nP, ss 1-5). and dark references will be collected automatically before each gain refs. Counting mode of K3 is equivalent to super-resolution mode of K2 with binning of 0.5.

SerialEM

- Start SerialEM server (SEM server) from quick start of microscope PC, start SerialEM from quick start of Gatan K3 PC.
- Move stage to square with intact film. Set microscope to SerialEM LD-V mode setting without turning on LD in serialEM: **2500x, mP, ss=8, IA=15um**. Move grid to eucentric height in SerialEM: *Tasks>Eucentric-Rough*. Align C2 aperture (70 um) at this mag.
- Set microscope to SerialEM LD-R mode setting without turning on LD in SerialEM: **37000x, nP, ss=5, IA=900nm**, Camera Record: Exp=0.4s, bin=2, autofocus to -0.5 um. Click Eucentric Focus on right control pad and perform *Tune>Direct Alignment* in microscope TUI for both uP and nP at 37000x: Beam Tilt PP, Beam Shift, Rotation Center.
- Do Obj Stigmatism and Coma-free Alignment in SerialEM with cross grating grid.
Focus/Tune>Correct Astigmatism by CTF and Coma-free Alignment by CTF. Do *Correct Astigmatism by CTF>Coma-free Alignment by CTF>Correct Astigmatism by CTF* in a closed loop since coma-free alignment may alter astigmatism alignment.
- Calibrate Imaging Shift VS Coma-free in SerialEM with LD-R mode setting without turning on LD in SerialEM: 37000x, nP, ss=5, IA = 900nm, Camera Record: Exp=1-2.5s, bin=2-4, autofocus=-0.5 um, IS=6-8um. *Calibration>Focus&Tuning>Coma vs. Image Shift*
Note: if calibration fails in this step, choose different camera exposure time (1s, 1.5s, or 2s), bin (2 or 4), and/or autofocus value (-0.5~-1.5 um).

Notes: it is highly recommended to do Imaging Shift vs Coma-free calibration in LD-R with LD on. Save settings after calibration is done thus it is saved with settings.

- Insert Obj Aperture (70 μm) and do alignment.

Prepare K3 Camera (staff job)

- Keep microscope to SerialEM LD-R mode setting without turning on LD in SerialEM: 37000x, nP, ss=5, IA=900nm, Camera Record: Exp=1s, bin=1.
- Take a single shot without specimen and do FFT. The FFT should show clean background without strong center cross or lines.
- Take a single shot with cross grating specimen: autofocus to -1 μm and do FFT. Check Thon Ring and make sure there is no obvious frequency cutoff, Thon Ring reaches the resolution as in good condition.

Collect Test Data Set (staff job)

Before moving forward to load your cryo-grid. It is always a good practice to collect a pattern (3by3) of test data with cross grating specimen.

- Keep microscope in SerialEM LD-R mode setting without turning on LD in SerialEM: 37000x, nP, ss=5, IA=900nm, Camera Record: Exp=5s, bin=1, check “Bin counting frames by 2 in hardware”, Dose Fractionation: frame time=0.05s, Set File Option and Set Folder.
- Run Sript22 Screen_MR to collect 9 (3by3) movies, upload movies to processing server and process them to CTF correction.
- Check all CTF images to make sure there is no obvious frequency cutoff, check micrograph_ctf.star file to make sure CTF correction reaches decent resolution.
- If all above are good, move to next session.

Make Low Mag Montage (LMM) Map/Grid Atlas (user&staff job)

In ASRC, we use K3 camera for all images. We do not switch between Ceta and K3.

- Insert/load your cryo grids. Keep LD mode off in SerialEM.
- Set mag at $\sim 51\times$, retract Obj Aperture, ss = 8, IA = 1.5mm, defocus = -732 μm (click “Autofocus” in SerialEM to set defocus to this value).
- Spread beam to cover whole K3 camera area (IA 1.5mm)
- Setup camera condition from SerialEM camera control setup: Record (e.g. bin=4, exposure=0.3, uncheck dose fractionation)
- Navigator menu -> Open
- Navigator menu -> Montaging & Grids -> Setup Full Montage, in the dialog window:
 - a. Check “Move stage instead shift images”
 - b. Check “ask about making map after each montage”Click “OK” to enter File Properties dialog, Click “OK” again to define montage filename to open and save montage file.
- Montage Control Panel
 - a. Uncheck “Treat as very sloppy montage”

- b. Check “Align pieces in overview”
 - c. Check “Show overview at end”
- Click “Start” to collect LMM.
- Once finished, click "Yes" in the pop-up window to make final overview of montage into a map item in Navigator.
- Copy map to buffer P (must be different from the default buffer in the macros) and save it as a jpeg file.
- Close the montage in File menu of SerialEM.
- Insert Obj Aperture (70um).

