UMass Cryo-EM Documentation

Release 1.2

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Oct 11, 2020
Contents

1 FAQ ......................................................... 1
  1.1 How do I ............................................. 1
  1.2 What is .............................................. 5

2 Training Contents and Materials .................................. 7
  2.1 Cryo-EM Training - Basic (level I & II) ...................... 7
  2.2 SerialEM Training - Basic, Tomography, Single Particle, Advanced 9

3 Instructions, Manuals and Protocols .............................. 13
  3.1 Making Graphene Oxide Grid ................................ 13
  3.2 Single Particle Data Collection Using SerialEM ............. 14
  3.3 Post Processing K2/K3 Frames from SerialEM Data Collection 18
  3.4 Align Movie Frames with SerialEM and IMOD Programs ....... 20
  3.5 Monitor Data Collection In The Fly ........................... 23
  3.6 Pixelsize and Distortion Info on K2/3 Cameras ............... 25
  3.7 Relion and cisTEM Docker Services .......................... 27
  3.8 cisTEM detaching and re-attaching sessions ................. 28
  3.9 Adding a Scalebar to Images .............................. 29

4 SerialEM Notes .............................................. 33
  4.1 SerialEM Note: Installation and Calibration .................. 33
  4.2 SerialEM Note: Make All LMM Maps Automatically .......... 38
  4.3 SerialEM Note: Setup Dummy Instance ....................... 40
  4.4 SerialEM Note: Setup LD with Mix of mP and nP Modes ....... 42
  4.5 SerialEM Note: More About Z Height ....................... 43
  4.6 SerialEM Note: Speed, Speed and Speed ..................... 47
  4.7 SerialEM Note: Tackle the Coma ........................... 51
  4.8 SerialEM Note: Utilize JPG File format ...................... 54
  4.9 SerialEM Note: Setup Email on K2 Computer ................ 57
  4.10 SerialEM Note: K3 is installed on Talos .................... 57
  4.11 SerialEM Note: Adding Point Items by Template Matching .............................................. 62
  4.12 SerialEM Note: Refine ZLP on Au Foil Grids ............... 66
  4.13 SerialEM Note: Flexible Timer ............................ 67
  4.14 SerialEM Note: More About X,Y Positioning ............... 68
  4.15 FastTomo: A Hybrid Approach to Speedup Tomography Data Collection .............................................. 70
  4.16 Index ................................................. 71
This is list of Frequently Asked Questions about Umass Cryo-EM. Some questions are technical, while others are more general.

1.1 How do I . . .

1.1.1 . . . get access to your facility?

We are core facility and open to everyone who wants to access including industry customers.

What is the logistics? Normally, you can start inquiry via email or phone call. If our facility is suitable for your projects, we will ask you to submit electronic forms for sample submission and request of instruments. The sample submission form is required by bio-safety office, for any sample coming to Umass Med Sch campus. After we receive your submitted forms and things seem to be in order, our administration will send you a quote for you to obtain a PO for the work or/and instrument time. And we usually can schedule time for you shortly. Our waiting time is usually long, so it is good to plan early.

We have price rate policy for each year. It is not published on the web page, but is available upon request.

1.1.2 . . . get to your facility location?

Please follow this site for main direction and the campus map - https://www.umassmed.edu/bmp/about/directions/

We are located at Medical School Building - A floor SA-106. You enter the “SCHOOLS” building from “medical school lobby” indicated on the campus map. After exiting elevator at A floor, turn left. Half way down the hallway, you will see a sign for “CryoEM Core Facility”. Call facility office # 774 455 4049 if you are lost.
For parking, you should use “South Road Parking Garage” on South Road, the opposite side of two flags.

For nearby hotel to stay, there are a couple of possibilities within walking distance.

1) https://www.beechwoodhotel.com/

2) https://www.marriott.com/hotels/travel/bosri-residence-inn-worcester/?scid=bb1a189a-fec3-4d19-a255-54ba596febe2

1.1.3 ... display the screened data on my own computer locally?

The data for screening is usually at three kinds.

1. Low Mag Montage (LMM) map (grid atlas), usually taken at mag from ~50X to ~150X. The data itself is in MRC 16-bit sign integer format. It is a MRC stack file containing about ~62 pieces if at 46X.

2. Medium Mag Montage (LMM) maps (square atlas) or some single shots. This is usually taken at lower range of M on Talos, such as 1750X. It is also MRC stack file.

3. High mag shots, usually taken at 22,000X or 28,000X. It is MRC stack file, each section is from an exposure. If K2 camera frame mode is used, the section is usually a single image from aligned movie stacks in-fly.

Note: The MRC files we saved here usually have filename extension as mrc, st (for stack file) and map. Regardless the extensions, they are all simply MRC files.

All the three kinds of data can be easily viewed using IMOD. For Windows, if you don’t want to install full IMOD package, a stripped version of display portion called “Windows3dmod” can be installed - from http://bio3d.colorado.edu/ftp/Windows3dmod/. For all the other platforms including Windows, a complete IMOD software package is available. IMOD User Guide can be found in http://bio3d.colorado.edu/imod/doc/guide.html.

Note:

• Since September 2017, most of screening images are also saved into JPG format at the same time when MRC files are saved. This gives you a quick feedback for your sample conditions. The small file size makes it easy for us to upload to Dropbox to share with users.

• After March 2018, all JPG files saved are true JPG instead of JPG compressed TIFF as before. They can be opened by almost any standard image software including ImageJ and Photoshop.

1.1.4 ... insert a scale bar into the screened image?

1. Open a MRC image with IMOD.

2. IMOD - Edit - Scale Bar ...
3. With proper scale bar displayed, press SHIFT+S. It will save a jpeg file with scale bar inserted.

4. Click “Done” to dismiss the setup window.

1.1.5 ... mount the data hard drive I received from you?

The hard drive for data should be in its original filesystem which is normally Windows NTFS. The interface on the hard drive should be USB3. It is in my mind that the drive should be most compatible possible.

If you plug the hard drive onto a Windows or Mac computer, the volume should automatically show up. And you can copy data out from the volume directly.

If you want to mount the hard drive directly onto a Linux computer, you have to connect and mount it manually.

After plugging the hard drive to USB port (USB3 preferred) on Linux computer, you should be able to see lines similar to these from `dmesg` command output on linux computer.

```
[334449.716558] usb 4-1: new SuperSpeed USB device number 2 using xhci_hcd
[334449.728460] usb 4-1: New USB device found, idVendor=0bc2, idProduct=ab34
[334449.728482] usb 4-1: New USB device strings: Mfr=2, Product=3, SerialNumber=1
[334449.728485] usb 4-1: Product: Backup+ Desk
[334449.728487] usb 4-1: Manufacturer: Seagate
[334449.728489] usb 4-1: SerialNumber: NA7H29DX
[334449.749996] usbcore: registered new interface driver usb-storage
[334449.752139] scsi host6: uas
[334449.752539] scsi 6:0:0:0: Direct-Access Seagate Backup+ Desk 040B PQ: 0
→ ANSI: 6
[334449.752586] usbcore: registered new interface driver uas
[334449.768013] sd 6:0:0:0: [sdc] Spinning up disk...
[334449.768023] sd 6:0:0:0: Attached scsi generic sg3 type 0
```

From this, you can see the logic volume is assigned to `sdc`.

On RedHat/RHEL7, CentOS 7 and Scientific Linux 7 and possibly later versions of Linux flavors, the NTFS filesystem is directly supported. For older version of Linux, you might have to install `ntfs-3g` package first. Therefore, you can mount the volume easily with a mounting command as below.

```
1.1. How do I ...
This command should not give you errors. After the command, you should be able to see the volume is mounted using 
\texttt{df} output

\begin{verbatim}
/dev/sdc2  4883638268  1418392  4882219876  1% /mnt
\end{verbatim}

and you should see a few more lines in \texttt{dmesg} output like this:

\begin{verbatim}
[334450.768547] .................ready
[334465.784580] sd 6:0:0:0: [sdc] 9767541167 512-byte logical blocks: (5.00 TB/4.54 TiB)
[334465.784585] sd 6:0:0:0: [sdc] 2048-byte physical blocks
[334465.817288] sd 6:0:0:0: [sdc] Write Protect is off
[334465.817294] sd 6:0:0:0: [sdc] Mode Sense: 4f 00 00 00
[334465.817451] sd 6:0:0:0: [sdc] Write cache: enabled, read cache: enabled, doesn't support DPO or FUA
[334466.214227] sdc: sdc1 sdc2
[334466.215286] sd 6:0:0:0: [sdc] Attached SCSI disk
[334626.393838] sdc: sdc1 sdc2
\end{verbatim}

1.1.6 ... know the image conditions of the data collected on your system?

1. From \textit{Frames.mdoc} file. This is a metadata file to record all the conditions for each frame stack file collected. It contains the most complete information including total dose, stage positions, frame dose, frame numbers and navigator label for this exposure.

A typical section of \textit{Frames.mdoc} file is as following:

\begin{verbatim}
[FrameSet = 0]
TiltAngle = 0.00249969
StagePosition = 24.2868 -41.113
StageZ = -29.8365
Magnification = 105000
Intensity = 0.114429
ExposureDose = 34.9645
PixelSpacing = 0.694291
SpotSize = 8
Defocus = -3.33245
ImageShift = -2.22045e-016 -7.77156e-016
RotationAngle = -94.0936
ExposureTime = 7
Binning = 0.5
CameraIndex = 1
DividedBy2 = 0
MagIndex = 31
CountsPerElectron = 35.3
TargetDefocus = -2.2
SubFramePath = X:\Anna_20171223\1015B_g1_0000_Dec23_13.20.21.tif
NumSubFrames = 35
FrameDosesAndNumber = 0.99898 35
DateTime = 23-Dec-17 13:20:32
NavigatorLabel = 84-1
\end{verbatim}

You might be interested only in total dose, pixelsize, frame dose and frame numbers etc., but it contains fairly complete information.
2. From Setup.png - an image file. This is snapshot for Camera Setup Dialog window and with frame data setup window. This image shows total dose, dose rate on camera, frame numbers, frame time etc..

3. From image header. You can get header information for MRC and TIFF image stack by an IMOD program header:

```bash
$ header image-stack.mrc
```

### 1.1.7 ... use the defect file for MotionCor2?

According to SerialEM helpfile - http://bio3d.colorado.edu/SerialEM/hlp/html/about_camera.htm, here are the step to convert defect map that MotionCor2 needs:

Finally, if you want to run MotionCor2 directly on the unnormalized data, you should give it a defect map file as well as the gain reference file. You can make a defect map from the text file with ‘clip defect’ in IMOD 4.10.7 or higher:

```bash
clip defect -D defects...txt fileWithFrames defects...mrc
```

where the fileWithFrames is used only to set the size of the output and can be any file of the right X and Y size. To make a compressed TIFF file, which will be much smaller, use:

```bash
clip defect -D defects...txt -f tif fileWithFrames defects...tif
```

### 1.2 What is ... 5

#### 1.2.1 ... service and charging details about your screening service?

Screening result usually includes 1) Low Mag Montage (LMM) maps at about 34X mag for entire grid atlas, 2) Medium Mag Montage (MMM) maps at about 2000X for a few promising meshes, 3) final mag shots for 10-20 holes. We also provide JPEG format too for convenient cloud reviewing.

For academic, the screening mostly happens on Talos. We split Talos 24 hours into two session - daytime and evening. If you have more than 8 grids, we will simply regard as daytime session. Less than 6, will be charged by per grid, which is less expensive than being regarded as a daytime session. So you may only screen 2-3 grids, for example, and we won’t charge by session in that case. We are very flexible at this and take the cost of customer into consideration.

The most common style to screen is to load 8-9 grids and start to screen, by 5 or 6PM of that day, one of the good conditions is located and we extend into full day (24 hours) session by collecting on the good grid into next morning 9 AM. We could get as many as 1000+ movie stacks. These are high quality shots on Gatan K2/K3 camera. People get about 3A resolution structures on our Talos with similar setup.

Please ask us if you have further questions.

#### 1.2.2 ... the data I will receive from your facility after imaging?

You received mainly three or four folders as below:

**rawTIFF folder:**

1. TIFF - compressed image stack containing multiple image frames, not gain normalized
2. pcm - IMID python command file for frame alignment using IMOD program
3. mdoc - adoc file that contains all the imaging condition information
4. log - it is log file of aligning result (after run pcm file to align frames)

alignedMRC folder: *_ali.mrc files - those are aligned of multiple movie frames, they are single images not movie frames

alignedJPG folder: JPG snapshot of the aligned MRCs together with power spectrum. These are for visually check image quality and sample condition.

SerialEM-Maps folder: all the control files for SerialEM software running for the session, it contains useful information each shot is from which grid, which region etc.. It is part of the session data, but less useful for end user to process the data.

1.2.3 . . . the Cs value I should use for CTF calculation for Talos and Krios?

2.7mm for both Talos Arctica and Titan Krios.

1.2.4 . . . the method I can get my data after collected at your facility?

UMass Med School has firewall and VPN in place. There is no way to “pull” data from our storage without establishing VPN first. However, outbound traffic - “push” is possible. There are a few ways we can send data to you.

- At Harvard Medical School, some labs ask SBGrids folks to setup a DropBox like account for their lab. With specific command, we can push data directly from our storage to HMS special DropBox location.

- If you setup a user account on a Linux box for us, we can transfer data via sftp or via rsync over SFTP protocol. We can also use your personal account without knowing your password but using SSH keys. We provide our public key to you and you put it in ./ssh/known_hosts, and we can establish connection using our private key at our end. You can remove that line to disable the possibility of connection.

- The data can be also sent to you after copying onto a portable HDD drive with USB3 interface. Default NTFS filesystem coming with most of the HDD is usually working fine.

- AWS. Cloud is becoming reasonable and attractive way to store and compute data. If you setup AWS S3 bucket, and share with us the keyID and secret key, we can upload onto AWS S3 bucket easily. The overall speed is not super fast, but fairly decent and faster than most of SFTP transfer to reginal institutions. For large filesize like a typical raw TIFF stack file about 300-400 MB, it can reach about ~68 MB/s. For external institutions who already have AWS, we recommend to use this way.

1.2.5 . . . What is difference between dose and dose rate? What condition should I use?

They are two different things, but related by the magnification of microscope.

Normally, dose means the total electrons hitting the specimen in a unit area. It usually has unit like e⁻/Å².

Dose rate means how strong the beam is, it is how many electrons hitting in one physical pixel area on the detector sensor for a unit time period. It usually has a unit like primary electrons/unbinned pixel/second. A proper dose rate is required for optimal performance of a camera.

Under a giving beam condition, your dose rate is fixed, you can change exposure time to obtain target total dose on specimen. Therefore, we should always determine the proper dose rate first.
2.1 Cryo-EM Training - Basic (level I & II)

(Each session is set 2 - 2.5 hours)

Author  Chen Xu
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Date    2016-07-27

Goal The goal of the training we offer is to help a new user coming to EM field to learn some basic knowledge and skill of Cryo-EM. Hopefully, at the end of these two levels of training sessions, you will be able to screen your negative stained specimen and cryo grids on a TEM scope without too much difficult.

Requirement You are not required to have experience with TEM operation. Certainly, we could progress faster if you have some basic knowledge about a TEM. The only requirement to you, the fresh trainee, is to have a spirit of commitment. You need to be determined to learn this new technique. If you think to have your data collected while in your training session and have your project done half way, you should not attend the training session that we offer. You will be wasting your own time, let alone ours. Usually, if an user doesn’t take good note during training session and doesn’t come back to practise on microscope, that is a good hint that the user is not serious at it. Operating a transmission electron microscope is very much like driving a car - you need to be serious and to practise if you really want to learn it!

This document lists the training contents that these two-level, 8 sessions will cover.

2.1.1 Level I - Basic TEM Operation (4 sessions)

Session I - 1

• Prepare continous carbon film grid
• Introduction to TEM; brief history; compare to X-ray as beam source
• Basic TEM Operation on Tecnai G2 system
• User Interface & Vacuum system

**Session I - 2**

• Specimen rod insertion and retraction
• Tuning microscope (Eucentricity, gun alignment, filament saturation, beam-tilt pivot points, rotation center)
• C2 aperture centering, C2 lens stigmation; Obj. aperture centering, Obj lens stigmation
• Correct side of beam spreading
• Demo and introduce CTF concept

**Session I - 3**

• Negative Stain sample preparation
• Refreshing microscope tuning procedure
• Introduce digital cameras - hardware component, background preparation, pixelsize at levels of detector and specimen
• Introduce DQE, and latest status of direct electron detectors
• Negative stain specimen imaging using CCD

**Session I - 4**

• Supervising practice of users for negative stain and scope operation
• Assist users’ operation and answer questions
• Demo and explain cryo-cycle procedure to condition the column

### 2.1.2 Level II - Cryo TEM Operation (4 sessions)

**Session II - 1**

• Prepare side-entry cryo holder, explain dry pumping station - warm-up & zeolite cycle
• Demo and explain FEI Virobot operation
• Explain holey grid pre-treament - bake, wash & electron beam irradiation
• Demo full procedure for Cryo freezing EM grids
• explain the Ethane tank operation

**Session II - 2**

• Low Dose TEM operation, explain low dose concept and setup
• Indroduce and explain shutter control on TEM system
• CCD imaging with Tecnai Low Dose software
Session II - 3

- Cryo grid loading to cryo holder on transfer station
- Cryo holder insertion & retraction, explain airlock pumping mechanism
- Demo and explain vacuum condition and how to quiet LN2 bubbling
- Go through whole process from sample loading to Low Dose imaging
- Explain ice condition

Session II - 4

- Supervising Cryo Session for user practice
- Debugging ice condition, cryo-cycle
- Supervising warm-up and baking cryo holder

2.2 SerialEM Training - Basic, Tomography, Single Particle, Advanced

(Each session is set 2 - 2.5 hours)

Author  Chen Xu
Contact  <chen.xu@umassmed.edu>
Date  2016-08-12

Goal  This is to provide hands-on training on SerialEM. I will teach basic functions of the program. And I will teach how to use the powerful program for electron tomography data collection, and for single particle application as well.

Requirement  You are required to have basic knowledge for TEM operation, preferred for Tecnai/Titan/Talos system. You should be able to operate scope independently to get a properly focused image. You are not required, however, to have pre-knowledge of SerialEM itself.

This document lists the training contents that are covered in the four categories - basics, tomography, single particle and advanced topic.

Note:  If you have any thought and suggestion to improvement the training, I love to hear them.

2.2.1 SerialEM - Basics (3 sessions)

Session 1

- Introduction to SerialEM, launch and exit the program. Explain system files and user setting file.
- Explain how SerialEM controls microscope and camera, and its relationship with microscope and camera control software interfaces.
- Explain SerialEM interface and layout - control panels and menus
- Camera setup, how to acquire an image from SerialEM interface
- Demo basic function such as Eucentricity (and shift beam to tilting axis)
Session 2

- Refresh Tune-scope procedure
- Prepare gain reference file in SerialEM
- Explain image buffers and how to save buffer image into file, explain MRC stack and modes
- Image Shift and Stage Shift
- Eucentricity, Autofocus and montaging
- Explain pixelsize and dose/dose rate.

Session 3

- Introduce Navigator - navigator items: map, point and polygon
- Demo full grid montage, Medium Map Montage (Using Image Shift and Stage Shift)
- Realign To Nav Item, demo and explain
- demo acquire map or image at multiple points . . .
- introduce script/macro

2.2.2 SerialEM - Tomography (5 sessions)

Session 1

- demo and explain how to collect a tilting series
- explain cooking resin specimen, defocus and other parameters (mag, binning etc.)
- explain proper sample preparation for platic sections (thickness, gold beads etc.)

Session 2

- Supervise user to acquire MMM maps and collect a tilting series, answer questions and comment on the condition used.
- Setup montage tilting series
- Setup batch mode for multiple locations

Session 3

- demo and explain dual axis tomography data collection
- demo how to rotate grid 90 degree and find the same location (registration transformation)

Session 4

- Low dose mode setup for Cryo Tomography applications
- refresh cryo sample and holder handeling
Session 5

- LMM, MMM in low dose mode
- bi-directional tilting series collection
- batch mode for cryo data collection.

2.2.3 SerialEM - Single Particle (5 sessions)

Session 1

- positioning X,Y, Image Shift and Stage Shift, backlash
- dragging to a new position, with Script/Macro
- positioning for preselected multiple location using RealignToItem and ZeroIS.
- positioning Z, using stage and using tilted beam image pair
- demo and explain scripts Z_byG and Z_byV
- demo center beam using keyboard and script

Session 2

- demo simple script LD, and explain actions
- refine hole centering using template
- draw grid point - manual picking and using hole finder
- introduce script LD-group and explain the ideas

Session 3

- K2/3 specific - image format(MRC, TIFF), Compression, 4-bit special for Super-res frames
- Asynchronize mode for K2/3 imaging, separate gain reference from raw image frame stack
- Consideration for dose - total dose, dose per frame, frame time
- In-fly frame aligning option

Session 4

- go through whole single particle procedure
- LMM, LD setup, MMM with “Z_byV2”, draw grid point, prepare hole template
- run LD-group

Session 5

- supervising user practise session to go through all the steps
- answer question
- explain script command to limit defocus changing range
2.2.4 SerialEM - Advanced Topics (3 sessions)

Session 1

• SerialEM installation and Calibration

Session 2

• Setup multiple accounts
• Setup multiple system files
• Setup executables for production and tests
• Setup Dummy instance to pick target holes while main instance is busy collecting

Session 3

• Explain SerialEM scripting interface and major functions
• Explain example script to control LN₂ refilling and obtain K2/3 hardware dark background
• Setup email alert system
• in-fly aligning frames using standalone GPU server computer
CHAPTER 3

Instructions, Manuals and Protocols

3.1 Making Graphene Oxide Grid

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Date-created 2018-3-10
Last-updated 2018-8-21

Abstract This protocol is based on MRC LMB one. We modify the protocol based on Xudong Wu’s method at Harvard Medical School.

3.1.1 Materials and Equipment Used:

1. GO Solution (Sigma:763705, 2 mg/ml)
2. ddH₂O
3. Tweezers
4. Pipette (1 µl, 3 µl and 20 µl)
5. Tabletop centrifuge
6. Parafilm
7. Whatman filter paper No1

3.1.2 Procedure:

1. Dilute GO solution 10x to 0.2 mg/mL with ddH₂O
2. Sonicate for 15s to break up aggregates
3. Spin for 30 seconds at 300 rcf to remove aggregates
4. Prepare a flat and clean working area (e.g. with parafilm)
5. Further dilute GO solution 10x to 0.02 mg/mL with ddH$_2$O
6. Glow discharge grids with carbon side up (easiGlow (PELCO, Discharge System); Quantifoil Cu 300 R1.2/1.3 120 seconds at 0.2 mBar and 15 mA)
7. Take up grids with anti-capillary tweezers
8. Place 1µl of the diluted GO solution (4) on the carbon side of the grid
9. Wait for the grid to dry out

Note:
1. This method is good for high coverage of GO, but maybe get multiple layers of GO on the grid.
2. Discharging conditions are varied between grids and discharge system.

3.2 Single Particle Data Collection Using SerialEM

Author Chen Xu
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Date-created 2016-10-18
Last-updated 2019-08-08

Abstract This document is to list step-by-step operations to perform single particle data collection using SerialEM. I often receive requests to provide script/macro for single particle data collection using SerialEM as control program. It is not very easy to explain that script/macro itself is only the small portion of whole operation steps. I realized that a brief but detailed protocol for whole process is perhaps more useful, specially for novice cryoEM users. It should be useful for more experience users as well as a quick checklist in case some step is forgotten. I wrote something similiar at Brandeis EM webpage, but here I rewrite this to reflect newer hardware of microscope and camera, and with updated SerialEM scripts/macros.

A Krios with K2 Summit camera and FEI Ceta camera is the base hardware setup for this protocol.

Note: This doc is a working progress. If you have comment and suggestion, please let me know. Thank you!

3.2.1 Check Scope Condition and Perform Tuning

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

- Check Gun Lens, Extracting Voltage, High Tension are set at correct values.
- Stare for a few seconds at the focused beam at the highest SA mag, to see if the beam has good shape and there is no shaking or jumping.
- From Direct Alignment, do gun tilt, beam tilt PP, Coma-Free alignment if needed.
- Check Thon Ring at roughly the same condition (mag, dose) as your image condition. Make sure there is no obvious frequency cutoff, and Thon Ring reaches the resolution as in good condition.
3.2.2 Prepare Cameras

For K2 camera, perform full procedure to prepare backgrounds from DM interface. This include software and hardware backgrounds. The hardware background file is for processor to use, while the software gain reference files sit in K2 computer for final software image correction. I was told the software gain reference was more stable than hardware background, but not sure this is still the case. Any way, just perform the full procedure following DM steps.

- After preparing camera, take a single shot with proper dose rate (~5-10 e/pix/s) for 1 second with no specimen and do an FFT. The FFT should show clean background without strong center cross or lines.
- For Ceta camera, do the same from FEI user interface.

3.2.3 Make Low Mag Montage (LMM) Map or Grid Atlas

It saves time with large area detector. Therefore, Ceta camera is probably better for this step.

- Select Ceta from Camera Control Setup
- Insert/load your cryo grids
- Set mag at ~87X, retract Obj Aperture
- Spread beam to cover whole Ceta camera area
- Start SerialEM if not yet
- Select Ceta from SerialEM camera control setup and FEI Camera ocx.
- Setup camera condition from SerialEM: Record (e.g. bin=4, exposure=0.4); a Record image gives proper counts (~2000)
- Navigator menu -> Open
- Navigator menu -> Montaging & Grids -> Setup Full Montage; define montage file to open
- Montage Control Panel -> Start
- Click “Yes” to make final overview of montage into a map
- Close the montage file

Tip: If you are not happy with the aligning of the pieces, you may check and uncheck boxes like “Treat sloopy…” and reload the map.

3.2.4 Setup Low Dose Condition

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

- Turn on Low Dose Mode from SerialEM Low Dose control panel
- Setup R beam first so that dose on detector and on specimen are all good.
- Defocus offset 100um for View is usually a good start.
- Always cycle “area” (low dose mode) in one directional looped fashion, i.e., V-F-T-R-V…
- Using the same spotszise for all the areas (low dose modes) is a good idea.
3.2.5 Make Medium Mag Montage Maps

- select K2 camera from Camera Control Setup (from now on)
- add a polygon (a mesh) in LMM map
- add points for good meshes at center
- add one landmarker such as a dirt point in LMM map
- take the landmarker into View image (you may use FlowCam to move that feature into middle first.)
- while landmarker point being current (highlighted), left click on the landmarker in View image, a green cross will appear
- Navigator menu -> Shift to Marker -> Yes (this will change all the coordinates for all the navigator items)
- highlighting polygon item on navigator window, so it is currently selected
- Navigator menu -> Montaging & Grids -> Setup Polygon Montage -> Check “using View …” in the dialog window -> define montage filename.
- Add flag “A” to all the interested mesh point items
- Navigator menu -> Acquire At Points … -> Check “Eucentric Rough” in Pre-action and “Acquire Montage Map” in main action
- When finished, the MMM maps should be added to Navigator windows. You perhaps can close the montage file now.

3.2.6 Draw Grids Points for Each Mesh

For each of the MMM map, do the following steps to add group points.
- add a polygon item to exclude bad area
- add 5 point items to define grid geometry
- make any of the 5 items in the group is currently selected
- Navigator menu -> Montaging & Grids -> Set Group Size (10um is a good start)
- Navigator menu -> Montaging & Grids -> Check “Devide point into Groups”
- Navigator menu -> Montaging & Grids -> Add Grid Points -> give polygon item number -> Flag “A” for all

3.2.7 Test Main Script to Run

Lets load the script “LD-Group” to script editor and try to run it.

