Batch tomography using SerialEM

Version 2.1 - 5/27/22 - Updated for SerialEM 4.0

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°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. 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.
   c. Go to "Camera" - "Prepare gain reference"
   d. Skip Linear mode, but collect for Counted.
   e. **Different for tomography:** Consider the dose to use for your gain reference. For thick samples, the beam hitting the detector can be much less than usual when going to high tilt. We often use 10-15 e/pix/s for the gain reference for tomography. If you're unsure, I recommend starting at 12. If the gain dose is too far from the acquisition dose, you will see a "swoopy" pattern in the images at high tilt. To change the desired dose rate:
      i. Check the "Expert mode" box
      ii. Change the dose rate to 12 e/pix/s (or whatever you are using)
      iii. Change the total counts from 7000 to 5000 (to save some time).
      iv. Proceed.

     f.   Adjust the intensity knob to set the desired beam intensity. If it reads 0 contact a manager! Click OK and wait for gain reference to complete (~5 minutes).

     g. **Different for tomography:** Put the gain reference settings back to default:
   - i.   Go again to "Camera" -> "Prepare gain reference"
   - ii. Skip Linear, collect for Counted
   - iii. Check "Expert" again and return the settings to 20 e/p/s and 7000 counts. Proceed
   - iv. When prompted to set the beam intensity, click "cancel".

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 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 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
   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:**

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. Remove the objective aperture for now.
            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. Focus the image: Set defocus target (Focus/Tune -> Set Target) to -0.5 um. Run autofocus.
    10. Coma-Free alignment: Focus/Tune -> Coma-free alignment by CTF.
    11. Insert and center your objective aperture: put the Fluscreen back in and click "Diffraction". Insert the **70 um** objective aperture and center relative to the diffraction pattern. Note: the 70um aperture is typical for cryo-tomography with a resolution cutoff around 2.4 Å - the increased contrast for tomography is worth the resolution tradeoff.
    12. Refocus the image: Set defocus target (Focus/Tune -> Set Target) to -0.5 um. Run autofocus.
    13. Focus/Tune -> Correct Astigmatism by CTF.
    14. If you are taking tilt series at 63kx or 79kx, 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"
            a. If you are using a higher mag, it usually works better to do tracking at 63kx. For this, choose the last person's 63kx settings and copy these to **Trial**. You can still make Focus match your Record setting.
    15. 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.
    2. In the pinned Excel sheet, go to the second sheet/tab. Input the following:
            a. Tilt scheme: (start, end, step) - usual settings for cryo-bio are -60,60,3
            b. Desired total dose - 150 e/$Å^2$ is typical
            c. Number of frames per tilt - usually something like 10 but depends on dose per tilt
    3. From the spreadsheet, read off the time per tilt and put this in "Exposure time" in SerialEM Camera Setup. Turn on Dose Fracitonation and put the frame time in the box.

Check "Save Frames" and make sure the number of frames matches what you expect, if not slightly adjust the exposure or frame time.

4. Save a copy of the spreadsheet to your data folder if desired by going to "File" - "Save As" - "Browse"

5. Set up the Record settings as follows

6. **Important: Make sure "Align Frames" is turned on**. Serial EM will align the frames of each tilt before writing them to the .mrc file that the tilt series saves into. This will prevent you from having to manually align the frames of each tilt.

7. Click "Set File Options" and set the filetype and name options. Use Tiff LZW. Suggested naming is sequential number, "Filename of current open file" and Tilt angle:
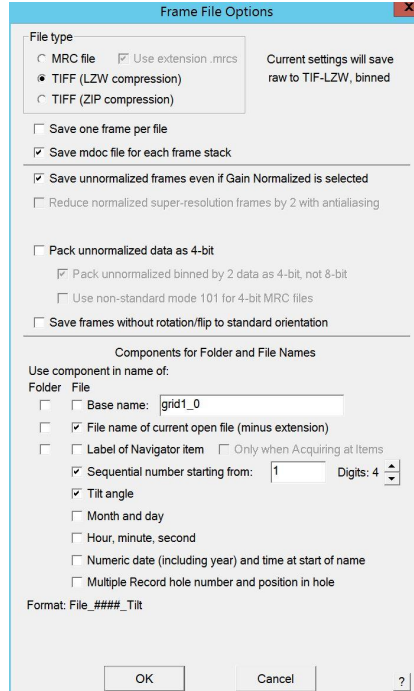
8. Click "Set Folder" and tell it where you want to save micrographs. Make sure you're saving to your own folder on the X drive - this drive is where images will save the fastest.
9. **You should write down & report in publication:**
   a. Pixel size (for 0.5x or 1x binning) & mag
   b. Total dose or 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. Tilt scheme
   h. Voltage (always 200), spherical aberration (always 2.7)
10. 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**.

