

Low dose serial EM setup for UTSW Titan Krios

Main steps

1. Align scope
2. Set up LMM shot on Standard grid
3. Insert sample grid, take practice shot, set up and start LMM.st
4. Navigator shift to marker at 540X
5. Set up and run several MMM.st
6. Set up Serial EM low dose mode
7. Set view shift offset
8. Navigator shift to marker at 15000X (or desired view mag)
9. Setup Multishot/Multihole parameters
10. Make grid of points
11. Set file and folder options, edit and run script 11 SPAK3 Multishot

Check health status of K2 camera, must be all green

After aligning scope, set up LMM shot and take a record

-SS6

-135X

-70 micron C2 app.

-no objective app.

-C2 100% spread

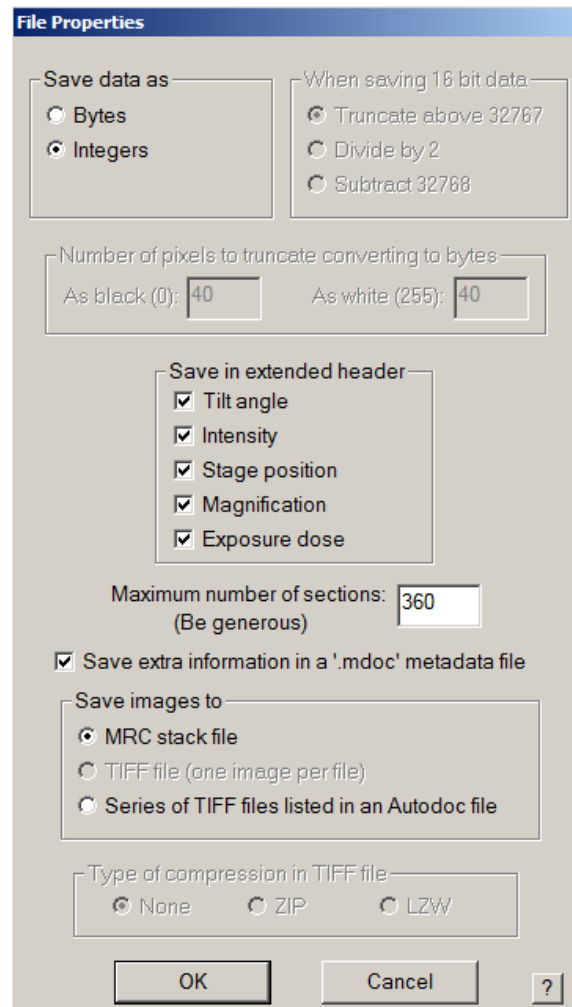
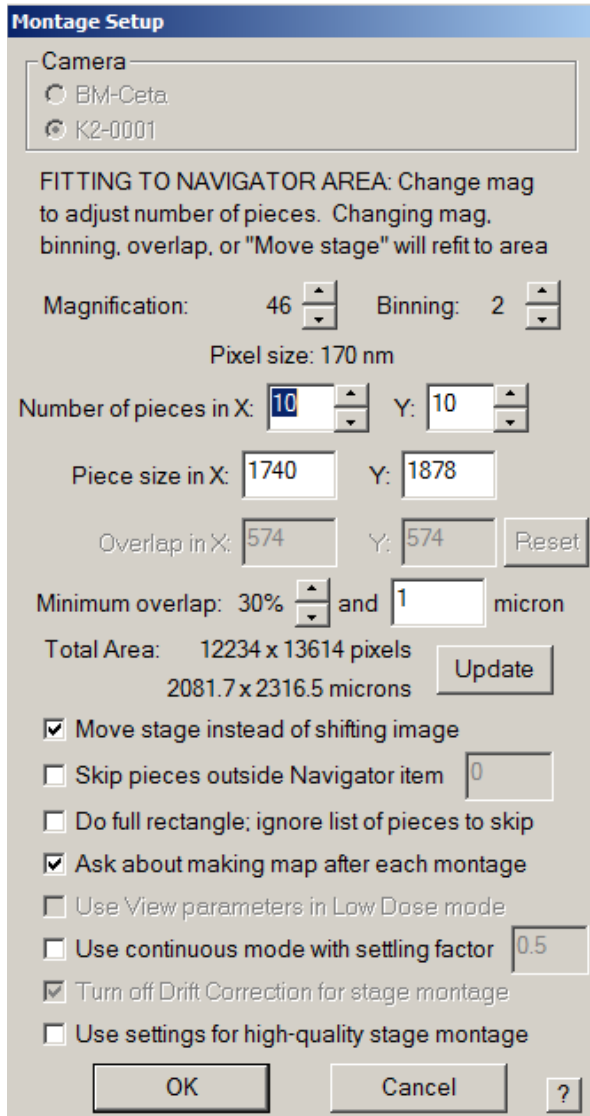
-record binning 4, counting mode, .1-.4s exposure, NOT DOSE FRACTIONATED

