# 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

Montage Setup	
Camera	
© K2-0001	File Properties
FITTING TO NAVIGATOR AREA: Change mag to adjust number of pieces. Changing mag, binning, overlap, or "Move stage" will refit to area Magnification: 46 - Binning: 2 -	Save data as       When saving 16 bit data.         Bytes       Truncate above 32767         Integers       Divide by 2         Subtract 32768
Pixel size: 170 nm	- Number of pixels to truncate converting to bytes
Number of pieces in X: TO + Y: TO +	As black (0): 40 As white (255): 40
Piece size in X: 1740 Y: 1878 Overlap in X: 574 Y: 574 Reset Minimum overlap: 30% and 1 micron Total Area: 12234 x 13614 pixels	Save in extended header ✓ Tilt angle ✓ Intensity ✓ Stage position ✓ Magnification ✓ Exposure dose
2081. / x 2316.5 microns	Maximum number of sections: 360
Skip pieces outside Navigator item	Save extra information in a '.mdoc' metadata file
<ul> <li>Do full rectangle; ignore list of pieces to skip</li> <li>Ask about making map after each montage</li> <li>Use View parameters in Low Dose mode</li> <li>Use continuous mode with settling factor</li> <li>Turn off Drift Correction for stage montage</li> <li>Use settings for high-quality stage montage</li> </ul>	Save images to MRC stack file TIFF file (one image per file) Series of TIFF files listed in an Autodoc file Type of compression in TIFF file None C ZIP C LZW
OK Cancel ?	OK Cancel ?

**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

Montage Setup	
Camera O BM-Ceta O K2-0001	Acquire at Items
Magnification: 940 🛉 Binning: 1 🛉	Initial Actions after Moving Stage
Pixel size: 4.44 nm	☐ Autocenter beam
Number of pieces in X: 5 + Y: 5 +	☐ Realign to item
Piece size in X: 3708 Y: 3838	Cook specimen
	Fine eucentricity
Overlap in X: 1112 Y: 1150 Reset	🗖 Autofocus 🗖 Only at start of group
Minimum overlap: 30% — and 1 micron	Run script SPAK2
Total Area: 14092 x 14590 pixels	Primary Task
62.6 x 64.8 microns	<ul> <li>Acquire map image or montage</li> </ul>
Move stage instead of shifting image	O Just acquire and save image or montage
	C Run script SPAK2
	C Acquire tilt series
Do full rectangle; ignore list of pieces to skip	
Ask about making map after each montage	Montages will be saved into the file:
After a montage, open message box asking if you want to r	Ghd_Training-T/May50_01.mrc
Use continuous mode with settling factor 0.5	Restore scope state after aligning to item
Turn off Drift Correction for stage montage	Close column valves at end
Use settings for high-quality stage montage	Send email at end
OK Cancel ?	GO Cancel ?

### 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 Astigmatisim 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**<sup>\*</sup> 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.

# 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.

Camera Parameters K2-0001	Frame File Options
Camera Selects active camera	File type
Parameter set C View/Search C Focus C Trial C Record C Preview	O TIFF (ZIP compression)
Parameters for Record     Copy from:     BM-Ceta     Positioning       Acquisition     © 0.5     Top 0       Continuous     © 1     Left 0       Processing     © 3     Attributed       Chark Subtracted     © 4     State       Gain Normalized     © 6     C	<ul> <li>Save one frame per file</li> <li>Save unnormalized frames even if Gain Normalized is selected</li> <li>Save normalized data as 16-bit with 100 times scaling of sum</li> <li>Pack unnormalized data as 4-bit (Super-Res) or 4/8-bit (Counting)</li> <li>Pack unnormalized Counting mode data as 4-bit, not 8-bit</li> <li>Use non-standard mode 101 for 4-bit MRC files</li> <li>Save frames without rotation/flip to standard orientation</li> </ul>
Exposure time       10       sec       Binned size: 7420 x 7676       Recenter         Drift settling       0.0000       sec       Area size       Swap X & Y         Drift settling       0.0000       sec       Quarter       Half       Full         Shutter mode       Wide Quarter       Wide Half       10% Less       10% More       A Bit Less	Use component in name of: Folder File File Base name: Training File name of current open file (minus extension) File name of current open file (minus extension) File name of current open file (minus extension) Sequential number starting from: 101 Digits: 4 Tilt angle Month and day Hour, minute, second Format Base ####
Force new dark reference next time only     Dose: Not calibrated     Take new dark reference each time     Average dark references     10     The second secon	OK Cancel 21
K2 Mode       Image: Construction of the section of the	
OK Acquire Cancel ?	

# 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 astigmatisim

### 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	
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	
Primary Task	
<ul> <li>Acquire map image or montage</li> </ul>	
<ul> <li>Just acquire and save image or montage</li> </ul>	
Run script SPAK2	
C Acquire tilt series	
Bestore scope state after aligning to item	
Class column volves at and	
_ Send email at end	
GO Cancel ?	

Updated 1/27/20 D.S.