Atlas

Load a grid, find a square near the center and do "Rough eucentric" at square mag (690-540). Go back to 86x imaging state, insert the 150 um aperture. Open a navigator.

Set up the Atlas under "Navigator" -> "Montaging and grids" -> "Set up full montage". Keep default settings (4x6) and click "OK". Save the file (I usually use "atlas.mrc"). The stage will move to the center of the grid. Start the Atlas by clicking the "Montage" button.

When the Atlas finishes, it will ask if you want to make a new map. Click "Yes" then **save your navigator.**

Map grid squares

Note: if your features of interest are large enough to see at low mag, you can map grid squares at 690/540x - see the single particle instructions for how to do this. This will save lots of time compared to the medium mag mapping procedure used here.

Switch to your desired Low Dose Record state. Then, switch to the 3K Low Dose view mag in Imaging States window. Set C2 to 50 um and re-insert the 70um OBJ aperture.

Check the Low Dose settings for tomography:
Under Buffer Controls, set Roll buffers A->H, nothing else selected.
Under Image Alignment and Focus panel, make sure "center image shift on tilt axis" is selected.
Click "Reset Image Shift".

Set the register for Low Dose:
1. In the Atlas, find a feature (usually a piece of ice) that will be visible at Low Dose View mag. It's best if this feature is near the area you plan to record in.
2. Click on the feature and click "Add marker" in the Navigator. Click "Go to XYZ".
3. Switch to Low Dose view mag (if not already there). Look for the same feature. Usually it's easiest to switch to continuous mode and drive the stage using the joystick.
4. Once the feature is in the View FOV, click to place the green marker on the feature in the view image. In the Navigator, highlight the marker that you placed in Step 2. Go to Navigator -> Shift to marker.
5. Select the middle options for both parts of the dialog
6. Click "Go to XYZ" and check that your feature is centered. Repeat "Shift to marker" if necessary.

Check that LD View is centered relative to LD Record:
1. Click "Preview" and center a feature with the stage (continuous mode + joystick works well if you don't initially see anything, or use shift+right click & drag).
2. Once the feature is centered, click View.
3. Right-click and drag the View image to center the same feature with image shift
4. In LD menu at left, click "Set" under "Offsets for View". This will remember the new image shift between View and Record. Repeat this process (center then "set") until the feature remains centered.

Calibrate Eucentricity by Focus:
1. Take a View image and make sure everything looks good (no beam edges, no slit)
2. Run "Tasks" - "Eucentricity" - "Fine Eucentricity".
3. Go to "Tasks" - "Eucentricity" - "Calibrate Focus Targets…" (at bottom)
4. Under "Calibrate With" click "Current Settings". Wait for calibration to finish.

Select squares to map:
Open the Atlas (double-click on it in the navigator). Click "Add points". Click in the **center** of each square where you might like to take a tilt series. Turn on "Acquire" in the navigator for all these points.

In the camera setup window, change the view parameters to 0.5s, bin2, single image.

Close any open files by going to "File" -> "close" (multiple times if necessary).

Click "Forget Prior State" in Imaging States window.

Select the first center point in the navigator and check the box for "New File at Item". Choose "Montaged images". Set up the dialog as follows:

Name the file MMM_01.mrc . Select "New file at item" for the rest of the center points (they'll be named MMM_02, 03, etc). We usually save these in their own directory.

Go to Navigator -> Acquire at items. Set up as in the screenshot below, or click "Read in parameters" and load "Documents/AcquireAtItems/MMM_tomo.txt". Click "Go" to start. This can take a while if you selected many squares - go grab a coffee or take a walk.