```
ScriptName LD-Group
# macro to skip points except the very first in the group.
# assume LD is setup.

# X,Y position
buffer = T
RealignToNavItem 0
Copy A $buffer # copy last image from Realign to buffer P
ResetImageShift
```

(continues on next page)
This script calls three functions - Relax, BufferShot and CycleTargetDefocus. The script that contains all the functions “MyFuncs” must be also loaded in one of the script buffers/editors. You can download the latest “MyFuncs.txt” here on github.com.

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

### 3.2.8 Final Checking

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

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

### 3.2.9 Run it!

Navigator -> Acquire at Points... -> Run Script “LD-Group” in Main action -> OK.
3.3 Post Processing K2/K3 Frames from SerialEM Data Collection

Author  Chen Xu  
Contact  <chen.xu@umassmed.edu>  
Date_Created  2017-02-13  
Last_Updated  2019-05-07  

Abstract  At UMASS Cryo-EM Facility, we use SerialEM to collect data for both single particle and tomography applications. And we do that on both Talos Arctica and Titan Krios with K2 cameras. 

For single particle, usually we save frames in compressed TIFF format without gain normalized (select Dark Subtracted in camera setup window). One of the advantages of doing this is to reduce data size. For Super-resolution frames, the raw frame data is in unsigned 4-bit. Pixel values are in the range of 0 - 15. For weak beam, there are a lot of zeros there too. With lossless compression methods, such data can be compressed into much smaller filesize without losing image information. Therefore, instead of applying gain normalized reference to all the frames, we leave the raw data compressed and saved to the disk and we later do post-processing to recover the full information of the image data.

In this doc, the procedures to do post processing are presented here for your reference.

3.3.1 For K2 camera on Talos Arctica

The DM camera configuration for camera orientation setup for K2 camera on Talos Arctica is 270 degree rotation and Flip along Y. The idea is that with a proper orientation setup, the image from camera is at the same orientation as on FluCam. This is initial condition for SerialEM setup.

However, when saving frames from single particle data collection, this orientation might not always be needed. As long as all the data is saved the same way for the entire session, it is fine with and without this orientation applied to all the frames before saving. This option is from a check box “Save frames without rotation/flip to standard orientation” in K2 Frame File Option dialog window.

If you saved frame as un-normalized TIFF, and you need to recover the image stack to a MRC format and apply gain reference file and mask out defects, here are steps.

1. check out the orientation from header of the file.

```$header YURI_B1_G1-SuperRes_636_Feb05_10.42.09.tif

RO image file on unit  1 : YURI_B1_G1-SuperRes_636_Feb05_10.42.09.tif  Size= 805815 K

This is a TIFF file.

Number of columns, rows, sections .....  7676  7420  80
Map mode ..........................  0  (byte)
Start cols, rows, sects, grid x,y,z ...  0  0  0  7676  7420  80
Pixel spacing (Angstroms)...........  0.8714  0.8714  0.8714
Cell angles ........................  90.000  90.000  90.000
Fast, medium, slow axes ............. X  Y  Z
Origin on x,y,z ...................  0.000  0.000  0.000
Minimum density ...................  0.0000
Maximum density ...................  15.0000
Mean density .......................  7.5000
Tilt angles (original,current) ......  0.0  0.0  0.0  0.0  0.0  0.0
Space group,# extra bytes,idtype,lens .  0  0  0  0
```

(continues on next page)
1. **Titles:**
   SerialEMCCD: Dose frac. image, scaled by 1.00 r/f 0

The last parameter in title line shows the orientation of imaging. Here is 0 - no rotation and no flip. In this case, Gatan gain reference file doesn’t need to do any rotation and flip. We simply convert it into MRC format.

2. Convert Gatan gain reference .dm4 into MRC format.

```bash
$dm2mrc GatanGainRef.dm4 GatanGainRef.mrc
```

3. Use “clip” to apply gain reference and deal with defects all in a single command line (later IMOD can take tiff file format as input directly). I quote a section from SerialEM helpfile here:

```plaintext
<helpfile>

Once you have the reference in the right orientation, you can use the program ‘clip’ in IMOD to apply gain normalization (and defect correction with version 4.8.6 or higher). In the following, ‘scalingFactor’ is the regular scaling factor applied to summed images, ‘fileWithFrames’ is the data file to normalize, ‘gainReference.mrc’ is the reoriented gain reference, and ‘normalizedFrames.mrc’ is the desired output file. The alternatives for GMS 2.3.0 or lower are:

Counting mode, not packed: The data need to be scaled to preserve precision after normalization. The command is

```bash
clip mult -n scalingFactor fileWithFrames.mrc gainReference.mrc
  → normalizedFrames.mrc
```

Super-resolution mode, not packed: The data need to be scaled to preserve precision after normalization. To have the same scaling by 16 that the plugin would apply, the command is

```bash
clip mult -n 16 fileWithFrames gainReference.mrc normalizedFrames.mrc
```

but if you want to apply the regular scaling factor, the output will need to be integers and the command is

```bash
clip mult -n scalingFactor -m 1 fileWithFrames gainReference.mrc
  → normalizedFrames.mrc
```

Counting mode, packed as bytes: The data need to be scaled to preserve precision and output as integers to preserve the range. The command is

```bash
clip mult -n scalingFactor -m 1 fileWithFrames gainReference.mrc
  → normalizedFrames.mrc
```

Super-resolution mode, packed as 4-bit numbers: By default, the data will be scaled by 16 when unpacking with normalization, so the command to get this scaling is just

```bash
clip unpack fileWithFrames gainReference.mrc normalizedFrames.mrc
```

but if you want to apply the regular scaling factor, the output will need to be integers and the command is

```bash
clip unpack -n scalingFactor -m 1 fileWithFrames gainReference.mrc
  → normalizedFrames.mrc
```

It is also possible to remove extreme values from the data at the same time with the ‘-h’ and ‘-l’ options. For example, adding ‘-h 6 -l 1’ after the ‘unpack’ will replace all values above 6 with 1.

