

Arctica data acquisition using SerialEM

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Starting your session

1. Pick a magnification to use for your experiment. Most common are 63kx and 79kx, with 100kx used for especially small or tricky samples. Review the [list of imaging conditions](#) (last page here).
2. Enable the microscope in [FOM](#).
3. Fill out the paper logbook.
4. Open SerialEM if it is not running:
 - a. Start “SEMserver” application on the microscope computer (pinned to the start bar).
 - b. Open SerialEM from the start bar or desktop of the Gatan PC. If not visible, open the Imaging States window from “Navigator”->“Open Imaging States”.
5. If you’ve just loaded samples, wait for all temperatures $<-165^{\circ}\text{C}$, then do the inventory. You can concurrently take gains/GIF tune with the inventory, just don’t close the column valves.

Preparing the K3 and GIF

1. Find a hole in the sample, or do the gain ref and tuning without a grid loaded on the stage.
2. Open the column valves (you’ll have to do this every time you load a new grid).
3. Take counted gain reference:
 - a. Insert 150um C2. In Imaging States, switch into 63kx “Counted gain ref” state. Magnification does not affect the gain reference.
 - b. Click on **Eucentric focus** on the microscope panel
 - c. Open Digital Micrograph. At the right side, click “1x” binning and “Counted”, then click “View”. You may have to wait for the K3 to insert. Make sure nothing is blocking the beam. Stop the view.
 - d. Go to “Camera” - “Prepare gain reference”
 - e. Skip Linear mode, but collect for Counted.
 - f. Use all the default settings for the gain reference (leave boxes unchecked).
 - g. Verify that the beam intensity is 20 e/pix/s. If it reads 0 contact a manager! Click OK and wait for gain reference to complete (~5 minutes).
4. Quick Tune the GIF:
 - a. Select the “Linear” button in the Technique manager window and switch to 1x binning.
 - b. In SerialEM, select the imaging state for your desired magnification labeled “GIF tune” (they are saved for 63,79,100kx).
 - c. In the Filter Control window in Serial EM, click “Clear Adjustment”.

- d. Click “View” in Digital Micrograph and verify that the dose rate is between 200-300 e/pix/s and nothing is blocking the beam.
- e. At the left side of the UI (under the microscope), expand the “Tune GIF” menu, click “Tune GIF” and select “Quick Tune”.
- f. Wait for tuning to finish (5 minutes). Contact a manager if there are any errors.
- g. Toggle back to counted mode, make sure that “View” is stopped in Digital Micrograph and minimize the program.

Beam Alignments

1. Find an intact square of film on the cross-grating or carbon film on a sample grid. Switch to 690x/other square mag in SerialEM imaging states.
2. Insert the 50 um C2 aperture.
3. Go to eucentric height using “Tasks” - “Eucentricity” - “Rough Eucentricity”.
4. Switch to your desired Low Dose Record imaging state. In “Low Dose Control” window click “Rec” button to switch the beam to record mode.
5. Lower the fluscreen (R1 button) and click “Eucentric Focus” on the microscope right hand panel.
6. In the microscope UI, go the the “Align” tab. Condense the beam using the intensity knob to the smallest spot. Do the following alignments with the beam condensed:
 - a. Center C2 aperture
 - You can either use the direct alignment or you can use Katie’s method. For Katie’s method, first center the beam in the green circle (GIF entrance) using beam shift. Then expand the beam clockwise from crossover until it is about the same size as the GIF entrance and then click ‘adjust’ on the Condenser 2 in the ‘apertures’ tab and adjust the aperture until the beam is centered with respect to the GIF entrance.
 - b. nP Beam tilt pp X and Y
 - c. Beam shift, click “done” when finished.
7. Set parallel illumination conditions:
 - a. Click “Record” again in SerialEM Low Dose Control window to get close to parallel.
 - b. Check the box next to “Continuous update” in the Low Dose window so SerialEM remembers the new intensity value.
 - c. Click into diffraction mode with “Diffraction” button on the right hand panel.
 - d. Insert an objective aperture, usually 100 um. **Adjust the focus knob until the edges of the aperture look sharp using the below steps.**
 - i. Use a focus step 4 or 5 to easily view changes in the aperture focus.
 - ii. Overlap the edge of the aperture with the central spot of the diffraction pattern. Look for a small bit of intensity “bleed” outside the aperture from the central spot.
 - e. Center the objective aperture relative to the diffraction pattern (not the screen). Use the 100 um objective aperture for single particle imaging. Smaller apertures give increased contrast at the expense of resolution (cutoff for 70um is around 2.4 Å).

- f. **Finely focus the diffraction pattern using the intensity knob.** It can help to look at the FFT (click “FFT” button below the center of the FluCam image).
 - g. Click out of diffraction mode (right hand panel).
 - h. Uncheck “Continuous update” in the Low Dose Control window.
 - i. In the Imaging States window, select your Record mag and click “Update” to use the new intensity for your Record images.
8. Use beam shift direct alignment (back in TEM UI) without condensing beam to center the record-mode beam on the camera (green circle).
9. Tune focus and objective stig (back in SerialEM):
 - a. Set defocus target (Focus/Tune -> Set Target) to -0.5 μm . Run autofocus.
 - b. Objective astigmatism: Focus/Tune -> Correct Astigmatism by CTF
10. Coma-Free alignment: Focus/Tune -> Coma-free alignment by CTF.
11. Repeat Focus/Tune -> Correct Astigmatism by CTF.
12. Calibrate coma vs image shift for multishot imaging:
 - a. Go to “Calibration” -> “Focus and Tuning” and run “Coma vs. Image Shift”.
 - b. Use a beam shift corresponding to the largest shift you expect to use for multishot: (For 1.2/1.3, 3x3 use 3.5 μm , 5x5 use 7 μm , 2/2s 3x3 use 6 μm).
 - c. If this fails, make sure you are away from a grid bar or look for for a flatter area - maybe a new grid square. Make sure to redo eucentric height and focus before re-trying.
13. Copy the Record beam settings to Focus and Trial areas using the buttons at the bottom of the Low Dose Control menu: “Copy current area settings to”
14. Save your settings under “Settings” -> “Save As...” to your data folder. **Do not overwrite the default settings file.**