Insert grid of interest in autoloader control panel by clicking "load"

Setup LMM.st

-Open navigator in serial EM

- Navigator -> montaging and grids -> setup full montage
- Set the number of tiles, mag, binning etc. 7X9 and 10% overlap works well.
- Click OK, save all information in extended header (check all 5 boxes)
- Select save as MRC stack file, click OK
- Save as LMM.st in KEEP folder



Note* you have now created directions to run LMM.st and should see the LMM.st file open at the top left of the screen, but you need to click START in the montage control panel to run the LMM.st. uncheck the “treat as very sloppy montage” box and click START. approximate run time 12-15 mins.

When LMM is finished SAVE NAVIGATOR as Nav1.nav

Do eucentricity

- Go to 540X
- Condense beam to cover 1 grid square then center with beam shift
- Tasks -> Eucentric Rough
- Update Z of the LMM and any points.

Find a feature and shift to marker

- Identify large recognizable feature in LMM
- In the navigator select Add Points, then add a point on the feature
- Select stop adding
- Highlight the point in the navigator and click 'Go to XY'
- Take a record image
- Right click and drag to move stage, taking record images until feature is found

* **Note:** If you do not find the feature, you can always click "go to XY" again, and go in another direction to search for the feature. If you still cannot find it, you click "go to XY", then you can put your screen down and move the stage with the joy stick. Remember, you should always try to avoid putting your screen down during data collection, as this will dose damage your sample.

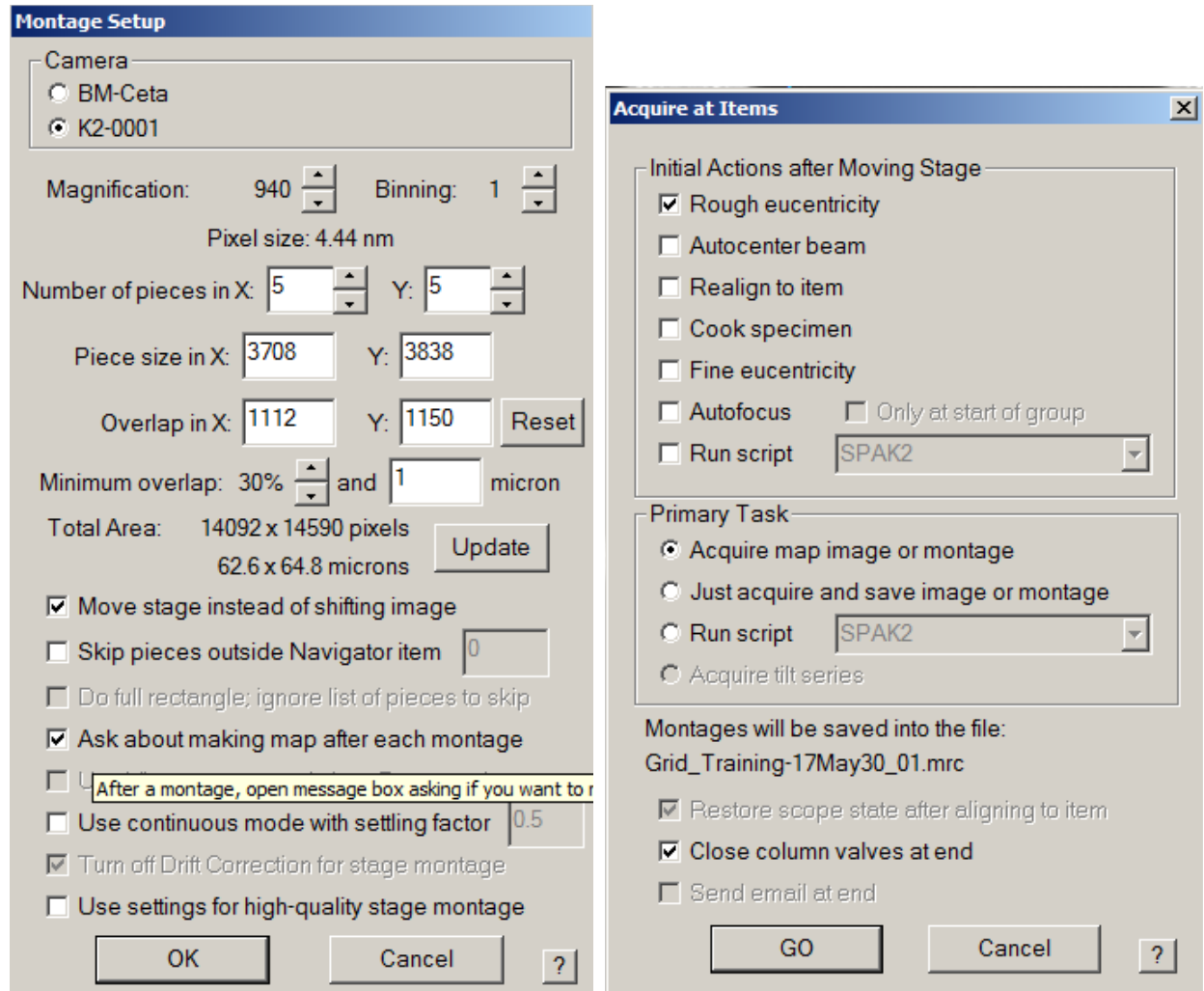
- When the feature is in the record image, put green marker (left click) in the feature
- Select Navigator -> shift to marker, verify that the shift is a reasonable distance, and click OK
- This process can be repeated until LMM and 540X are well correlated