3.3. Post Processing K2/K3 Frames from SerialEM Data Collection
To apply defect correction to files from GMS 2.3.1 or higher, add `-D defects...txt` before `fileWithFrames` in the appropriate command, where `defects...txt` is the file saved by the plugin.

In IMOD version 4.8.41 or higher, all programs can read 4-bit files directly. The `clip unpack` command has thus been changed so that it can be used for normalizing any kind of data, and it can also be invoked as either `clip unpack` or `clip norm`. A command that works for all of the above cases is

```
clip norm -n scalingFactor -m 1 fileWithFrames gainReference.mrc
→ normalizedFrames.mrc
```

Where the default scaling factor is 16, extreme values can be removed with `-l` and `-h` options, and `-D defects...txt` would be added for files from GMS 2.3.1 or higher. With IMOD 4.9.2/4.10.1 or higher, you can add add `-R -1` and use the DM reference directly instead of a rotated reference.

For K3 frames, you should specify a scaling factor of 32.

</helpfile>

## 3.4 Align Movie Frames with SerialEM and IMOD Programs

**Author**  
Chen Xu

**Contact**  
<chen.xu@umassmed.edu>

**Date**  
2017-12-17 last update

**Abstract**  
IMOD can align movie frames nicely and very quickly. In late versions of IMOD, the program AlignFrames also utilizes GPU and can efficiently read in compressed TIFF frame images and decompress them, apply gain reference to normalize image frames, deal with defects and align all the frames - all at once.

To align small movie stack in-fly during tilting series data collection might be among motivations that David M developed this. One can easily see how useful and nice it is that every tilt is aligned for small movie stacks and return to SerialEM automatically in the background. It could save user huge mount of processing time unless you want to redo the movie alignment again yourself. For this purpose, the same function and code of IMOD program are also included into SerialEM program so that the alignment can be done during data collection.

I personally find this in-fly aligning capability extremely useful for single particle applications too. With SerialEM setup properly, I can get aligned image for an exposure directly. This not only provides feedback immediately, but also does it without changing low dose imaging conditions. I don’t have to change back and forth the low dose record exposure time, counting to Linear mode etc..

**Framewatcher**, an IMOD python script program, makes it very easy to align all the movie frames in a changing directory. No need to bother with cron job and file lock etc.. It watches for any unprocessed image stack in the directory and align them for you.

In this document, I try to tell you how I use them.

### 3.4.1 Alignframes

This program takes many options as command line arguments. For details, please read the man page with example usages [http://bio3d.colorado.edu/imod/betaDoc/man/alignframes.html](http://bio3d.colorado.edu/imod/betaDoc/man/alignframes.html).

As usual, the long command line can be run with a command file. Here is an example of a python command file YURI_B1_G1-SuperRes_2967_Feb04_01.14.57.pcm.
One can run this command file like this:

$subm YURI_B1_G1-SuperRes_2967_Feb04_01.14.57.pcm

### 3.4.2 Framewatcher

*framewatcher* is a python script to run *alignframes* at batch process. One feature I like a lot is that it can watch a growing directory and process new coming frame files. For details usage, please refer man page [http://bio3d.colorado.edu/imod/betaDoc/man/framewatcher.html](http://bio3d.colorado.edu/imod/betaDoc/man/framewatcher.html).

If frame stack files are with their command file *.pcm*, then one can just run it by issuing command in the directory:

$framewatcher

This will start to align all the frame files in the same directory, until you do Ctrl_C.

If there is no *.pcm* existed for each file, and you just want to align them using the same parameters, then you can do that using a master pcm file to take care all the files you wanted to align. Here is an example of master.pcm:

$alignframes -StandardInput
UseGPU 1
StartingEndingFrames 3 42
MemoryLimitGB 20.0
PairwiseFrames 20
GroupSize 1
AlignAndSumBinning 6 1
AntialiasFilter 4
RefineAlignment 2
StopIterationsAtShift 0.100000
ShiftLimit 20
MinForSplineSmoothing 0
FilterRadius2 0.060000
FilterSigma2 0.008574
VaryFilter 0.060000
ModeToOutput 2
InputFile YURI_B1_G1-SuperRes_2967_Feb04_01.14.57.tif
OutputImageFile YURI_B1_G1-SuperRes_2967_Feb04_01.14.57_ali.mrc
ScalingOfSum 37.549999
CameraDefectFile defects_YURI_B1_G1-SuperRes_358_Feb01_07.52.46.txt
GainReferenceFile SuperRef_YURI_B1_G1-SuperRes_001_Jan31_15.48.35.dm4
RotationAndFlip -1
DebugOutput 10

(continues on next page)
As you can see, this is the same as individual pcm file, except without InputFile and OutputImageFile defined in the command file. In this case, you tell the program to use this master.pcm file:

```
$framewatcher -m master.pcm
```

The program will go through all the individual files and generate their individual pcm file based on master.pcm and align each one.

Since `framewatcher` can flexibly define output location, we can utilize it to save all the raw files and as well as aligned result files into a network drive from local SSD drive. Sometimes, directly saving on network drive and also aligning frames there could cause slowdown of SerialEM data collection. This works as a neat way to empty X or Y drive on K2 computer, they will never fill. For example, following command will move all the new files saved by SerialEM and aligned files on X drive to the network drive Z.

```
$framewatcher -w X:\MyData -o Z:\Storage\MyData -pr Z:\Storage\MyData
```

`framewatcher` can also output aligned sum together with power spectrum into a single image in JPEG format. This is ideal to send to remote user who wants to check image quality during data collection session. The file is small and can be opened with any image viewer.

```
$framewatcher -w X:\MyData -po 1024 -o Z:\Storage\MyData -pr Z:\Storage\MyData
```

You can even simply move all the raw files without aligning them.

```
$framewatcher -w X:\MyData -noc -pr Z:\Storage\MyData
```

Interestingly, `framewatcher` will also copy (not move) Gatan gain reference file and Defect file to Z drive too.

If you also want pcm file to move together with raw file, you can use “-after” option:

```
$framewatcher -w X:\MyData -noc -pr Z:\Storage\MyData -after 'mv %{rootName}.pcm % →{processedDir}'
```

You can even do ctffind and plot the curve using the “-after” option, if you installed Albert’s `ctffindPlot` program. The command is like this:

```
framewatcher -gpu 0 -bin 2 -po 1024 -dtotal 46.6 -after 'ctffindPlot %{outputFile}'
```

From November 23, `alignframes` and `framewatcher` also have options to do dose weighting. This is still in alpha version, but perhaps will be IMOD main production soon. Here I demo a couple of options to use with `framewatcher`:

```
$framewatcher -w X:\MyData -po 1024 -dtotal 39.8 -Vt 200 -o Z:\Storage\MyData -pr

Z:\Storage\MyData
```

where the total dose on sample is 39.8 electrons/A^2, accelerating voltage is 200kV.
If on the storage tank, we have a few subfolders to make things more organized, and we use K2 computer to align, we
cando someting like this:

Note: Very often, people get confused by the terms “dose” and “dose rate”, partially because there seems to have
no official definition here. As per my understanding, “dose” means electron dose on specimen and usually has
unit electron/A², while “dose rate” means beam intensity level for detector and usually has unit electron/unbinned
pixel/second. Dose rate is a reference value for the performance of a detector. In the case of K2 Summit counting or
super-resolution mode, this value is usually choosen between 5 - 10. Much higher than 10, the performance of K2
camera is likely to be worse. Once this value is fixed under current microscope conditions, we select exposure time
and frame time etc. to satisfy the total dose on the sample and frame dose (also on sample) within the frame time for
movie alignment purpose.

### 3.4.3 Using GPU

To my understanding, the code for `alignframes` is optimized to utlize GPU and paralellization as well. Reading in and
decompressing TIFF stack file is also very efficient. On my linux box with Xeon(R) CPU E5-2650 v3, with 256GB
memory and Nvidia M4000 GPU, it aligns a 50 Super-resolution frame file in about 22 seconds with GPU option.

### 3.4.4 On K2 Computer

Since K2 computer comes with pretty high-end hardware, it could be used to align the frames in background. All I had
to do is to install a decent GPU card. I replaced the ATI video card that comes with the K2 box and install a M4000
GPU card in with 8GB memory on the card. One advantage for this card is that it is single slot high, not like most
Nvidia cards which occupies two PCI slot space. This makes the replacement simple and easy.

Now, after installing IMOD with Cygwin, I align all the movie frames right off the K2 computer box.

### 3.4.5 Align using SerialEM directly

Beside aligning frames at the background separately with IMOD, we can also use SerialEM plugin to align the frames
directly. From camera setup page of SerialEM interface, you can define to let SerialEM Plugin to align the frames.
Slightly different from using IMOD which aligns as separate process, SerialEM Plugin aligns all the frames from
an exposure and returns the aligned average to SerialEM main instance. This is very handy for us to obtain sample
information quickly and conveniently.

### 3.5 Monitor Data Collection In The Fly

**Author** Chen Xu  
**Contact** <chen.xu@umassmed.edu>  
**Date-Created** 2018-08-02  
**Last-Updated** 2018-09-10  
**Abstract** We already routinely align movie frames during data collection so we can check images from time to time.
We did most directly using K2 camera computers. This works very nicely. However, there are still a couple
things we feel missing. 1) we need to see the defocus range and phase shift values computated and plotted out.
2) we need to do this with no delay and without slowing down the data collection itself.
We decided to align movies and perform CTF determination using a dedicated workstation with dual GPU. Our Summer Student, Albert Xu, made this completely automatic. During data collection, a plot is showing on web browser and refreshing itself.

This document is mainly for ourselves as a check list. Hopefully, it can also be useful for your setup.

For Albert’s `ctffindPlot` project, please see https://github.com/alberttxu/ctffindPlot.

### 3.5.1 Setup for Shipping, alignframes and CTFfindPlot

1. **Ship raw data from K2 local SSD to storage tank.**
   
   Assuming storage tank is CIFS mounted onto K2 computer, as \W:\, and we have a new folder call ChenXu_20180802. We create a folder on local sd drive X:\ usually using the same folder name. We collect everying off camera onto this local SSD folder X:\ChenXu_20180802 first including all LMM, MMM maps etc. and raw TIFF data as well. We use IMOD program `framewatcher` to ship the raw data, pcm parameter files, defect file and gain reference file to storage.

   From cygwin shell terminal on K2 computer, go into local folder X:\ChenXu_20180802 and do this:

   ```
   $ framewatcher -nocom -pr W:\ChenXu_20180802
   ```

   From today - Sept 09, 2018 the latest package (currently in nightly builds) supports multiple processed folders so the collected files can be shipped into several folders. This is very useful to align them parallely by running alignframes from inside of each folder separately. A example is below:

   ```
   $ framewatcher -nocom -pr W:\ChenXu_20180802\tmp1 -pr W:\ChenXu_20180802\tmp2 -pr W:\ChenXu_20180802\tmp3 -pr W:\ChenXu_20180802\tmp4
   ```

   This will move all the raw files onto storage location, so local SSD never fills.