Measure dose for Record beam

1. Find an area with no film. Go there and take a Record image of at least 1s. Note the value in green at the top of the image in Serial EM (something like 25 e/ubpix/s). This is your dose rate per physical K3 pixel. Convert this to electrons per square Angstrom per second by dividing by the **1x** pixel size squared. You can open the spreadsheet in Excel on the K3 computer to help with this calculation and find updated pixel sizes.
2. Decide what total dose per area to use, and determine the acquire time by dividing total dose by the dose rate. Also choose how many frames to use. A usual starting point is 50 $\text{e}/\text{\AA}^2$ total, with 50 frames - if you change things dose should probably go downward and frames upward (recently tested 20 $\text{e}/\text{\AA}^2$ and 50 frames with good results). Divide the total time by the number of frames to get the frame time (use the spreadsheet).
3. You can save a copy of the spreadsheet to your data folder if desired by going to “File” - “Save As” - “Browse”
4. Select ‘Setup’ under Camera & Script Controls in SerialEM.
5. In Record settings enter your calculated exposure time and frame time - see screenshots below:

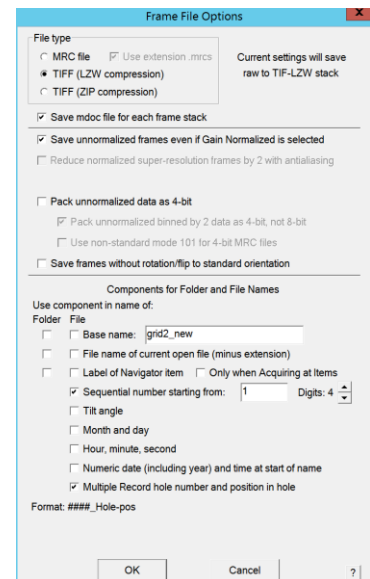
0.5x binning:



1x binning:



5. As in the screenshot, select “Unnormalized” under “Processing”. This will further reduce the file sizes. Serial EM automatically copies the gain reference to your data directory in dm4 format for 0.5x-binned data or mrc for 1x-binned. If needed, convert this to mrc using sbgird/imod program [dm2mrc](#).
6. Click “Set File Options” and set the filetype and name options. Use Tiff LZW. Suggested naming is sequential number plus multishot suffix. Typical options are at right.
7. Click “Set Folder” and tell it where you want to save micrographs. Make sure you’re saving to your own folder on the X drive - this drive is where images will save the fastest.
8. **You should write down & report in publication:**
 - a. Pixel size (for 0.5x or 1x binning) & mag
 - b. Total dose or frame dose
 - c. Number of frames
 - d. Electrons per pixel per second over vacuum
 - e. Objective aperture size
 - f. Slit width (20 eV unless you have changed it)
 - g. Multiple record scheme (3x3, 5x5 etc)
 - h. Voltage (always 200), spherical aberration (always 2.7)
9. In the Imaging States window, select your Record state and click “Update” to save the new exposure parameters when going in and out of low dose. **Save your settings file again.**



Setting up data acquisition in SerialEM

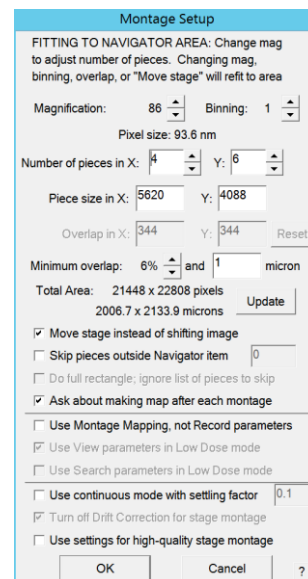
Load your specimen in the Autoloader menu of the Microscope UI. The microscope will automatically retract the objective aperture and close the column valves. Wait for the loading process to complete (a few minutes). The microscope UI will display “Cartridge loaded” when loading is finished. Open the column valves to proceed.

Take an Atlas/Grid Map

Optional: [Atlas several grids while you get coffee using the mapgrids script](#) (notes at the end of this document)

To take a single Atlas:

1. Choose the 86x/atlas imaging state. Put in the 150 um C2 aperture.
2. Go to eucentric height using “Tasks”->”Eucentric Rough”
3. Open a Navigator by selecting “Navigator” -> “Open”.
4. Select “Navigator” -> “Montaging & Grids” -> “Setup Full Montage”. The settings should match the screenshot at right.
5. Click OK. In the next window set file details, then select a folder and filename for the atlas.
6. Press “Start” under brown Montage Control window (or use Ctrl-M).
7. When the montage is complete, click “Yes” to save it as a map. It should now show up as the first entry in your navigator.
8. Save your navigator by going to Navigator -> Save.



Montage Setup

FITTING TO NAVIGATOR AREA: Change mag to adjust number of pieces. Changing mag, binning, overlap, or "Move stage" will refit to area

Magnification: 86 Binning: 1

Pixel size: 93.6 nm

Number of pieces in X: 4 Y: 6

Piece size in X: 5620 Y: 4088

Overlap in X: 344 Y: 344 Reset

Minimum overlap: 6% and 1 micron

Total Area: 21448 x 22808 pixels
2006.7 x 2133.9 microns Update

☒ Move stage instead of shifting image

☐ Skip pieces outside Navigator item 0

☐ Do full rectangle: ignore list of pieces to skip

☒ Ask about making map after each montage

☐ Use Montage Mapping, not Record parameters

☒ Use View parameters in Low Dose mode

☐ Use Search parameters in Low Dose mode

☐ Use continuous mode with setting factor 0.1

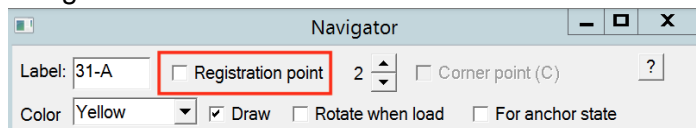
☒ Turn off Drift Correction for stage montage

☐ Use settings for high-quality stage montage

OK Cancel ?