Note: If you are not happy with the aligning of the pieces, you may check "Treat sloppy..." and reload the map.

- From this point on, uncheck “Move stage for big mouse shift”
 - a. For imaging shift, right mouse click and drag
 - b. For stage shift, hold SHIFT key, do right mouse click and drag
 - c. For stage shift, alternatively, do image shift as above and click “Reset Imaging Shift”
 - d. Pay attention to the IS values in SerialEM interface

Setup Low Dose Condition (staff&user job)

You should have known how to setup Low Dose condition already. Here are some tips.

- If you plan to use mix of microprobe (mP) and nanoprobe (nP) for LD, read document *SerialEM note 03: Setup LD with Mix of mP and nP Modes*.
- Follow instructions in the above document to turn on Low Dose Mode in SerialEM, load settings saved in Preparation step, then set up each LD modes and camera parameters using the two table below. Make sure that LD-V and LD-R should be exactly the same as in preparation step.
- Setup R beam first (check “continue update...”, remember to uncheck after done) so that dose on detector and on specimen are all good. Choose proper beam spot size and intensity.
- Take image with R mode without sample, calibrate electron dose, check “Keep Focus and Trial Identical”, Copy current area mag & beam to “F”.
- Defocus offset (-200um) for V is usually a good start. Align LMM, V and R to make sure the same feature is centered in images in three settings. Use image shift offset in V to align V and R. Use “shift to marker” to align V and LMM.
- Always cycle "area" (low dose mode) in one directional looped fashion, i.e., V-F-T-R-V...
- Take image with V mode, setup F image area relative to R image area.

LD modes

Mode	Beam type	Mag (x)	Spot size (ss)	Illumination area (IA)	Dose rate (e/unpix/s)
View	mP	2500 (12.3 A/pix)	9	15 um	~2.4
Focus	nP	37000	5	900 nm	~15
Trial	nP	37000	5	900 nm	~15
Record 1	nP	37000 (0.87 A/pix)	5	900 nm	~15

Record 2	nP	28500 (1.055 A/pix)	5	1.05 um	~15
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Camera parameters

Mode	Mag (x)	Exp time (s)	Binning	Size	Dose frac
View	2500 (12.3 A/pix)	0.2	4	Full	No
Focus	37000	0.2	4	Wide half	No
Trial	37000	0.2	4	Full	No
Record (map)	37000	0.4	2	Full	No
Preview	37000	0.2	4	Full	No
Record 1 (collection)	37000 (0.87 A/pix)	4-5	0.5 (bin by 2)	Full	Yes
Record 2 (collection)	28500 (0.87 A/pix)	4-5	0.5 (bin by 2)	Full	Yes

Note: K3 always save frames in super-res mode (bin 0.5) regardless of bin selection. If you wish to save frames in non-super-resolution mode (bin 1) please check “bin by 2 at hardware” in Camera Setup. Remember to check dose fractionation, give frame file name and set folder path

Save Accurate Imaging Shift (staff&user job)

Multiple Record requires saving accurate image shift values for holes.

- If you plan to use Multiple Record, read document *SerialEM note 04: Multiple Record Setup Dialog*.
- I recommend using regular 3 by 3 pattern.

Make Medium Mag Montage Maps (staff&user job)

- Select K3 camera from Camera Control Setup (from now on)
- Add a polygon (a mesh/square) in LMM map
- Add points for good meshes/squares at center
- Add one landmarker (green cross) such as a dirt point in LMM map by adding point
- Take the landmarker into View image (you may use fluoroscreen to move that feature into middle first.)
- While landmarker point being current (highlighted), left click on the landmarker in View image, a green cross will appear
- Navigator menu -> Shift to Marker -> Yes (this will change all the coordinates for all the navigator items)
- Highlighting polygon item on navigator window, so it is currently selected
- Navigator menu -> Montaging & Grids -> Setup Polygon Montage, in the dialog window:
 - c. Check "using View parameters in Low Dose mode"
 - d. Check “Move stage instead shift images”
 - e. Uncheck “ask about making map after each montage”

Click “OK” to enter File Properties dialog, Click “OK again to define montage filename and save montage file.

- Add flag "A" to all the interested mesh point items

- Navigator menu -> Acquire At Points ... -> Check "Eucentric Rough" in Pre-action and "Acquire Image/Montage Map" in main action
- When finished, the MMM maps should be added to Navigator windows. You perhaps can close the montage in File menu now.

Draw Grids Points for Each Mesh (staff&user job)

For each of the MMM map, do the following steps to add group points.