2. **ssh login GPU computer as you and su to “guest”, make new folders and align movies**

   ```
   $ ssh xuchen@gpu
   [xuchen@gpu ~]$ su - guest
   [guest@gpu ~]$ cd /mnt/Titan/ChenXu_20180802
   [guest@gpu ChenXu_20180802]$ mkdir rawTIFF alignedMRC alignedJPG
   [guest@gpu ChenXu_20180802]$ framewatcher -gpu 0 -bin 2 -po 1024 -pr rawTIFF -thumb alignedJPG -dtotal 46.5
   ```

   This will move raw data files (TIFF, dm4, defect, pcm) into `rawTIFF` and `_powpair.jpg` into `alignedJPG`. You can also add an option “-o alignedMRC” to move all the aligned MRC files into that folder `alignedMRC`.

   As mentioned above, one can also run a few jobs of framewatcher from multiple directories separately, so speed thing up, with or without a GPU card. You can manually run the command from tmp1, tmp2, tmp3 and tmp4. You can also ask a simple shell script to do that. The only disadvantage might that you have to “kill” when you need to stop `framewatcher` because you cannot do Ctrl_C from shell interactively in this case.

   ```
   #!/bin/bash
   for dir in tmp{1..4} ;
   do
   cd $dir
   framewatcher -bin 2 -po 1024 -o ./ -pr ./rawTIFF -thumb ./alignedJPG -thr 4 -dtotal 46.5 &
   cd ..
   done
   ```
3. Copy and edit ctffind parameter file (as “guest”, in the same folder; we usually create a new terminal from tmux by “Ctrl_B C”).

```bash
[guest@gpu ChenXu_20180802]$ cp /usr/local/ctffindplot/test/ctffindoptions.txt .
[guest@gpu ChenXu_20180802]$ vim ctffindoptions.txt
```

edit to fit your situation. The file looks like this:

```bash
ctffind << EOF
(filename)
(basename)_ali_output.mrc
1.059
300.0
2.70
0.07
512
30.0
5.0
5000.0
50000.0
100.0
no
no
no
yes
0.0
3.15
0.5
no
EOF
```

4. plot

```bash
[guest@gpu ChenXu_20180802]$ ctffindPlot
```

This will generate a plot and continuously update a file called `ctf_plot.png` which can be loaded into a web browser and let it refresh periodically. All the aligned MRC files will be moved into `alignedMRC` by the plot program after done.

For convenience, there are a few parameter files for common conditions which you can directly use with option “-t”.

```bash
[guest@gpu ChenXu_20180802]$ ctffindPlot -t /usr/local/ctffindPlot/ctffind_params/Titan_130k_NoVPP.txt
```

### 3.6 Pixelsize and Distortion Info on K2/3 Cameras

**Author** Chen Xu  
**Contact** <chen.xu@umassmed.edu>  
**Date_Created** 2017-2-23  
**Last_Updated** 2019-3-28  
**Abstract** We have K2 on Talos Arctica and GIF/K2 on Krios.

This doc lists pixelsize information for K2 cameras so you can decide which magnification you want to use for your final image. I also try to give information about image distortion on these cameras.
3.6.1 On Talos Arctica

According to FEI document http://www.fei.co.jp/_documents/DS0189-10-2014_Talos_Arctica_WEB.pdf, the Cs value for Talos Arctica is 2.7mm.

Below are pixelsizes on K2 for a few magnifications.

Table.1 Pixelsize (Å) of K2 camera on Talos

<table>
<thead>
<tr>
<th>Magnifications (X)</th>
<th>Counted (Å)</th>
<th>Super resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17,500</td>
<td>2.425</td>
<td>1.213</td>
</tr>
<tr>
<td>22,000</td>
<td>1.888</td>
<td>0.944</td>
</tr>
<tr>
<td>28,000</td>
<td>1.485</td>
<td>0.742</td>
</tr>
<tr>
<td>36,000</td>
<td>1.169</td>
<td>0.584</td>
</tr>
<tr>
<td>45,000</td>
<td>0.914</td>
<td>0.457</td>
</tr>
<tr>
<td>57,000</td>
<td>0.719</td>
<td>0.360</td>
</tr>
</tbody>
</table>

Also the distortion information at these few mags. This mag distortion is believed due to stretch on projection lens system. The measurement and correction programs are used and available from http://grigoriefflab.janelia.org/magdistortion.

Table.2 Mag Distortion Parameters for K2 camera

(Note: this is for images saved by SerialEM directly. So this is after rotation and flip applied. Please do not use this with raw frame saved by plugin without rotation and flip, as it will have different but related values.)

<table>
<thead>
<tr>
<th>Magnifications (X)</th>
<th>Dist.Angle(degree)</th>
<th>Major Scale</th>
<th>Minor Scale</th>
<th>Totat Distortion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,500</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22,000</td>
<td>65.9</td>
<td>1.016</td>
<td>1.000</td>
<td>1.56</td>
</tr>
<tr>
<td>28,000</td>
<td>24.5</td>
<td>1.015</td>
<td>1.000</td>
<td>1.51</td>
</tr>
<tr>
<td>36,000</td>
<td>22.5</td>
<td>1.015</td>
<td>1.000</td>
<td>1.51</td>
</tr>
<tr>
<td>45,000</td>
<td>21.3</td>
<td>1.016</td>
<td>1.000</td>
<td>1.56</td>
</tr>
<tr>
<td>57,000</td>
<td>22.6</td>
<td>1.017</td>
<td>1.000</td>
<td>1.66</td>
</tr>
</tbody>
</table>

3.6.2 On Titan Krios

Table.3 Pixelsize (Å) of GIF K2 camera on Titan Krios - BEFORE Sept. 27, 2017

<table>
<thead>
<tr>
<th>Magnifications (X)</th>
<th>Counted (Å)</th>
<th>Super resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26,000</td>
<td>4.505</td>
<td>2.253</td>
</tr>
<tr>
<td>33,000</td>
<td>3.588</td>
<td>1.794</td>
</tr>
<tr>
<td>42,000</td>
<td>2.939</td>
<td>1.470</td>
</tr>
<tr>
<td>53,000</td>
<td>2.274</td>
<td>1.137</td>
</tr>
<tr>
<td>64,000</td>
<td>1.863</td>
<td>0.931</td>
</tr>
<tr>
<td>81,000</td>
<td>1.485</td>
<td>0.742</td>
</tr>
<tr>
<td>105,000</td>
<td>1.170</td>
<td>0.585</td>
</tr>
<tr>
<td>130,000</td>
<td>0.910</td>
<td>0.455</td>
</tr>
<tr>
<td>165,000</td>
<td>0.720</td>
<td>0.360</td>
</tr>
<tr>
<td>215,000</td>
<td>0.569</td>
<td>0.279</td>
</tr>
</tbody>
</table>

On September 2017, Gatan serviced GIF/K2 system on Krios and they removed a housing block between column and GIF, so the pixelsize changed. The new set of values is below in Table. 4.
### Table 4: Pixel size (Å) of GIF K2 camera on Titan Krios - AFTER Sept. 27, 2017

<table>
<thead>
<tr>
<th>Magnifications (X)</th>
<th>Counted (Å)</th>
<th>Super resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>64,000</td>
<td>2.147</td>
<td>1.087</td>
</tr>
<tr>
<td>81,000</td>
<td>1.714</td>
<td>0.857</td>
</tr>
<tr>
<td>105,000</td>
<td>1.353</td>
<td>0.677</td>
</tr>
<tr>
<td>130,000</td>
<td>1.059</td>
<td>0.529</td>
</tr>
<tr>
<td>165,000</td>
<td>0.832</td>
<td>0.416</td>
</tr>
<tr>
<td>215,000</td>
<td>0.654</td>
<td>0.327</td>
</tr>
<tr>
<td>275,000</td>
<td>0.516</td>
<td>0.258</td>
</tr>
</tbody>
</table>

From March 10, 2019, K3 was upgraded on Krios to replace the K2. GIF alignment has changes too. The pixelsizes changed a lot.

### Table 5: Pixel size (Å) of GIF K3 camera on Titan Krios - AFTER March 10, 2019

<table>
<thead>
<tr>
<th>Magnifications (X)</th>
<th>Counted (Å)</th>
<th>Super resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81,000</td>
<td>1.060</td>
<td>0.530</td>
</tr>
<tr>
<td>105,000</td>
<td>0.830</td>
<td>0.415</td>
</tr>
<tr>
<td>130,000</td>
<td>0.650</td>
<td>0.325</td>
</tr>
</tbody>
</table>

### 3.7 Relion and cisTEM Docker Services

**Author** Albert Xu  
**Contact** <albert.t.xu@gmail.com>  
**Date-created** 2019-01-08  
**Last-updated** 2019-02-14

**Abstract** This is a quick information how to use Relion3 and cisTEM dockers on two Linux workstations in CryoEM Core Facility. One of the potential advantages to use docker is to avoid CUDA version conflict.

Some of the dockerfile information can be found at [https://github.com/alberttxu/cryoem_dockerfiles](https://github.com/alberttxu/cryoem_dockerfiles).

#### 3.7.1 Relions

1. on Eagle

```bash
ssh -Y relion3@172.18.8.143 -p 30003  
password = ********
```

2. on Falcon

```bash
ssh -Y relion3@172.18.8.136 -p 30003  
password = ********
```

#### 3.7.2 cisTEM

1. on Eagle
ssh -Y cisTEM@172.18.8.143 -p 30001
password = *******

2. on Falcon

ssh -Y cisTEM@172.18.8.136 -p 30001
password = *******

Note: Let other people know if you are using either eagle or falcon. For each machine there is only one account for Relion3 and one for cisTEM.

3.8 cisTEM detaching and re-attaching sessions

Author Albert Xu
Contact <albert.t.xu@gmail.com>
Date-created 2018-06-13
Last-updated 2018-06-13

Abstract In cisTEM when you close the main window, all running jobs are terminated. Xpra is a window server that saves the state of graphical applications so that you can detach from your cisTEM session and have it run in the background.

https://xpra.org/ “Xpra is an open-source multi-platform persistent remote display server and client for forwarding applications and desktop screens. ... it allows you to run programs, usually on a remote host, direct their display to your local machine, and then to disconnect from these programs and reconnect from the same or another machine, without losing any state.”

3.8.1 Installing Xpra on Linux

1. Download the Xpra repository information into your system’s package manager folder.

For our Centos 7 system,

```
sudo bash -c "curl https://xpra.org/repos/CentOS/xpra.repo > /etc/yum.repos.d/xpra.repo"
```

Xpra also supports Fedora, Debian, and Ubuntu. For Debian and Ubuntu, the package manager folder is /etc/apt/sources.list.d

2. Install

For Centos and Fedora,

```
sudo yum install xpra
```

For Debian and Ubuntu,

```
sudo apt install xpra
```
### 3.8.2 Usage Examples

1. Log into the cisTEM computer from your remote computer. You will need less strict X forwarding with the -Y option.

   \[\text{ssh} \ -Y \ \text{username@ipaddress}\]

2. Start a cisTEM process using xpra. You will need to choose a sessionID number. I arbitrarily chose 100.

   \[\text{xpra} \ \text{start} \ :100 \ --\text{start-child}=\text{cisTEM}\]

   Hit enter one more time, and now the session has been created in the background. You will have to attach to it.

3. Attach to the session. From the cisTEM computer,

   \[\text{xpra} \ \text{attach} \ :100\]

   and the cisTEM window should open. If there is only one session, you don’t need the :100

4. Detach from the session. From the command line, hit Ctrl-C and the window will disappear.

   **Note:** when re-attaching: Usually remote connections from outside the local network are laggy. Fortunately, Xpra has compressions to lessen the amount of bandwidth. To enable compression when reattaching, do

   \[\text{xpra} \ \text{attach} \ --\text{encoding}=\text{rgb} \ --\text{compress}=1\]

   This is the recommended way from Xpra.org.