Optional: If you reloaded a grid that you already have an atlas from (maybe from the mapgrids script), you can transform the atlas to account for any shift/rotation of the grid that may have occurred from unloading/reloading ([source](#)):

1. Add at least two points (on something remarkable, like a crack or large piece of ice) far from each other on the original Atlas.
2. Mark each of these points as Registration Points using the button at the top left of the navigator window:



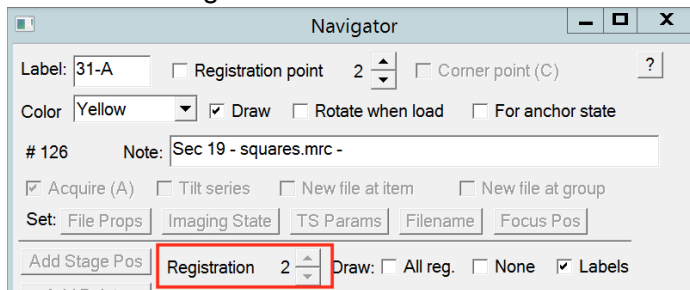
Navigator

Label: 31-A ☒ Registration point 2 ☐ Corner point (C) ?

Color: Yellow ☒ Draw ☐ Rotate when load ☐ For anchor state

They should now have an indication of which registration and which point they are in the “Reg.” column (e.g. 1R1, 1R2)

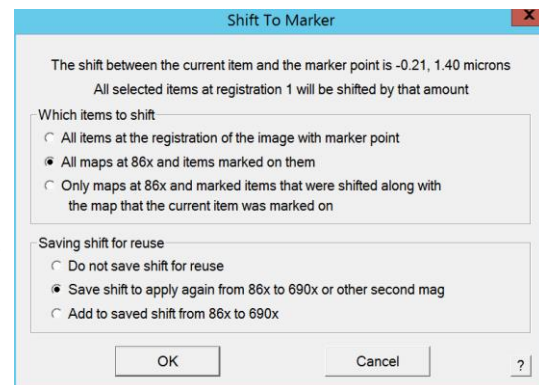
3. Increase the registration from 1 to 2:



4. At 86x, visit each navigation point from the original navigator: select the old navigation point, click “Go to XYZ”, take a View image. Locate the feature in the new image, and add it as a point (“add marker” or “add points”) in the new image. Again, select “Registration point” as before, and make sure the number matches the point in the previous registration (1R1 should match 2R1 and so on).
5. After repeating for all registration points, in Navigator menu select “Transform Items” to transform the atlas and any old points from the old registration to the new one. You can now proceed normally.

Square Selection and Overviews

1. Choose a magnification to use for square mapping (690, 540, or 410x). Click “View” and center one square. Check that the mag you choose shows the entire area of the thinnest squares on your grid, otherwise zoom out.
2. Insert the 50um C2 aperture, 100um OBJ aperture, and click “Autofocus”.
3. If your atlas was acquired at Z-height of zero (if you collected it using the script or didn't set eucentric before), run “Tasks” - “Eucentricity” - “Rough Eucentricity”, then highlight the Atlas in the navigator and click “Update Z”.
4. Adjust the register between atlas and square overview magnifications (recommended to do near the center of the grid):
 - a. Open your Atlas (double click on it in the Navigator). Add a navigator point on a notable feature by clicking on it and clicking “Add Marker” in the navigator window. It can be useful to open your atlas in a floating window using “Window” - “New Window” to remember where this marker is.
 - b. Click “Go to XYZ” to move the stage close to the feature.
 - c. Click “View” and locate the same feature in the new image. Click on it to center the green marker on the feature.
 - d. Make sure the navigator point selected is the one you just added on your feature.
 - e. In the “Navigator” menu at the top, select “Shift to Marker”. Under “Which items to shift” select “All maps at 86x and items marked on them”. Under “Saving shift for reuse” select “Save shift to apply again from 86x to 690x” (see screenshot).



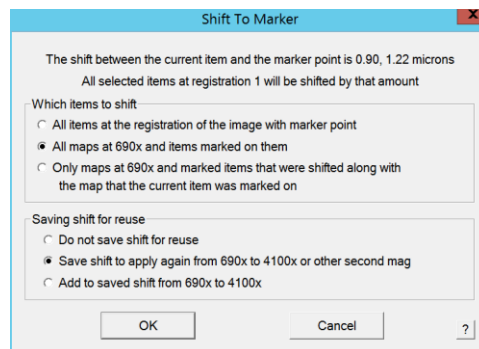
- f. Click OK.
 - g. Check that this was successful by clicking “Go to XYZ” again and then “View”. The feature and marker should both be centered in this new image. If not, repeat the procedure.
5. Once the registration has been fixed, select all of the desirable squares for the data collection session:
 - a. Open the Atlas
 - b. Select ‘Add Points’ and click on the **center** of each square that appears intact and has good ice thickness.
 - c. Choose squares - 2-3 for a quick dataset, 20 or more if you want to collect overnight.
 - d. Select the “Collapse” checkbox in the navigator window, then select “Acquire (A)” to mark all the new squares for acquisition.
 - e. Open a file for the square maps: Make sure you’re at the square mag, then go to “File” - “Open New”. Create an mrc file for the square maps (like squares.mrc).
6. Save a map for each square:
 - a. Select “Navigator” -> “Acquire at Items”.
 - b. Select “Mapping” at the top left.
 - c. Set parameters as follows, or click “Read in Parameters” and load Documents\AcquireAtItems\square_maps.txt

- d. A square overview image will be taken at each point and saved as a map entry in the Navigator window. This only takes a few seconds per square.
7. **Save your Navigator.**
8. Inspect your squares - examine each square overview image by double clicking in the Navigator window. Rate each one by the criteria: good – blue (default), ok – yellow, bad

- red, change the 'Color' value accordingly. This will help you prioritize good areas when setting up points for data collection.
- 9. If you find you don't have enough good squares, you can repeat this process with a new set of points.