- add a polygon item to exclude bad area
- add 5 point items to define grid geometry (L shape, three points in a row)
- make any of the 5 items in the group is currently selected
- Navigator menu -> Montaging & Grids -> Set Group Size (10um is a good start for 1.2/1.3 grid)
- Navigator menu -> Montaging & Grids -> Check "Divide point into Groups"
- Navigator menu -> Montaging & Grids -> Add Grid Points -> give polygon item number -> Flag "A" for all

Test Main Script to Run (staff job)

Let's load the script "LD_MR" or "LD_group_MR" to script editor.

These scripts call several functions in "MyFuncs". The script that contains all the functions "MyFuncs" must be also loaded in one of the script buffers/editors.

This is a good time to test run this script on one of the point items in navigator windows, to make sure it runs fine.

Final Checking (staff&user job)

Now we should check to make sure all the conditions are good for batch data collections for hours and days.

- Low Dose beams lined up for all the modes (area is the term SerialEM uses)
- Record beam has proper intensity
- Objective aperture is inserted and centered
- Objective Stigmation is good
- Thon ring with R beam on carbon area shows good scope condition
- Ensure that dose rate, total exposure time, frame time, binning, output file options, frame saving folder etc. are all-good and are recorded.

Run it!

Navigator -> Acquire at Points... -> Run Script "LD_MR" (script 21) in Main action (no pre-action) -> GO.

Macros

Script5 MyFuncs
Script21 LD_MR
Script22 Screen_MR
Script23 LD_group_MR

Appendix I

How to setup Low Dose in SerialEM

In SerialEM (SEM), choose K3 for all camera modes (V/F/T/R). Go to saved position in FEI TUI. Add current position to stage in SEM by AddStagePos in navigator window and label it as "LowDoseSetupinSEM". Setup Low Dose (LD) in SEM in the following order or load LD settings from previous saved settings in Settings > Open. Eucentricity (Task > Eucentricity Rough) and focus must be roughly set at medium mag (~15-20kx) before LD setup.

Record/Preview: turn on "continuous update mag and beam", [28500x (1.08 A/pix) or 37000x (0.88A/pix), nP, bin 1, ~ 15 e/unpix/s. spot size 5-6, total dose 60-90 e/A2, 0.04-0.06 s/frame]. Center beam with Direct Alignment, then use spot size and intensity to choose proper dose rate and check it out in Preview, make sure the beam size is not too large to cover neighboring holes and not too small to show Fresnel fringe in images. Preview should be the same as Record except bin 4 and short exposure time (0.2s). check "Keep Focus and Trial Identical", Copy current area mag & beam to "F".

View: turn on "continuous update mag and beam", [2500x, mP, spot 9, IA ~15 um, ~2.4 e/unpix/s, 0.2s, C2 aperture 70um, obj aperture 70um, bin4 for K3, -200 um defocus offset]. The dose of each image is most important (it need to be as low as possible to protect samples and need to be high enough to give good image contrast, which can also be increased by defocus offset and obj aperture). Choose mag to show several holes in image and should be in the same range with record (2500x is the lowest mag for SA range).

Set proper offsets for View mag, first defocus offset and then image shift (IS) offset: do IS offset in view in LD (center one feature in Record, switch to View and right mouse drag centered feature in Record mode to the center of the screen in View image, click **set** to setup IS offset in View mode) to match the center in record, **check additional beam shift in LD**, center beam with track ball, use spot size, exposure time, and intensity to choose proper dose.

Note for View mode:

- For gold support film, spot 8, IA 80 um, 2500x, bin 4 for k3, -200 um defocus offset, 0.4s. Adapted (target dose per tile is < 0.01e/A2).
- For Carbon support film, Spot 11, IA 20 um (2-3 times of the K2 size), 2500x, bin 4, -150 um, 0.3-0.5s. result dose rate 2.5 e/unpix/s (Not tested yet. target dose per tile is < 0.02e/A2). This is adapted from established case (1700x, spot 11, bin 4, IA ~20 um, 0.5s, 7x7 to 15x15) from other EM facility.

Trial/Focus: turn on "continuous update mag and beam",[same as Record]. Make sure turn on "keeps Focus and Trial identical". Focus area=half; Trial area =Full. (The beam size should slightly smaller than camera area so the edge of the beam can be seen on T image. This is needed for "CenterBeamFromImage" routine to work nicely.)

Note for LD:

- a. Check “continuous update mag and beam” when planning to change beam intensity and/or size.**
- b. “Additional beam shift” must be checked when doing beam centering in View and Focus/Trial, use track ball for beam shifting.**
- c. “Additional beam shift” must not be checked when doing beam centering in Record.**