### 3.9 Adding a Scalebar to Images

**Author**  Christina Ouch

**Contact**  <Christna.Ouch@umassmed.edu>

**Date_Created**  2020-01-02

**Last_Updated**  2020-01-06

#### 3.9.1 Overview

Stack file images taken with SerialEM can be processed and labeled using Imod and ImageMagick.

I have written a simple shell script that will add a scale bar to the lower right-hand side of an image. This script first converts your stack file into a series of tifs using mrc2tif (Imod), scales the image by 50%, normalizes, adds a scale bar and converts to jpg using ImageMagick.

#### 3.9.2 Prerequisites

This program requires that you have both Imod and Imagemagick installed on your computer.

On linux, you can use your package manager to install Imagemagick. Download and run the install packages Imod.
On Windows, you can use the cygwin environment to install both Imod and ImageMagick. Follow the instructions on Imod’s website to install cygwin and Imod. After the installation, rerun Cygwin installer, then select the internet as a source. Type in magic into the search bar and install the ImageMagick package.

After installation, both Imod and ImageMagick should be installed. Check that each program is install by running ‘convert –version’ and ‘imod’.

### 3.9.3 Script Download


Extract the sh-script to the directory with your stack files.

### 3.9.4 Editing the Script

The script has a few fields that needs to be updated for your particular microscope setup. Edit the top of the file for the magnifications and pixel size. For this case, we have three magnifications, 1250x, 17.5k and 45k with pixel sizes 32.91, 2.27 and 0.87 angstroms respectively. Edit these to match your microscope setup. It is important that these values match the pixel sizes for your images for each mag. Verify it by running ‘header’ command on the stack file.

| RO image file on unit 1: btx2-g2-Car7-R3-45k-edge.st Size= 690529 K |
|----------------------------------|-----------------|-----------------|-----------------|
| Number of columns, rows, sections .. | 5760 4092 15    |
| Map mode .................................. | 1 (16-bit integer) |
| Start cols, rows, sects, grid x,y,z .... | 0 0 0 5760 4092 15 |
| Pixel spacing (Angstroms)............. | 0.8700 0.8700 0.8700 |
| Cell angles ............................ | 90.000 90.000 90.000 |
| Fast, medium, slow axes .............. | X Y Z |
| Origin on x,y,z ...................... | 0.000 0.000 0.000 |
| Minimum density ..................... | 0.0000 |
| Maximum density ........................ | 6621.0 |
| Mean density .......................... | 922.64 |
| tilt angles (original,current) ....... | 0.0 0.0 0.0 0.0 0.0 0.0 |
| Space group, # extra bytes,idtype,lens . | 0 3072 0 |

This script captures the pixel size from the header and generates a scale bar based on that pixel size.

The other parameters to edit is scale_length, scale_label and scale_label_offset. The length is in nanometers and the offset is used to center the scalebar label. Adjust this value to center the label. Alternatively, set the scale-bar_label_offset to 0 to left-justify the label. The scale_x2 parameter defines the right-most position of the scale-bar. The current value of 2750 works well for K3 micrographs binned 2x. For K2 data or other cameras, you may need to reduce this value so that it is within the bounds of the bin2 image. Adjust y_anchor and y_text to position the scalebar and label respectively. The options for the header are shown below.

```
# Magification #1
mag1="1750"
apix1="32.91"
scale_length_1="1000"
scale_label_1="1000 nm"
scale_label_offset_1="10"

# Magification #2
mag2="17.5k"
apix2="2.277"
scale_length_2="100"
```

(continues on next page)
scale_label_2="100 nm"
scale_label_offset_2="55"

# Magnification #3
mag3="45k"
apix3="0.87"
scale_length_3="50"
scale_label_3="50 nm"
scale_label_offset_3="100"

# Scale bar location options
# We will use coordinate 2750,1900 as an anchor point for the right-most position on our scale bar. Labeling will be relative to that point.
scale_x2="2750"
y_anchor="1900"

# Fonts will be positioned at a y value of 1940
y_text="1940"

3.9.5 Running the Script

Place the script in the same directory with all of your stack files that need to be labeled. These stack files need to have a .st extension to be recognized by the script. Open a linux terminal or Cygwin and cd to that directory. Run the edited script. This script will create subdirectories using the mag variables defined in the header.

Note: When editing the script in windows, hidden newline characters may be added to the file. These newline characters prevent the script from running. Please run this command in cygwin to remove these hidden characters. Replace input with your original script and output for the fixed script

Command syntax:
tr -d '\r' <input >output

Example:
tr -d '\r' <labeling_script_new_v4.sh >labeling_script_new_v4-fixed.sh

Use a coding editor or notepad (do not use wordpad or office) to make future changes to the file.

3.9. Adding a Scalebar to Images
4.1 SerialEM Note: Installation and Calibration

**Author**  Chen Xu

**Contact**  <chen.xu@umassmed.edu>

**Date_Created**  2017-11-12

**Last_Updated**  2020-10-01

**Abstract**  When I helped a few sites to install and calibrate SerialEM, I had impression that most new users felt this process was very hard. I felt the same way when I initially learned to install and calibrate SerialEM by myself. I even got frustrated and had to call David for a few times. When I think back about all the troubles I had to install and calibrate SerialEM, I believe I would have an easier time if I had a brief guideline document for what steps to follow in order, and what to do in each step. The helpfile from SerialEM is very complete to provide almost all information needed, but it is perhaps a lot to read and not clear where to start for a beginner.

I wanted to list some steps here to guide you through this initial installation and calibration phase. It is like a crush list. For more detailed information, you should always find it from helpfile.

### 4.1.1 Installation

Here are steps to follow.

#### Step 1

- Ask David for the initial system file. Normally, you would fill out a “questionnaire” available at the ftp server - [http://bio3d.colorado.edu/ftp/SerialEM/questionnaire.txt](http://bio3d.colorado.edu/ftp/SerialEM/questionnaire.txt) and send it to David. David or Guenter Resch will then create a framework file on the same ftp server for you to download. This framework file is a zip file, you can download it to local like Desktop and unzip it by double clicking on the file. Beside a sub-folder “Admin” created under “C:\ProgramData\SerialEM”, the most important file in the framework is one initial system file
called “SerialEMproperties.txt”. You must have this file to get started. Please refer to the SerialEM webpage for the latest information regarding this.

**Step 2**

- Make sure your camera computer and microscope computers are on the same local network. For example, K2/3 computer can be configured to have a network interface with IP address 192.168.1.2, and FEI scope with 192.168.1.1. And they should be able to ping each other.

- You might be confused by Gatan’s DM already being able to communicate with scope, as it can detect magnification change of scope. However, this DM connection to scope is usually via serial port by a direct serial cable. SerialEM uses standard TCP/IP to communicate to a remote computer and therefore requires a standard network setup in place.

- If you configured the local network, you should have DM communicate to scope (via Gatan’s RemoteTEM) using TCP/IP too. It is a lot more robust. But this is not a concern for SerialEM operation here.

**Step 3**

- Decide which computer to install SerialEM. In theory, you can install SerialEM on either computer - camera or microscope. For K2/3 camera, SerialEM should be normally installed on the K2/3 computer, as K2/3 image returning to SerialEM locally is usually faster than via network.

**Step 4**

- Decide which type of executable to use. SerialEM builds for both 32 and 64-bit platforms. Unless you have to run it on a Windows XP, you should choose 64-bit.

**Step 5**


**Step 6**

- Unzip the installation package file downloaded. You can double click on this file, it will unzip the program into C:\Program Files\SerialEM. The folder “SerialEM” will be created automatically if there isn’t one already. The new package content will be unzipped into a new sub-folder, e.g. SerialEM_3-6-13_64.

**Step 7**

- Quit Gatan DM if it is running.

**Step 8**

- Right click on a file called install.bat in the package folder C:\Program Files\SerialEM\SerialEM_3-6-13_64 and select ‘Run as Administrator’. This will copy some files into upper folder which is C:\Program Files\SerialEM, register DM plugin file and copy it to the Gatan plugin folder at C:\ProgramData\Gatan\Plugin.
Step 9

- Manually copy a file called `FEI-SEMServer.exe` from C:\Program Files\SerialEM on K2/3 computer to C:\Program Files\SerialEM on scope computer. This is a bridging program to control scope by passing the scope function calls between SerialEM main program on remote K2/3 computer and the scope scripting interface. Run the program by double clicking on it (it needs to run or SerialEM cannot control scope). This is 32-bit application, runs on both 32 and 64-bit Windows platforms. So there is only one such executable to run on Windows 7, XP or 2000 Windows OS.

Step 10

- On K2/3 computer, Edit `SerialEMproperties.txt` file in folder C:\ProgramData\SerialEM to have proper lines in general property area to define network properties.

```markdown
#GatanServerIP 192.168.1.2
GatanServerIP 127.0.0.1
GatanServerPort 48890
SocketServerIP 1 192.168.1.1
SocketServerPort 1 48892
```

Step 11

- On K2/3 computer where SerialEM is to be installed, define a system environment variable `SERIALEM-CCD_PORT` with the value 48890 or other selected port number, as described in the section in helpfile.
- If everything goes well, you should be able to start SerialEM and it should connect to “see” both scope and DM. Congratulations!

4.1.2 Calibration

Although most of calibration results will be written into another system file `SerialEMcalibrations.txt` when you save the calibration from Calibration menu, there are a few places you need to manually edit the `SerialEMproperties.txt` to take in the calibration results. These include pixelsize and tilting axis angle - they are more like instrument parameters.

For pixelsize calibration, it is best to use standard 2160 line waffle grid. For all other calibration like Image Shift and Stage Shift, it would make things a lot easier to use a non-periodic sample. Please see the NOTE at the end of this document.

Step 0

- Determine camera orientation configuration. Make sure the image orientation from camera shot agree with that of on large screen or FluCam. If it doesn’t, try to adjust the camera orientation of Gatan K2/3 camera from Camera - Configuration. You can use beamstop to help. You should add a property entry to reflect the DM configuration so SerialEM takes care of it even someone might have changed DM configuration.

```markdown
DMRotationAndFlip 7
```

Step 1

- Edit property file to define the camera configuration information about orientation determined by step 0. SerialEM will return to main display with proper orientation. This is initial starting point for all the calibrations.
Step 2

- SerialEM - Calibration - List Mag. Scope will go through all the mags and list them on log window, from lowest to highest. Check it with what are in SerialEMproperties.txt, update that if needed.

Step 3


Step 4

- Start with lowest magnification above LM range. On Talos, it is 1250X. At close to Eucentricity, and close to eucentric focus.

Step 5

- Take a T shot with 2x binning on a K2/3 camera, make sure the counts are neither too low nor too high.

Step 6

- Take a T shot, then Calibration - Pixel Size - Find Pixel Size. The log window shows both mag index and pixel size. Edit SerialEMproperties.txt to add a line like below in K2/3 camera property section.

```
# MagIndex DeltaRotation (999 not measured) SolvedRotation (999 not measured) →
   Pixel size (nm, 0 not measured)
RotationAndPixel 17 999 999 3.396
```

Here, 17 is mag index for 1250X, and 3.396 is pixel size in nm just calibrated.