Fixing the Register for Low Dose mode

1. Pick one square map to use for focus position, hole template, multi-shot setup. Go to that map using "Go to XYZ" in the navigator. Set Eucentric height using "Tasks - Eccentricity - Rough Eucentricity" - then click "Update Z" in the Navigator window with the map selected.
2. **New:** Choose a Low Dose View mag. There is a new list of View settings in the imaging states window. Hole finding works better at higher mag than our usual 4100x. First, switch into the imaging state for your Record mag to go into Low Dose mode. Then switch into the correct imaging state for View depending on your grid:
 - a. For 1.2 um holes, use 11.5k
 - b. For 2 um holes, use 5.6k
 - c. In general, when you take a View image with a hole centered you want to see all of one hole and only a little of the neighbors.
3. The stage should be at your last square map. Double click the map in your navigator, then open it in a new window using "Window" - "New Window".
4. Open the column valves if they are closed and click "View". Look for a notable feature in your view image (maybe a big piece of ice). If there isn't one in the field of view, switch to continuous view mode and drive the stage around with the joystick until you find a feature. Stop the View.
5. Look at your square map and locate the feature. Click on it, then click "Add Marker" in the Navigator window to place a point on it. Uncheck "Collapse" in the Navigator window if it is checked.
6. Locate the same feature in your View image. Click on it to place the green marker.
7. Go to "Navigator" - "Shift to Marker". Again, select the middle option for both (see screenshot at right) and click OK.
8. Click "Go to XYZ" and View again. If the feature and marker are not both centered, repeat the procedure.
9. **Important:** Next align the Record mag with the View mag as follows:
 - a. Center a feature in the View image. Take a Preview image.
 - b. Center the feature in the Preview image with stage shift (hold shift, right-click, and drag) or by moving the stage with the joystick.
 - c. Take another Preview image.
 - d. Repeat steps 6-7 until the feature remains centered in the Preview image.



- e. Once centered take a View image. Center the feature with image shift (right-click and drag), then select 'Set' under 'Offsets for View' in the Low Dose Control window.
 - f. Repeat this until the feature remains centered.
10. Select your Low Dose View state in the Imaging States window and click "Update".

Medium Magnification Montage of Collection Squares

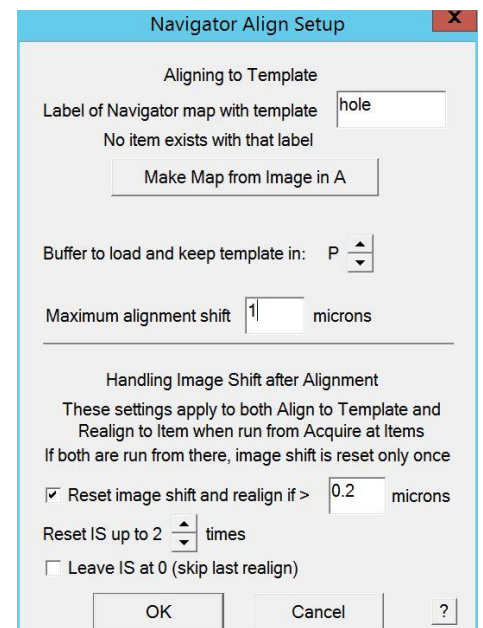
Medium mag montages help to very accurately move the stage to a hole. They're rarely required so [these instructions have been moved to the end](#). Usually just using the hole template is sufficient. Try using them if you find that SerialEM is having difficulty moving to the correct hole. There is a new lower mag imaging state called "uP LD View" that can be used for speeding up medium mag mapping - ask a manager for help using this.

Setting Focus Position

1. Take a View image over a hole. If you can't see any neighboring holes, temporarily use a lower-mag low-dose View state (4100x)
2. Carefully center the hole with image shift (Right-click and drag). Take another view image. Repeat until the hole is centered in the image.
3. Define the focus area:
 - a. In the "Low Dose Control" window, under "Define position of area" select Focus.
 - b. Click to place the yellow focus position box onto carbon neighboring your hole.
 - c. If you are using stage tilt, you can force the Focus area to be on the tilt axis by unchecking "Rotate interarea axis" and setting value to 0 - this will ensure the height of the focus area is the same as the record area.
4. If you reduced the View mag, restore it to the original mag you were using (5600, 11500 etc) by double-clicking the corresponding imaging state.

Setting the Hole Template

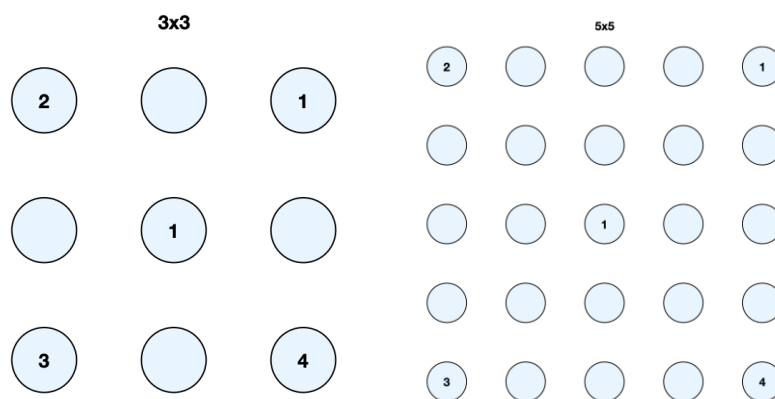
1. Look for a clean hole of average ice thickness - try to find a spot with no ice or dark regions around the hole. The image you just took to set the Focus position might work well.
2. Carefully center the hole and take a View image. I recommend temporarily increasing the binning to 8 and time of 0.2 s.
3. Crop the image to show only this hole:
 - a. Hold "Ctrl" + "Shift" and draw a box slightly larger than the hole. Make sure the hole is centered within the box, if not carefully re-draw it.
 - b. Go to "Process" - "Crop Image".
4. Go to the "Navigator" Menu and select "Setup Align/Realign" (near the bottom).
5. Verify that Buffer P is selected.



- Click "Make Map from Image in A". Save the file (template.mrc). This will save a new map in your Navigator with the hole template. I usually give it the label "hole".
- Set the Maximum alignment shift to 1 μm for 1.2/1.3 or 2 μm for 2/2 (suggested is $\frac{1}{2}$ the hole spacing). Leave the other options as in the screenshot.
- Under "Buffer Control" box at left, click on "P" under "Copy Active Image to buffer". This stores the hole template in buffer P.