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   [Prepare Squares ▼]

Primary Action-related options
- ☐ Cycle defocus target in autofocus, drift wait & TS
  - From [-2] to [-1] um in 10 [⇅] 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

MONTAGES will initially be saved into the file:
mmm01.mrc

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 [5]
- ☐ No message box when errors occur
- ☑ Close column valves at end
- ☐ Retract cameras at end
- ☐ Send email at end
- ☐ Hide unselected options

[Save Parameters to File] [Read in Parameters]

Tasks before or after Primary Action
- ☐ Manage Dewars/Vacuum   Every item   [Setup]
- ☐ Coma-free Alignment   Every item   [Setup]
- ☐ Correct Astigmatism   Every item   [Setup]
- ☐ Condition Phase Plate   Every item   [Setup]
- ☐ Update Dark Reference   Every item
- ☐ Refine ZLP   Every item
- ☐ Rough Eucentricity   Every item
- ☑ Eucentricity by Focus   Every item   [Setup]
- ☐ Autocenter Beam   Every item   [Setup]
- ☐ Realign to Item   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]
- ☐ Run Script before Action   Every item
  - Script to run: [# 58 ▼]
- ――― **Primary Action inserts here** ―――
- ☐ Run Script after Action   Every item, after
  - Script to run: [MyFuncs ▼]

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

Position in sequence
- [Move Up]
- [Move Down]
- ☐ Run after Primary Action

☐ Run at nearest item with ◉ label ○ note starting with [   ]

☐ Hide unused tasks   [Restore Default Order]

[GO] [Postpone] [Cancel] [?]

Set up positions

Define the Focus position: Take a View mode image, select "Focus" under "Define position of area". At the bottom of Low Dose Control, uncheck the box next to "Rotate inter area axis" to keep the Focus position constrained to the tilt axis. Click in the image where you'd like to Focus (usually I use the very top or bottom, as long as it's on the carbon).

Optional: Switch to a higher View mag (5600x) - this can allow you to see more detail when picking positions. 3000x will also work fine but it's harder to see gold particles. If you change View mag, re-do shift to marked between your medium mag maps and the new view mag, and recheck that View is aligned with Record (basically, repeat the procedure on page 6 at "Set the Register for Low Dose").

Check camera settings: Make sure binning for View and Preview is the same (I usually use bin4). I usually set View to 0.2s and Preview to 0.05s.

Set defocus: Go to an unimportant part of your square map, set Autofocus target to -5 um and run autofocus.

Add points: From the Navigator window, open each of the medium mag montages. Click "add points" in the navigator and click where you might want to set up tilt series.

Open a file for maps: "File" - "Open new", open a file called allignimages.mrc to save your Preview & Anchor maps.

Make maps for alignment: For each of the points you added above, select in the navigator and click "Go to XYZ". Take a View image, center the area of interest, then take a Preview image and center if necessary. Remember this is your area of interest, so too many Previews may cause damage (try to limit to 1-2). If you want to use this area for tomography, click "New Map" in the navigator. The first time it will ask if you want to save the image, click "Yes". For the first map, click "View" once without moving the stage. Click "New map", then check the box labeled "For Anchor State" near the top right of the navigator window. This saves the View settings to use for future anchor maps.