Step 7

- You might want to change to a grid without repeating features, please see “note” at the end of this document.
- Calibration - Image & Stage Shift - IS from Scratch.

Step 8

- Calibration - Image & Stage Shift - Stage Shift.

Step 9

- Calibration - Administrator, turn it on.
Step 10

• Calibration - Save Calibration.

Step 11

• Take the tilting axis value (e.g. 86.1) from step 8 - stage shift calibration, edit it into the 2nd “999” in SerialEM-properties.txt like below.

\[\text{RotationAndPixel 17 999 86.1 3.396}\]

Note: The pixel size and tilting axis can just be done for a couple of switching mags such as the lowest M and the highest LM. SerialEM uses these a couple of calibrations and all the Image Shift calibration to interpolate to obtain the pixelsizes and tilting axis angles for all other magnifications. This is very cute.

Step 12

• Increase Mag by 1 click and do Calibration - Image & Stage Shift - Image Shift

Step 13

• Repeat above step to cover all the magnification till the highest to be used such as 100kX.

Step 14

• Decrease Mag by 1 click and do Calibration - Image & Stage Shift - Image Shift

Step 15

• Repeat above step to cover all magnification till the lowest to use like 46X.

Step 16

• At about 20kX, do Autofocus calibration (only need to do at single mag).

Step 17

• Beam Crossover calibration

Step 18

• Start with most used spotsize like 7, do Beam Intensity calibration

Step 19

• repeat Beam Intensity Calibration for all other spot sizes likely to be used: 3, 4, 5, 6, 8, 9.
Step 20

- At one mag like 5000X, using spot size 9, do Beam Shift Calibration (only need to do at single mag).

Step 21

- Usually, people use the lowest M mag for Low Dose View beam and with large defocus offset such as -200 or -300 microns. You need to the calibrate High-Defocus Mag for this View mag. This will make stage shifts still good for such large defocus, as they are interpolated for the defocus offset.

Note:

- Calibrations needed to be done for both mP and nP mode include: beam crossover, beam intensity, beam shift and autofocus.

- Waffle grating grid is good and handy for pixel size calibration, but it is not ideal for Image Shift and Stage Shift calibrations, as the waffle pattern might screw up the correlation in the calibration procedures. I found the normal Quantifoil grid with some 10nm Au particles absorbed onto can be very good for normal calibration purpose. I glow discharge a Quantifoil grid and add 1 ul deca-gold solution on the grid and let it dry.

- I found that standard PtIr grid for TFS to perfom Thon Ring test also works very well for calibration purpose.

- Most of SerialEM actions are cross-correlation based, including calibrating. Therefore, a clean and recent preparation of camera gain reference file is desired, because it will help to have less screw-up due to fixed noise pattern dominating the cross-correlation.

4.2 SerialEM Note: Make All LMM Maps Automatically

Author  Chen Xu
Contact  <chen.xu@umassmed.edu>
Date_Created  2018-04-03
Last_Updated  2018-04-04

Abstract  We found that it was extremely useful to be able to make Low Mag Montage (LMM) maps for all the grids in autoloader cassette automatically. Since it can take a while for multiple grids, you should give yourself a good break while scope is busy working without feeling guilty.

4.2.1 Procedure

Here are steps to follow.

1. Dock the cassette. After temperature in the autoloader recovers, do Inventory.

2. Setup image condition. I do it inside or SerialEM Low-Dose mode. I use Search area for the job. On our Krios with GIF/K2, I set mag for Search as 220X (I cannot go lower as wish, because some hardware piece in lower portion of column will start to cut into image.).

3. Setup proper exposure and binning for Search parameter from camera control panel. I usually use binning 2, exposure 1 seconds, and in Linear mode (mP mode, Spotsize 8).

4. Take a Search shot, make sure the count value is proper, no beam/aperture edge in the image.
5. Navigator - Montaging & Grids - Setup Full Montage. Make sure **Search** is checked in the montage setup dialog window. Define a file like LMM.map.

6. Edit script **Cars** to reflect cartridge and sample information, like below:

```plaintext
ScriptName Cars

## parameter of 1) folder 2) Car and 3) sample name
## to be called by LMMCars and other

# define where to save
SetDirectory X:\Munan_20180402

## define cartirges and sample names
cat = { 2 3 4 5 6 7 }  
name = { 56-g1 56-g2 56-g3 56-g4 54-g2 54-g4 }

Here you define folder location, cartridge #, and sample names. The map filename will have the info in it, such as LMM-Car2-56-g1.st.

7. Now run the Script **LMMCars** as below:

```plaintext
ScriptName LMMCars

# LMM for multiple cartriges, assumes the montage file opened.

####################################
# navigator must be open
####################################
Call Cars

##### No editing Below ###########
CallFunction LMMCars

## in the end, rise mag to settle temp & Close the valves
#GoToLowDoseArea  V
SetColumnOrGunValve 0

####################################
Function LMMCars 0 0
Loop $#cat index
LoadCartridge $cat[$index]
#SetNavRegistration $cat[$index]
SetColumnOrGunValve 1
MoveStageTo 0 0
OpenNewMontage 0 0 LMM-Car$cat[$index]-$name[$index].st
Montage
NewMap
CloseFile
EndLoop

EndFunction
```

4.2.2 Convert LMM maps into JPEG format

For easy display and small file size, we usually convert all the maps in MRC format to JPEG.

- Set Bin Overview to 1 on Montage control panel (default is usually higher than 1 with montage from command)
• Load the map file, the overview will be displayed in a specific buffer such as Q
• Run a small script

```plaintext
scriptName LMM->JPEG
# convert to JPEG format for easy display
SetDirectory X:\Munan_20180402
# reduced image for good JPEG density range, reduced one will be in A
ReduceImage Q 2
SaveToOtherFile A JPEG JPEG LMM-Car2-56-g1.jpeg
```

Note:
• The JPEG image generated from above script is true JPEG file, not a JPG compressed TIFF file as before. Compressed JPG cannot be displayed properly by Photoshop and ImageJ, although preview, paint and webbroser can show them nicely.
• You can also convert MMM maps and single shot MRC image the same way.

4.3 SerialEM Note: Setup Dummy Instance

**Author**  Chen Xu  
**Contact**  <chen.xu@umassmed.edu>  
**Date_Created**  2017-12-16  
**Last_Updated**  2019-05-23  
**Abstract**  Dummy instance of SerialEM can be very useful in two cases: 1) to be used on the same computer while main instance of SerialEM is busy collecting data; 2) can be used on a remote computer, e.g., a home computer to pick particles. Here I list what is needed to setup dummy instance in these two cases.

4.3.1 On the same computer

Since SerialEM is installed and working, this is very simple.

1. make another alias (shortcut) from main instance icon.
2. edit new shortcut’s property to add “/DUMMY” at the end of the Target line, as below.

**Fig.1 Property Widows for Dummy Instance**
4.3.2 On a remote computer

1. install SerialEM onto a remote computer, as described in Installation and Calibration.

2. get property and calibration files from a working scope and put them in the default location C:\ProgramData\SerialEM, on a Windows 7 or 10 system.

3. make a shortcut from the executable and edit the property of the shortcut to add “/DUMMY” to the end of Target line, as above.

4. repeat the last two steps for a different scope.

4.3.3 Make Multiple Dummies For Different Scopes

If we want to make multiple dummies on the same computer, say, one for Talos and one for Krios, we modify above procedure slightly different.

1. put two sets of systems files (property and calibration files) to two folders, such as C:\ProgramData\SerialEM\Krios and C:\ProgramData\SerialEM\Talos.

2. Edit both property files to insert two lines into each one.

```
NoScope  1
NoCameras  1
```

3. Instead of /DUMMY, the /Krios or /Talos is used in the Target line.

Fig.2 Property Windows for Talos Dummy Instance
4.3.4 On a Mac computer

Thanks to Pranav Shah <p.shah.lab@gmail.com> who shared with me how he setup SerialEM on a Mac computer using Wine. It turns out fairly straightforward, as long as you know which wine to install. The nice tutorial Installing Wine on Mac written by David Baumgold is easy enough to follow.

4.4 SerialEM Note: Setup LD with Mix of mP and nP Modes

Author  Chen Xu

Contact  <chen.xu@umassmed.edu>

Date-created  2018-03-31

Last-updated  2020-09-21

Abstract  There are cases and situations that people want to use nanoprobe(nP) mode, but nP is not comfortable for lower mag range such as first a few magnifications just above LM, because the beam doesn’t spread wide enough to cover entire camera area. This forces us to use mP for View and nP for rest of LD areas, namely F, T and R etc.

However, most people find it hard to setup LD conditions with the mix of nP and mP modes. I had frustrated time doing so too. This is, I think, mainly because nP and mP don’t share the same origins for beam shift and defocus (and beam tilt too) - they have their one origins. In SerialEM, all the LD conditions are linked together. Therefore, the separate origins of focus and beam shift for mP and nP modes give extra hard time setting up LD in this mix use of nP and mP.
SerialEM already has a way to deal with this problem. I hope this doc makes it clearer and easier to follow practically.

### 4.4.1 Procedure Setting Up LD with $mP$ and $nP$

In my case, I use $mP$ for View area with -300 microns focus offset. All other areas - F, T and R are with $nP$. I usually use the same spot size for everything.

0. Before LD is turned on, make sure beam is centered for both $mP$ and $nP$ beam. I usually use Direct Alignments to do this with $mP$ and $nP$ beam. That is, turn $mP$ on, Directly Alignments - Beam Shift (multi-function to center) - done. Repeat with $nP$ mode.

1. Turn on SerialEM LD.

2. Lower Down large screen or insert screen.

3. From Task - Specialized Options, make sure the “Adjust Focus on Probe Mode Change” is NOT checked.

4. Set View Defocus Offset to 0 using dial Up-Down button on SerialEM LD Control Panel.

5. Select R area (radio button) on LD control panel.

6. On microscope right panel, press “Eucentric Focus”.

7. Reset Defocus (L2 button on our current setup for soft buttons, yours could different), this makes defocus display 0.

8. Select V area (radio button) on LD control panel.

9. wait 6-7 seconds to allow scope to switch to this mag and $mP$ mode.

10. On microscope right panel, press “Eucentric Focus”.

11. Reset Defocus (L2 button on our current setup for soft buttons, yours could different), this makes defocus display 0.

12. Set View Defocus Offset to target value (-300 in my case) using dial Up-Down button on SerialEM LD Control Panel.

13. From Task - Specialized Options, make sure the “Adjust Focus on Probe Mode Change” is NOW checked.

That’s it.

### 4.5 SerialEM Note: More About Z Height

**Author** Chen Xu

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**Date** 2017-12-09

**Last Updated** 2020-10-11

**Abstract** This is the first of a series of SerialEM notes I have wanted to write for a while. An application of using SerialEM, even a simple one, could be very useful and “handy” for a SerialEM user. I try to give more explanation for what I did, rather than to just present plain lines of codes (yes, SerialEM scripting code) so that it can be helpful for a SerialEM user, especially a new comer to understand better how SerialEM works.

Quickly and accurately moving specimen to eucentric height is a frequently needed task. Everything is going to be easier if specimen is at eucentric height and objective lens at eucentric focus. I wrote a little document before
how to use tilted-beam method to do this using SerialEM “SerialEM HowTo: Positioning Z”. In this note, I give you an improved version and hopefully it is easier to use and more robust too.

4.5.1 Background Information

SerialEM has built-in task function to do eucentricity using stage-tilt method. It is robust, but slower than beam-tilt method. Beam-tilt method is opposite to autofocus function. When specimen is at eucentric height and objective lens at eucentric focus, the tilted beams produce precise overlapped image pair. This is how autofocus works. This principle can also be used to adjust specimen height, because if we preset objective at eucentric focus, cross-correlation between two tilted beam images also provides information how much specimen is away from eucentricity. Specifically, beam-tilt method used here does a few things:

- it sets scope objective lens to eucentric focus value
- and measures the defocus value for current specimen height using tilted-beam image pair,
- it then changes stage position to that reported value but in opposite direction,
- moves stage to determined direction and distance,
- and it iterates until the reported defocus value is close enough to zero.

The beam-tilt method works very nicely most of the time and it is pretty quick. However, there are a couple of things making it less perfect. First, the signal becomes very weak when stage is already close to eucentricity. We all know the contrast is the lowest when focus matches z height. We can use focus offset to increase the contrast, but non-linear property causes some inaccuracy. The calibrated standard focus value could also change a little with time and scope condition. All these together make it less robust.

When we use SerialEM Low-Dose mode, we often give large focus offset such as -200 microns to View area (I call it View beam) to make the View image good contrast. If we can use this large defocused View beam to obtain tilt-beam pairs for measuring defocus value accurately, that would be ideal.

4.5.2 Z_byV2 Function

The function code is below.