Set up Multiple Records

- On one of your square maps, add 5 navigator points in two groups: one point in the center of your pattern (Click "Add points", click center, click "Stop Adding") and one group containing the four corners - (Click "Add points", click each corner going clockwise or counterclockwise, click "Stop adding"). See diagrams below:



- Move the stage to the center hole by clicking on its navigator point and selecting "Go to XYZ"
- Go to Low Dose View mode.
- In the Low Dose panel, change the defocus offset for view mode from -100 to 0. If the procedure is done at high defocus, the saved image shifts will not be accurate.
- Take a View image. If necessary (if the image is blocked) - remove the slit for view mode: Check "continuous update" in Low Dose Control with beam in View mode. In "Filter control" box, uncheck "Slit in". You should no longer need to remove the OBJ aperture with new higher view mags.
- Note, this image usually looks ugly, (beam off center, brightness too high), but as long as you can see the edges of the holes these settings will work.
- Go to "Navigator" -> "Montaging and Grids" -> "Set Multi-Shot Parameters"
- Uncheck "Do multiple records within each hole". Make sure "Do Records in multiple holes" is checked. Select "Regular pattern of holes: n by n". Set the values to 3x3/5x5, whatever you are planning to use.
 - Actually, "Do multiple records within each hole" can work for 2/2 grids - one image at the top of the hole and one at the bottom. In that case, use two images per hole, offset by 0.65 μm to 0.8 (recommend 0.7), with no image at center.
- Save the values for the image shift at each corner:

- a. Carefully center the hole at the center of your pattern.
- b. Click “For Corners of Regular Pattern” to begin saving image shifts at the four corners.
- c. Select the first corner in your Navigator and click “IS to Nav Pt”. This should bring the first point close to the center using image shift and automatically highlight the next point in the pattern. Click “View” and carefully center this first hole using right click and drag. When the hole is centered, click “Save image shift”.
- d. Press “IS to Nav Pt” again to image shift to the second hole. Repeat c for each of the four holes. When you click “Save image shift” for the fourth corner the process will complete.

10. Set remaining options to match the screenshot at right.

11. Click “OK”

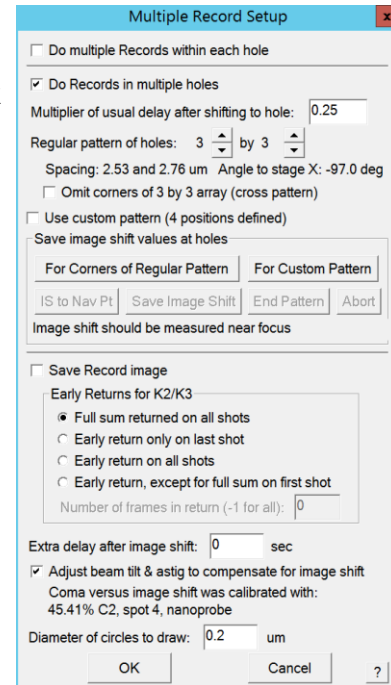
12. Re-insert the objective aperture if you removed it.

13. Change the defocus offset for View back to -100.

14. Re-insert the slit: Click “Slit in” in Filter Control window and uncheck “Continuous update” in LD Control.

15. Take a View image and check that things look normal - it’s common to have to re-run Refine ZLP here after re-inserting slit.

16. Save your settings again (“Settings” - “Save”



Set Z-height for each square and pick points

Notes: If the hole finder does not work well for your sample (not enough contrast), see the instructions for manually adding a grid of holes [at the end](#).

If you can’t find a good set of slider positions, you can first set all the Z-heights like we used to (using script Update Z), then manually run the hole finder for each square map.

1. Calibrate Eucentricity by Focus:
 - a. Take a View image and make sure everything looks good.
 - b. Run “Tasks” - “Eucentricity” - “Fine Eucentricity”.
 - c. Go to “Tasks” - “Eucentricity” - “Calibrate Focus Targets...” (at bottom)
 - d. Under “Calibrate With” click “Current Settings”. Wait for calibration to finish.
2. Set up Hole Finder conditions:
 - a. Load your most average square map by double clicking on it in the Navigator window.
 - b. Go to “Navigator” -> “Montaging and Grids” -> “Find holes in regular grid”.
 - c. Adjust the parameters to match your grid - for example, a 1.2/1.3 grid has 1.2 um hole size and 2.5 um spacing.
 - d. Click “Find holes”. If the settings are good, all the holes in your image should be marked with a pink or blue +. Make sure “Show included holes” and “Show excluded holes” are both checked. If many holes were not found, you should try

- changing the settings for hole finding “Maximum error”, “Filters to try”, and “Edge threshold”. [More information is here](#) (SerialEM help - “Hole Finder Dialogue”).
- e. Pink holes will be kept and blue discarded. Adjust the sliders for “Lower Mean Cutoff”, “Upper Mean Cutoff”, “SD Cutoff” and “Black pixel % cutoff” until only the holes you want to keep are marked in pink.
 - f. Under “Layout of points” select “Zigzag” if you are using multiple records and “Away from focus” if using one record per stage move.
 - g. Do NOT click “Make navigator points”! We will do this momentarily when we run the script.
 - h. Check that the slider settings for the hole finder also work well for some of your other square maps - load a thicker square, or one with empty holes, and see if the holes you want are included/excluded without having to change the slider settings too much. If you find you want to change the sliders between squares, you should pick the points by hand for each map instead of through the script.
3. Reorder your square maps if desired - the points will be imaged in the order you add them here, so you should prioritize your best squares first - if you color coded them earlier, add the greens and blues first, then the yellows.
 4. Prepare your square maps for data acquisition:
 - a. For each good square map, turn on Acquire in the navigator.
 - b. Go to “Navigator” -> “Acquire at items”. Select Mapping and set parameters as follows in the screenshot (next page), or click “Read in Parameters” and load Documents\AcquireAtItems\prepare_squares.txt
 - i. Run script: Prepare Squares updates Z height and runs the hole finder on each map - if you’d rather run the hole finder yourself for each square, see the old instructions at the end of these notes, and choose “Update Z” script instead of “Prepare Squares”
 - ii. Checking “Skip Z moves in initial move and Realign” should save a little bit of time since the stage will stay at the last found eucentric height.
 - iii. Make sure “Eucentricity by Focus” is checked on the right.
 - iv. **Select “Do subset from index” and keep the default indices.**
Otherwise the script will run infinitely as it adds new points marked for acquisition.
 - c. The script will update the Z-height for each square and pick points for acquisition using the parameters you defined in the Hole Finder.
 - d. Click “GO”, this can take a while if you have many squares.