For all the following points, once the area is centered in Preview image, click "New map" to save high mag map, then Anchor map to save View mag map. SerialEM uses these maps during acquisitions to reliably return to the tilt series positions.
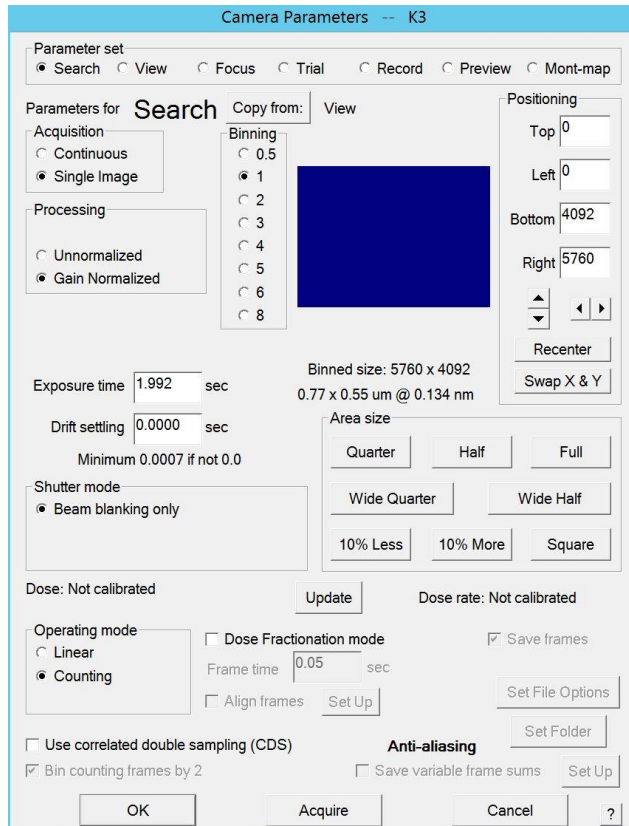
Precise Z-height for each area: (Note: Katie thinks you can get away with skipping this and rely instead on the Z-height per square but Florian does not) Once complete, toggle on "Acquire" for all the new Preview maps in the navigator. Go to Acquire at Items, under Primary Action select "Run Script" and specify the "Update Z" script. Under pre-actions, select "Eucentricity by Focus" (could also use "Fine Eucentricity" if you don't trust the beam tilt method but it will probably take 5x longer). Run Acquire at items to update the Z height of each position.

Set up Search
Switch beam to your Record state. Click "S" under "Copy current area settings to" to copy Record settings to search.

Under "Offsets for" click the button next to "search" and verify the defocus offset is zero.

In Camera Setup menu, set parameters for Search as in the screenshot below. **Make sure the camera is set to "Counted" mode** - for some reason in Search it likes to switch to Linear.

Camera Parameters -- K3

Parameter set
● Search ○ View ○ Focus ○ Trial ○ Record ○ Preview ○ Mont-map

Parameters for **Search** Copy from: View

Acquisition
○ Continuous
● Single Image

Processing
○ Unnormalized
● Gain Normalized

Binning
○ 0.5
● 1
○ 2
○ 3
○ 4
○ 5
○ 6
○ 8

Positioning
Top 0
Left 0
Bottom 4092
Right 5760

Recenter
Swap X & Y

Binned size: 5760 x 4092
0.77 x 0.55 um @ 0.134 nm

Exposure time 1.992 sec
Drift settling 0.0000 sec
Minimum 0.0007 if not 0.0

Shutter mode
● Beam blanking only

Area size
Quarter Half Full
Wide Quarter Wide Half
10% Less 10% More Square

Dose: Not calibrated   Update   Dose rate: Not calibrated

Operating mode
○ Linear
● Counting

☐ Dose Fractionation mode
Frame time 0.05 sec
☐ Align frames Set Up

☑ Save frames

Set File Options
Set Folder

☐ Use correlated double sampling (CDS)   **Anti-aliasing**
☑ Bin counting frames by 2   ☐ Save variable frame sums   Set Up

OK   Acquire   Cancel   ?

Set up tilt series

Close any open files ("File" - "Close" until it's greyed out).

Check the camera setup - set View mode to bin4, 0.1s and check that Record settings are still correct.

Go to a sacrificial area and check objective aperture centering and stigmation. Test Autocenter beam (Tasks - Autocenter Beam). Run RefineZLP in Serial EM.

Switch into "Rec" in Low Dose Control window. Click "Forget prior state" in Imaging States window.

Select the first high-mag map in the navigator and click shift-T. Select the last map and press shift-T again. This will mark all maps as locations for tilt series. Save as Single image..

Define where to save the tilt series and how to name the files, the usual convention is to make a directory called "tomos" and in it name the first file TS_01. The numbers will increase by 1 for each tilt series.