Note* If the shift is not correct, you can always undo this by clicking Navigator -> undo last shift.

Setup and run several MMM.st

- Add Nav points in the centers of desirable squares
- Make all points “acquire” points by ticking the acquire box in the navigator
- Go to first point and tick the “new file at item” box
- Set up the MMM.st file (1X1 depending on mesh) bin 1, .3s exposure counting mode *** deselect “ask about making map after each montage” box.
- Click OK
- Save all info in extended header
- Click OK, save as MMM.st in KEEP folder
- Click Navigator -> acquire at points
- Tick the rough eucentricity box and select acquire map image, select “close column valves at the end”.
- Click GO

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When LMMs are done SAVE THE NAVIGATOR

Go to undesirable area (using LMM or preferably on a bad MMM) and turn on Low Dose Mode

Set up Low Dose Mode in serial EM

-Remember to check “continuous update”

View: 15000X (8700 for multishot), .2s-.4s, bin 4, full

Trial: 105,000X, .2s-.4s, bin 4, full

Focus: 45,000X, .2s-.4s, bin 2-4, wide half

Preview: 105,000X, .05s (or less), bin 4-8, full

**Record: 105,000X, 1-2s, bin .5-1 (super res. or counting), 720nm
Illumination area (parallel beam) dose fractionation on, .05-.1s frames.**

**-Insert and center objective aperture in diffraction mode (WITH SCREEN
INSERTED!!!!!!!!!!!!!!!!!!!!)**

**-Take a view and set the “define position” of area for Focus and Trial, can
also check “Keep Focus and Trial Identical”**

-Insert objective aperture and center if necessary

**-Center all beams, starting with the preview/record beam, then trial/focus,
then View. Go to bad area and put screen down to center beams if
necessary.**

**Note* never offset the record/preview beam, only add offsets to the F/T/V
beams.**

Set View Shift Offset

-Center feature in Preview image

-Take View image

-Check continuous update

**-Uncheck “move stage for big mouse shifts” in the Image alignment and
focus control panel.**

-right click and drag to center feature in view crosshair

- Under offsets for view click “set”

-Take preview to insure feature is centered, repeat if necessary

**Do the Navigator-> shift to marker procedure on a MMM, finding a feature as
described above for the low dose view mag.**

Setup multishot/multihole collection parameters

(9 holes)-Add a nav point in the center of a group of 9 holes (for best results, the edge of the hole can be used for exact template centering) and go to it

Note* make sure it is a flat area of the quantifoil film. You can check to see if it is flat by tilting to 60 degrees at low mag.

-Tune the Coma and Objective Astigmatism of the Record beam (use continuous preview, or the automated functions in serialEM that use CTF under the focus/tune tab)

-Run the Coma Vs. Image shift calibration under Calibration -> focus Tuning -> Coma vs. Image shift.

Note* This calibration needs to be done at your exact record conditions and alignment. If anything other than beam shift has been changed since the last session, this needs to be recalibrated.