Final adjustments

1. Move to a sacrificial area of film.
2. Click "Rec" (In Go to: section of "Low Dose Control"). Lower the fluscreen.
3. Click into diffraction mode (right hand panel button) and re-center your objective aperture. Go out of diffraction.
4. Usually the beam shift looks wrong here, but I recommend skipping aligning it at this point - it seems to settle back into place after data collection starts.
5. Optional: If it's been a few hours since you aligned and you're using carbon films you can do Focus/Tune-> Correct Astigmatism by CTF.
6. Optional: RefineZLP in Filter Control if it has been a while since the slit has been centered.
7. **In camera setup, make sure "Save Frames" is checked and all other parameters are as you want them.**
8. In Autoloader flap-out (where you run the Inventory) make sure that "Turbo Auto Off" is selected to reduce vibration.

Starting Data Acquisition

1. Go to "Navigator" - "Acquire at Items". Select "Final Data" at top left.

2. Edit the “PreSPA” Script (#6) - specify the minHoles variable - the script will skip any navigator point with fewer than this amount of holes to increase efficiency.
3. Set up as in the screenshot below, or “Read in Parameters” from Documents\AcquireAtItems\take_data.txt.
4. Set your desired defocus range under “Cycle defocus target” on the left.
5. You can tweak other settings/timings by clicking on the action and changing parameters at the bottom right. Some actions have an additional “Setup” menu. The settings selected here mirror the actions taken in our usual data acquisition script. See [“Notes about new Acquire at Items”](#) for more details about each action.
6. Click “GO” to start collection. SerialEM may pop up a warning about ignoring the stage move - as long as you are running “PreSPA” script you can click “Yes” - the PreSPA script will explicitly move the stage for any point that has more than “minHoles” positions.
7. Monitor data collection and make sure everything is working properly. To make any changes, stop collection by pressing “Stop” under “Camera & Script Controls”. Depending on what you were doing when you stopped, you may need to also click “End All”. Make any changes, open the column valves, and restart the script.

Acquire at Items

Parameters for: **Mapping** **Final Data**

Primary Action

☐ Acquire and save image or montage

☒ Make Navigator map

☐ Skip saving to file

☒ Acquire multiple Records **Setup**

☐ Acquire tilt series

☐ Run script **Z beam tilt**

Primary Action-related options

☒ Cycle defocus target in autofocus, drift wait & TS

From **-0.4** to **-1.4** um in **5** steps

☐ Adjust beam tilt & astig for image shift

☐ Do early return with sum of **0** frames

☐ Acquire non-montage images in Low Dose with

☒ Record ☐ View ☐ Search

NO SAVING: "Save Record" not on in multishot

Task-related options

☐ Do hybrid Realign to Item/Align to Template

☒ Relax stage after final movement

☐ Skip Z moves in initial move and Realign

☐ Skip stage move to item if possible

General options

☐ Do subset from index **1** to **307**

☒ No message box when errors occur

☒ Close column valves at end

☐ Retract cameras at end

☐ Send email at end

☐ Hide unselected options

Tasks before or after Primary Action

☐ Manage Dewars/Vacuum Every item **Setup**

☒ Run Script before Action Every item

Script to run: **PreSPA**

☐ Coma-free Alignment Every item **Setup**

☐ Correct Astigmatism Every item **Setup**

☐ Condition Phase Plate Every item **Setup**

☐ Rough Eucentricity Every item

☐ Eucentricity by Focus Every item **Setup**

☐ Cook Specimen Every item **Setup**

☐ Fine Eucentricity Every item

☐ Align to Template Every item **Setup**

☒ Autofocus Every item **Setup**

☒ Wait for Drift Every item **Setup**

☐ Realign to Item Every item **Setup**

Primary Action inserts here

☐ Run Script after Action Every item, after

Script to run: **MyFuncs**

☒ Autocenter Beam Every 60 min, after **Setup**

☒ Refine ZLP Every 240 min, after

☒ Update Dark Reference Every 120 min, after

General controls for task: Cook Specimen

How often to run task

☒ Every **1** items

☐ Only at start of group

☐ Only at end of group

☐ Every **15** minutes

☐ When moved > **40** um

☐ Run after Primary Action

☐ Run at nearest item with ☒ label ☐ note starting with

☐ Hide unused tasks **Restore Default Order**

Position in sequence

Move Up

Move Down

Save Parameters to File **Read in Parameters** **GO** **Postpone** **Cancel** **?**

Before you leave:

Start your data transfer, fill out the grid form for the next user, or unload any grids you aren't collecting on - make sure to check "Leave cartridge on compustage" if you do this. If you unload some grids, make sure to re-load the cassette & inventory (even if empty) so the next user can unload your grid.

Stopping Data Collection

1. Press "Stop" under "Camera & Script Controls", if stopped in the middle of the script also go to "End all".
2. The column valves should close, if you need to take additional images remember to re-open the column valves.
3. If you are finished with the data collection session:
 - a. Save the Navigator file, go to "Navigator" -> "Save"
 - b. Save the Log file, go to "File" -> "Save Log..."
4. Disable the microscope in FOM. If you're not watching when data collection finishes and need a manager to adjust your time, send an email.
5. Let the next user know if you want to save any of your grids. Make a note in the "Arctica Loading" logbook, and leave labeled grid boxes and tube. You can unload your own grids if there is at least a 3 hour window before the next user (to allow loading stations to dry in the oven).
6. If no other user is scheduled directly after you, start a K3 anneal. Go to "Camera" -> "Temperature" in GMS. Type in 50. The settings for the anneal cycle will pop up. Change the time to 10 hours (or the number of hours until the next user minus 2), and click "Start". The camera will automatically cool back to -20 when the cycle is complete.