Set up the tilt series controller. A recommended starting point is the following:

Tilt Series Setup

Tilt Angle Specifications

Tilt to `60` End at `-60` [Swap] Base increment `3`

☐ Increment proportional to cosine of angle Total # of tilts: 41

☑ Run series in two directions from `0` ☐ Return with Walk Up

☐ Magnification for anchor: `20000` 8.2 um ☑ Use View for anchor

☑ Use dose-symmetric scheme [Set Up]

Delay time after tilting by basic increment: `3` seconds

☐ Change some settings as a function of angle [Set Changes]

Size, Image Shift, and Recentering Specifications

Magnification: `63000` Binning: `1` Pixel size: 0.127 nm

☐ At start and when needed, track at magnification: `5600`

Limit image shift to `15` um at start

☐ Recenter beam from Low Dose tracking images

☐ Autocenter beam every `60` minutes

☐ Autocenter beam in second half of series at `50` degrees

☐ Refine the zero-loss peak every `15` minutes

Beam Intensity or Exposure Control

☐ Change Record exposure time instead of beam intensity

◉ Keep beam intensity constant

◯ Vary intensity as 1/cosine of tilt angle to the 1/1 `` power

◯ Set intensity to keep mean counts of Record images at `5000`

The image in buffer A implies a Record zero-tilt mean of 89

☐ Taper counts down to `4000` above +/- `50` degrees

☐ Keep intensity below current value (use if intensity already set up)

☐ Do not increase intensity above value for first saved image

☐ Use current intensity at zero tilt (increase to start series)

Total dose: 41.0 x dose at 0 deg; 41.0 x dose at 60 deg

☐ Change other exposures proportional to Record change

☐ Change drift settling proportional to exposure change

Camera and Acquisition Parameters

☐ Average Record dark references

☐ If taking frames, get initial partial sum of `1` frames

Autofocus Control

Defocus target: `-6` um Beam tilt: `4` milliradians

Autofocus offset: `0` um Iteration threshold: `0.1` um

Do autofocus at least every `3` degrees ☐ Skip Autofocusing

☐ Focus every time above `50` degrees

☐ Repeat autofocus if focus differs from prediction by > `0.25` um

Do autofocus when error in focus prediction exceeds `0.2` um

☐ Check autofocus, stop if measured < `0.25` X actual defocus

☐ Stop if autofocus exceeds absolute focus limits

☑ Stop if autofocus change exceeds `10` um

Initial and Final Actions

☑ Align to image now in Buffer A

☐ Refine eucentricity

☑ Leave mid-tilt anchor near plus or minus `40` degrees

☐ Use mid-tilt anchor that you have left in buffer T

☐ Close column valves at end of series

Tracking Control Parameters

☐ Wait for drift to settle [Set Up]

☐ Repeat Record if percentage of field lost is more than `5`

☐ Stop and ask before repeating Record in Low Dose mode

☐ Stop if Autoalign shifts more than `30` % of image size

Get tracking image when error in X/Y prediction is > `0` % of field

Track: ◉ Before ◯ After ◯ Before & after Autofocusing

☐ Stop after aligning tracking image to allow manual adjustment

☐ Align only with tracking images, not Record images

☐ Align with Preview before getting new track reference

Get new track reference if Record alignment differs by > `2` %

[Previous] [Next] [First Half] [Second Half] [Full]

[GO] [Single Loop] [Postpone] [Cancel] [?]

You can ignore the Defocus target - this will be varied by the Acquire at Items menu.

Changing Focus position for tilt series:
If you need to adjust some of the focus positions, you can change them per-item using the navigator maps. For each position you want to change:
1. Double click the View map in the navigator
2. Select the Preview map
3. Select Edit Focus in the Navigator window (Low Dose needs to be on!)
4. Click in the View map to set the Focus/Trial position for this tilt series
5. In the navigator the item should be marked with "TSP" instead of TS.

Run Batch Tomograms

If desired, set up "PostTomo" (script #12) - this script will save a high-defocus image of each location after the tilt series has finished. If you're using it, you must specify an absolute path to where you want these images to save - I usually make a directory called "High_def_ims" within my data directory for each grid. Paste the full path (starting with X:/) into the script. Make sure to leave a space after the "=" - so the script should say:

```
dir = X:/:path_to/folder
```

If you don't want to save the high defocus maps, uncheck the box next to "Run Script after Acton".

Go to Navigator -> Acquire at Items. Set up as in the screenshot below. You can also click "Read in Parameters" and load Documents/AcquireAtItems/Take_tomos.

You will want to customize the defocus range and step - typical values are 1.5-3.5 in 4 steps.