- Make sure record beam is at -1-2 microns defocus. Go to 0 defocus offset with the view beam, (usually -20 microns is used for view at 15000x)

-Uncheck the “move stage for big mouse shifts box”, make sure image shift is zeroed and right click and drag to add points to the neighboring holes. Either four corners of your pattern, or all points in the pattern i.e. “for corners of a regular pattern” or “Custom pattern”.

-Add points to LMM at four corners.

(Points could also be added using the view beam with image shift, (right click and drag).

Note* having features in your template setup area is helpful. If there are no features, you can always burn in a feature with the beam to use as a waypoint. Do this with the screen down by condensing your view or record beam.

-Click Navigator-> montaging and grids-> set multi-shot parameters

-select “Do multiple records within each hole” 2-4 no center shot

-select “do records in multiple holes”

-select “Use record illuminated area for circles in low dose”

-Select regular pattern of holes, 3X3

Note* If you cannot see the circles of your pattern click the center of your screen. Note, the holes may not match up between the view mag and MMM mag, we will test for accuracy later so don't worry about this now.

-Select the first of the four (or nine) nav points in the navigator window.

-Click "for corners of a regular pattern" or "Custom pattern (for all 9 holes)" button in the multishot setup menu

- Make sure you "Click IS to NAV pt" and the second point will turn yellow

-Take a view shot (15000X) and center the hole by right clicking and dragging to image shift. Then retake the view to ensure that the hole is centered

Note* To improve accuracy of the image shift, one can go through a second round of template setup, centering template on the edge of the holes, taking a preview image after shifting the view beam to insure that the edge of the hole is in view. If additional image shift needs to be added, add it here before saving IS. This has not been necessary for single shots/hole.

-Click "OK" to exit the setup window

-Return view defocus offset to -20 (or whatever is desired).

Note* If your rotation center/coma free alignment are done accurately, there should be almost no movement between 0 and -20 defocus offset with the view beam.

-Save NAV.

-Recheck "Move stage for big mouse shifts"

-Test template by running the macro on your center point, change this point to be an acquire point, Navigator -> acquire at points.

- You must make sure that Multiplerecords is present and not commented out in the SPA macro for multishot to work. This script is number 10 on the Krios (SPAK3 Multiplerecords)

Add grid of points

-Add 3 corner and two end points of rectangle on MMM

-Navigator-> add grid of points, then select option to turn Acquire on for all points

Note* You may use a polygon to designate which area you want acquisition points to be placed in. Simply add a polygon, then add the five points of the pattern inside it, then note the polygon item number (green number) after (Navigator-> Montaging & grids-> add grid of points) is selected. This can be used to exclude obstructions such as broken areas or large ice crystals. Additionally, after the first square pattern has been made, adding a polygon to successive squares and placing a single point inside the polygon will allow the addition of new points inside that polygon via (Navigator-> Montaging & grids-> add grid of points). This is much faster than adding the grid manually for each square. Additionally, after selecting holes on 1 or two squares you can start your data collection. Then open the "DUMMY" version of serial EM on the desktop, open your navigator inside the dummy version and pick the rest of your points on your maps. Save the .nav as something different and then merge it with the .nav that is currently running in the real version of SerialEM, this is just to save time and is totally optional. Additionally, if doing multihole acquisition keep in mind that the grid of points will be set in the same pattern on every other group of 4 holes.

```
X  o  o  o  o
o  o  o  o  o
o  o  o  o  o
o  o  o  o  o
X  o  o  o  o
X  X  o  o  X
```

Test V align to P

-Go to clean hole that will not be collected (or center of four holes for multihole).

-Center view image

-Save to P buffer

-Make image not centered

-Run script 2 V align to P. This should center the hole

Calibrate/calculate electron dose in Empty area in SerialEM with 1s bin1 exposure at record C2 and mag