Appendix

Image Acquisition Parameters

EFTEM Magnification	minimum C2 Aperture	1x pixel size (Å)	0.5x pixel size	Field of view (nm)	Spot size
100 kx	50 um	0.81	0.405	466.56	2
79 kx	50 um	1.04	0.52	599.04	3
63 kx	50 um	1.31	0.655	754.56	4
49 kx	70 um	1.71	0.855	984.96	4

Screening many grids using the Mapgrids Script

Switch to the 86x atlas imaging state, insert the 150um C2 and remove the objective aperture. Set up an atlas as usual using “Navigator” - “Montaging and grids” - “Setup full montage”. Choose a file to save the atlases into (they will all save into the same mrc stack).

Optional: in the Autoloader flap-out in the scope UI (where you run the Inventory), toggle the vacuum selector to “Turbo Always On”. This will make the grid exchange faster during the script. **It is very important that you turn this back to “Turbo Auto Off” after mapping.** If the turbo is running during data collection, it will add vibration to your data.

Go to “Script” - “Edit” - “#3”

Look for the comment #List cassette slots you would like to montage, and list your desired slots in the array in the next line - for example cat = { 1 5 3 } will montage slots 1, 5, and 3 in that order as long as all three slots are occupied.

If you want to montage a grid already on the stage, make sure that the next four lines are uncommented, otherwise comment them out if you don’t want to do this.

Check that no vacuum process is running - in the Autoloader menu, make sure “Load” is not greyed out - if it is, wait until “Load” is clickable. In the SerialEM script editor click “Run”.

If you want to end the script early, clicking the “End Loop” button will allow the current atlas to finish.

Notes about new Acquire at Items dialogue

https://bio3d.colorado.edu/SerialEM/hlp/html/hidd_navacquire.htm

I’ve set up the Acquire at Items for data acquisition to closely mirror the SPA script we used in older versions of SerialEM.

The basic steps are:

1. Checks: Are the dewars filling? This is done in new PreSPA script, and it actually triggers the dewars to fill if there are fewer than 5 minutes on the timer so that filling doesn't start during your multiple records.
2. Checks: How many holes does the current Navigator point have? PreSPA will skip this position if it has fewer than the minHoles variable defined at the start of the script. This is designed to make collection a bit more efficient - if you've picked way too many points to finish during your reserved time, you might as well only visit the points that have 9 holes to collect in (so you spend relatively less time centering & focusing, compared to time spent collecting images).
3. Aligns to the hole template: I am still doing this through the old "AlignToP" script that is run by PreSPA. I'm not sure why, but it seems a little more robust than using "Align to Template" in SerialEM.
4. Autofocus - self-explanatory, hopefully. Focus targets are defined on the left under "Cycle defocus targets"
5. Wait for Drift - like the old SPA script this is configured for 3 Å/s, but you can tweak this if desired by clicking "Setup"
6. Acquire multiple records - this is the Primary Action
7. Various post-actions - Autocenter beam
8. HW dark
9. ZLP

Index of Scripts

Number	Name	Description
01	MyFuncs	List of functions callable within other scripts
02	WaitForRefilling	If dewars are filling, update hardware dark and wait for filling to finish
03	MapGrids	Atlas many grids
04	UpdateZ	Updates Z height of navigator item - use after some Eucentricity type in Acquire at Items
05	Prepare Squares	Updates Z height, loads square map, runs hole finder and combines points for square maps. Use with a Eucentricity pre-action in Acquire at Items
06	PreSPA	Pre-actions for SPA acquisition - exclude points with fewer than minHoles, fill dewars if there's less than 5 minutes until next fill, and center hole for items that aren't skipped. This is a pre-action in Acquire at Items during SPA data acquisition

07	AlignToP_withskipfail	Called by PreSPA - hole template centering which will fail if shift is too big or hole not found in 5 iterations
08	AlignToP	Old hole-centering script - not currently used
09	unused	
10	Functions	EMBL functions file for tomo scripts - not sure any of these are ever actually called
11	FillDewarsTS	Checks if dewars are due to fill in one hour or less - if so fills them. Called as pre-action when running tilt series through Acquire at Items
12		Takes high defocus image after tilt series. Make sure to define directory to save into.
13-19	Unused	Save for more tomo scripts
20	SPA	Old SPA collection script - if you prefer to use this instead of Acquire at Items dialog, you would turn off every checkbox in Acquire at Items and just make this the Primary action.
23	SPA-Kellogg	
24	SPA-Eshan	
25-29	unused	
30	CheckShutter	Checks if automatic beam blanking is working - takes View, changes to Record beam, unblanks without taking image, 30s wait, takes another View image - if you see no burn mark then the shutter is working
31	IS-Pixel	Used for doing pixel size calibrations during SerialEM setup
50		Thickness measurement script from: https://doi.org/10.1107/S205979832100334X
60	RecordMany	I think this is Mike C's it just takes a bunch of Records in a row.

Medium Magnification Montages

1. Retract the objective aperture if it is in (it blocks some of the "View" camera area).
2. Open a representative good square overview map with fairly thin ice.
3. Select "Add Polygon" and draw a polygon at the boundary of the collectible area. Be a little permissive here to get more of the area of squares that may not be perfectly centered.

4. Go to "Navigator" -> "Montaging & Grids" -> "Setup Polygon Montage"
5. Make sure View mode is selected for collecting the montage (recommend uP view - 3000x). Select appropriate montage parameters and select filename for montage maps.
6. Toggle "Acquire" at each square overview map in the Navigator where you want to create a montage map.
7. Go to "Navigator" -> "Acquire at Points". For the Primary Task, select "Acquire map image or montage". If you plan to leave the room during montage collection (it takes ~1 hour for 15 squares) check "Close column valves at end". Click "Go".
8. A montage map made of View images (4100x), will be created for each square. This allows precise placement of acquisition points. The montage map images can also be used as reference images for re-centering at each acquisition point during scripted data collection.