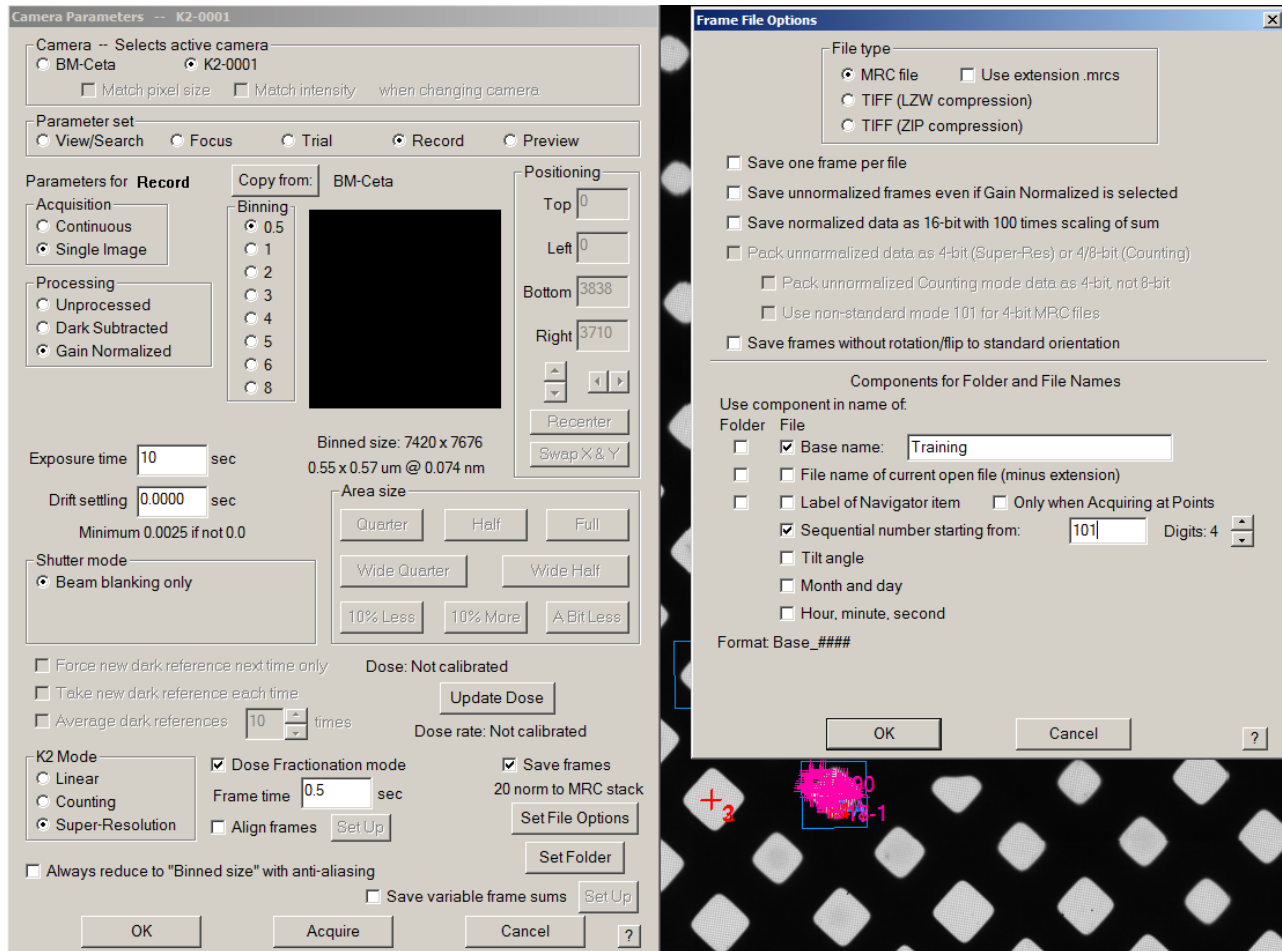
Click Calibration -> electron dose and follow prompts, your dose will now update in real time. You can use this information to calculate the record exposure time.

Set file and folder options in the setup window for the dose fractionated record beam.

-Save as TIFF (LZW compression) in Images-Disc folder

*****Note*** To save space, make sure to select LZW compressed TIFF, and “do not normalize images even if gain normalized is selected” file type in the setup. But in the file options select “Save unnormalized even if gain normalized is selected”. Make sure to copy a copy of the gain reference (In the C -> Program Data -> Gatan -> Reference Images (all of them should be copied to your Images-Disc folder.**

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Edit the SPA Multishot script, adding your X:/...Images-Disc/ file path and click OK

Save the Navigator

Do coma free alignment X, Y, center the OBJ aperture, and do the obj astigmatism

Navigator -> acquire at points

- Deselect eucentric rough
- Under Primary task select run script
- Select Close column valves at the end
- GO
- Wait for first few images to ensure all is well.

Acquire at Items [X]

Initial Actions after Moving Stage

- Rough eucentricity
- Autocenter beam
- Realign to item
- Cook specimen
- Fine eucentricity
- Autofocus Only at start of group
- Run script SPAK2 [v]

Primary Task

- Acquire map image or montage
- Just acquire and save image or montage
- Run script SPAK2 [v]
- Acquire tilt series

Restore scope state after aligning to item

Close column valves at end

Send email at end

GO Cancel ?

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