Manual point-picking method

1. Open one of your medium-mag montages (or square overview maps if no medium mag) by double-clicking it in the Navigator window. Start with the best areas because the points will acquire in the order you pick unless you manually reorder them at the end.
2. If most of the holes in the square look suitable, you can have SerialEM fill in a grid with their locations:
 - a. Select 'Add Polygon'. Left click to add vertices of the polygon and surround the area of good ice on the grid square. Select 'Stop Adding' to close the polygon.
 - b. Select 'Add Points'. Left click on the precise center of 5 holes, starting at a corner, with one in each direction immediately adjacent to the corner and one further down the line of holes in each direction. This establishes the geometry of the holes.
 - c. Go to Navigator Montaging & Grids Add Grid of Points. Enter the number of the polygon (this can be found on the overview map image or the Navigator window) For the next prompt, select 'Away from Focus Area' to select points away from the Focus area to prevent beam damage on exposure areas prior to imaging. For the last prompt, select 'Yes' to turn on Acquire for every point.
 - d. A grid of points will appear on the square overview, which should be centered well over all of the holes within the polygon. If this is not the case, select the points in the Navigator window and select 'Delete Item' to remove them. Then, readjust the centering of the 5 points used for the geometry of the holes and repeat step c.
 - e. Select 'Edit Mode' in the Navigator window. This allows you to select (left-click), add (middle-click), remove (select then press Backspace), and move (select then right-click) points. Remove any bad positions (covered in crystal ice, empty, or blocked), add holes that were excluded, and reposition points as necessary.
2. If you are picking around empty areas, thick areas, or ice chunks and there isn't a large continuous area of holes it may be faster to add points one by one:
 - a. Choose "Add points" in the navigator. This will add the points in a group (so it's easier to toggle "Acquire" on all of them).

- b. Remember where you defined your focus area. Click at the center of the holes you want to use, moving away from the focus region so as to not damage an area before recording in it. (For example, if your focus area is up and to the left, start at the top-left corner of the square and pick down and to the right).
 - c. Click “Stop Adding”, “Collapse Groups” and check the Acquire box.
3. Repeat this procedure for each of the good square overviews. Start with the blue ones and then add the yellow ones, as necessary, to give yourself enough points to run through the entire data collection session, or until you will add more points at a later time.

Note: The data collection will run from top to bottom on the Navigator window, so you can rearrange the points by left-click and dragging to change the collection order as desired.

Adding Additional Points Using Dummy SerialEM

On the desktop of the Gatan PC there is another SerialEM shortcut titled “DUMMY”. This version of the software doesn’t connect to the microscope, but will allow you to edit your navigator, so you can pick more points while the collection script runs.

1. Save your navigator in the main version of SerialEM.
2. Open the dummy version from the desktop of the Gatan PC.
3. Load your navigator by going to “Navigator” -> “Read and Open”, twice (for some reason the first time it opens a blank navigator; the second time it will prompt for the file you want to open).
4. Save the navigator with a new name (“Navigator” -> “Save As”)
5. Load your settings file “Settings” -> “Open”
6. Add points as usual
7. Turn on “Acquire” for all your new points.
8. Save this navigator.
9. Close Dummy SerialEM.
10. Stop data acquisition in the main version of the software (see step 5 in the previous section).
11. Go to “Navigator” -> “Merge File” and select the new file. The new points will now be included in your navigator.

Using Hole Finder map-by-map

1. Open your first square map in the Navigator.
2. Go to “Navigator” -> “Montaging and Grids” -> “Find holes in regular Grid”.
3. Adjust the parameters to match your grid - for example, a 1.2/1.3 grid has 1.2 um hole size and 2.5 um spacing.
4. Click “Find holes”. If the settings are good, all the holes in your image should be marked with a pink or blue +. Make sure “Show included holes” and “Show excluded holes” are both checked. If many holes were not found, you should try changing the settings for

hole finding “Maximum error”, “Filters to try”, and “Edge threshold”. [More information is here](#) (SerialEM help - “Hole Finder Dialogue”).

5. Pink holes will be kept and blue discarded. Adjust the sliders for “Lower Mean Cutoff”, “Upper Mean Cutoff”, “SD Cutoff” and “Black pixel % cutoff” until only the holes you want to keep are marked in pink.
6. Under “Layout of points” select “Zigzag” if you are using multiple records and “Away from focus” if using one record per stage move.
7. Click “Make Navigator Points” and click “Yes” if asked to turn on Acquire for the new points.
8. If you are using multiple records, use the hole combiner to merge the found holes into points for the center of your multiple record pattern:
 - a. Go to “Navigator”->“Montaging and Grids”->“Combine holes for Multi-shots”
 - b. Select “On image in current buffer”
 - c. Click “Combine Points”. The points on your image will be reduced to show only the centers of your multi-shot group. You may notice some points appear to be too close to the edge or each other, but SerialEM will remember to take fewer images at these points - in other words it will not record in the same hole twice, even if it looks like your patterns overlap.
9. Repeat this for all your square maps in the order you want to acquire them or follow the directions below to automatically pick points for all maps:

Data collection with stage tilt

5/20/22

Don't try this on carbon films.

Note -you cannot move/insert/retract objective aperture with the stage tilted - any time you need to adjust it must tilt to zero, move/insert the aperture, then tilt back up.

1. Atlas as usual at 0° (eucentric height first)
2. Pick squares, set Z-heights, and map:
 - a. Switch to LD View & set register with Atlas mag
 - b. Calibrate eucentricity by focus
 - c. Acquire at items - run Update Z script & Eccentricity by Focus at each point (no mapping yet)
 - d. Switch to square mag
 - e. Set register with Atlas mag
 - f. OBJ in
 - g. Tilt to desired angle
 - h. Take map of each square
 - i. Open file for saving
 - ii. Acquire at items: "Acquire map image or montage" only. Make new map checked.
3. The rest of collection set up is mostly normal but done with the stage tilted:
 - a. Go back to LD state
 - b. Set register from square mag to View mag
 - c. Set image shift from View mag to Record mag
 - d. Set focus position (try to keep on tilt axis or remember there will be a little offset between what you ask for and what the actual defocus is)
 - e. Set up multishot
 - f. Set hole template
 - g. Pick points: some tips for the hole finder:
 - i. Try a larger maximum error since the holes won't be regularly spaced on the tilted maps - go to 0.2 or even 0.5. After the first successful round of hole picking use the "Set Size/Spacing" button to get a more accurate average size and spacing for the tilted maps.
 - ii. Should be able to pick points as normal.
 - h. Run the acquisition as usual with the stage tilted. Be sure to monitor your defocus range (process on the fly) and tweak as necessary if your focus area is not on the tilt axis.