```
Function Z_byV2 2 0 iter offset
Echo ===> Running Z_byV2 ...

# for defocus offset of V in Low Dose, save it
GoToLowDoseArea V
SaveFocus

## set objective lens
SetStandardFocus 0
ChangeFocus $offset

## Adjust Z
Loop $iter
Autofocus -1 2
ReportAutofocus
If ABS $repVal1 < 0.3
    Echo Close enough, break...
    Break
Endif
Z = -1 * 0.72 * $repVal1
Z = ROUND $Z 2
```

(continues on next page)
The real difference between this and previous version \texttt{Z\_byV} is an additional line inserted after \texttt{SetEucentricFocus}:

\begin{verbatim}
SetStandardFocus 0
ChangeFocus $offset
\end{verbatim}

These two lines will set objective lens to a specific strength; the large defocus offset also offers good contrast. A special thing here is to measure defocus value using a LD\_View beam. The advantage of this command is to use high contrast image.

\begin{verbatim}
Autofocus -1 2
\end{verbatim}

This function should be called in script like this way:

\begin{verbatim}
CallFunction MyFuncs::Z\_byV2 3 -288.32
\end{verbatim}

Obviously, the -288.32 is to pass to variable $offset in the function, and 3 to iteration variable $iter.

Now question is how to determine this offset value for accurate Z height for and under current scope condition.

\subsection*{4.5.3 Find the Offset Value using Script \texttt{FindOffset}}

If we found the good “offset” value, it will be good for some time, at least this session. So this like a short term calibration. Here is how to find it:

- Adjust specimen to Eucentricity, using FEI interface tool or SerialEM task function
- run script as below.

\begin{verbatim}
ScriptName FindOffset
# script to find proper offset value to run Z\_byV2
# assume specimen is ON the eucentricity
## Eucentric Z
##
#Eucentricity 3
ReportStageXYZ
Z0 = $repVal3
#Z0 = 187.81
SetCameraArea V H
ReportUserSetting AutofocusBeamTilt BT
echo BT = $BT
SetUserSetting AutofocusBeamTilt 1.6
## now find the offset
# for initial offset, get a close value from current setting
ReportUserSetting LowDoseViewDefocus
\end{verbatim}
offset = 0.72 * $repVal1  # or  
# offset = -153         # some starting value from previous run

Loop 10
  CallFunction MyFuncs::Z_byV2 1 $offset
  ReportStageXYZ
  Z = $repVal3
  diffZ = $Z - $Z0
  echo $diffZ
  If ABS $diffZ < 1
    offset = ROUND $offset 2
    echo >>> Found "offset" is $offset
    echo >>> run "Z_byZ2 $offset"
    Break
  Else
    offset = $offset + $diffZ
  Endif
EndLoop

X = { 0 0 0 0 0 0 0 0 0 0 0 }
Y = { 0 0 0 0 0 0 0 0 0 0 0 }

temp_offset = $offset - 10

Loop $#X i
  Echo $i
  Echo $X
  Echo $Y
  CallFunction MyFuncs::Z_byV2 1 $temp_offset
  ReportStageXYZ
  Z = $repVal3
  diffZ = $Z - $Z0
  Y[$i] = $diffZ
  X[$i] = $temp_offset
  temp_offset = $temp_offset + 2
EndLoop

LinearFitToVars X Y
echo $repVal1 $repVal2 $repVal3 $repVal4
real_offset = - $repVal3 / $repVal2
echo =====> $real_offset
SetUserSetting AutofocusBeamTilt SBT
RestoreCameraSet

It uses function Z_byV2 to see which offset value to recover the Z height determined early by other method. It first find an offset value that recovers Z height within 1um (you can define 0.5), then it uses a fitting method to refine this value to make it more accurate. If this script runs and gives offset value as -153.51, then you should use the function with this value.

Note: This offset value changes when V beam size changes. Therefore, it makes sense to do this “calibration” of finding offset value after all the Low Dose area conditions are set and fixed. With the “good” offset value that gives good results, the program works very reliably, if the V beam doesn’t change. For example, on our Krios, the V beam (called Low Dose area V) illumination area stays the same, the script works very well.
4.5.4 Note about Damping Factor

You might have noticed I used 0.72 in the value of Z movement:

\[ Z = -1 \times 0.72 \times \text{RepVal1} \]

This is to compensate the non-linear behavior of autofocus measurement, with the condition of large defocus offset used. For example, when the stage Z position is -100 microns off from the eucentric height, the autofocus measurement gives something like -136 microns. Therefore, using a proper damping factor (100 / 136 ~ 0.73 here) can make the Z movement more accurately to the target. Since this is a non-linear behavior, this damping factor changes with Z. For example, when Z is off very little, say 5 micron, the factor can be larger like 0.85. One would naturally try to find the curve so to use a more accurate damping factor value in interpolating fashion dynamically. However, if you think about backlash of stage movement, it is the best to avoid any overshoot. Using a single, slightly smaller value could help to keep stage move with backlash corrected when iterating a few times. 0.72 is found to be a good number in our situation.

What exactly the damping factor value should you use? I suggest you move your stage 200 microns away, and you calculate the the ratio of 200 to autofocus measurement value $\text{RepVal1}$ after \text{ReportAutofocus} (damping factor $= 200 / \text{RepVal1}$) and use the result.

If setting correctly, even your stage is more than 150 microns away, calling the function with three rounds of iteration can bring the stage to eucentric height within 0.5 microns in a few seconds. Amazing to me.

4.6 SerialEM Note: Speed, Speed and Speed

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Created 2017-12-26
Updated 2018-08-23

Abstract Speed of data collection is important, specially for a facility running 24/7 and for whole year long. If we can save a few seconds for an exposure, which might not sound much, it becomes a lot accumulated in a year time. We could collect more data, help more users if we are more efficient.

Thanks to the author and developer, David Mastronade, SerialEM is always under active development and improvement. I believe efficiency has been one of the goals for development. For example, positioning routine - Realign earlier always run two rounds: first round to align to the center of a map or a center of a piece of a map; and second round to align to the actual target. Later version of SerialEM can skip the first round of center align if it is done recently. This saves huge mount of time for single particle application.

In this document, I want to mention a few tips which you might or might not be aware of. Some of the things are related to newly added features of SerialEM. Some are from my personal experience. It would make me happy if they can also save you a few seconds.
4.6.1 Minimize Mag Switch

Switching between mags takes time. You can definitely feel the slowness of mag switching, say bewteen 1250X and 130kX. You might think of turning off some lens normalization via FEI interface, but I always worry the stability of the system might suffer. I am not trying to save time from there.

However, I found that I could save time from this positioning actions:

```
RealignToNavItem 1
Copy A P
ResetImageShift

View
AlignTo P
ResetImageShift
```

This little script uses the last image of Realign routine which has some image shift in it, as reference to do another round of aligning and ResetImageShift to get rid of image shift. It seems to be flawless and it is actually working. But I noticed the scope switched from View mag to Record mag for short period of time and then switch to View mag again during the actions. There is an extra switch there! At first, I was very puzzled, then I realized that I had been using a wrong command!

The problem is caused by the argument 1 in command line:

```
RealignToNavItem 1
```

The argument “1” here means scope will resume to the state before realigning routine. And that state is high, record mag from exposure of last navigator point. Therefore, with above script, scope switch to View mag to perform realign function and then it switches back to record mag. It then switches to View mag again when at line of

```
View
```

If I put “0” as argument for “RealignToNavItem” like here:

```
RealignToNavItem 0
```

then scope stays in View mag. It at least saves 5 seconds!

4.6.2 Order of Actions

When we use “Acquire at points . . .” to collect single particle data, the default action of control mechanism is to move stage to the new item’s stage position. And then it starts to run the actual collecting script like “LD”. If the first action in the “LD” script is RealignToNavItem, the scope changes to the map mag, usually is View mag. Therefore, there are two physical actions here involved - stage move and mag switch.

For whatever reason, before stage movement finishes, scope can not do anything. Since “RealignToNavItem” will also introduce stage movement, if we ask RealignToNavItem to take care of mag switching and stage movement, it can move stage while mag switching is happening. This can initiate two actions at the same time; therefore, saves time.

This is new feature added not long ago. In late versions, there is a check box “Skip initial stage move” in “Navigator Acquire Dialog” window for this very purpose.

4.6.3 Using Beam Tilt for Z Height Change

We all know how important is to have Z height close enough to eucentricity. If there is 10 micron off, then everything won’t work quite right. SerialEM’s built-in function “Eucentricity” is a robust function, straightward to use. However,
it takes some time to run due to stage tilting and settling time required. I wrote two scripts (functions) “Z_byG” and “Z_byV” to use beam tilting pair for the same job. They do not use stage tilt and takes less images, therefore, it runs faster. You do have to get calibration done for Standard Focus value though.

In single particle data collection, sometimes, we have to make MMM maps from many meshes. The very first thing we do after getting to the center of a mesh is to fix the eucentricity height before map is collected. Using beam tilting method, it can save bit of time in this process.

From my own experience, doing the eucentricity using beam tilting method even works fairly well in low range of magnifications. It seems to be accurate enough for parallel beam capable scope like Krios.

4.6.4 Relaxing Stage After Moving to Target

For high quality movie stacks, even we use short frame time, the stage drift rate is still needed to be monitored. Some people use longer frame time due to worry the signal within frame being too weak for frame aligning later. In this case, drift control needs to be in place seriously, as stage naturally drifts and it can have different speeds at different time.

SerialEM can ask stage to move with backlash retained or imposed. After such movement, relaxing stage stress by moving backwards a small distance can help stage settle down much faster, at least to a normal behaviour stage. This feature has been implemented into SerialEM now. I have found it saves us huge mount of time for our routine data collection. I strongly recommend to upgrade to later version for this reason.

The feature is used this way:

```
ResetImageShift 2
```

2 means moving stage with backlash imposed or retained, and moving backward 25nm distance in the end. This small distance doesn’t actually move the stage location, but helps relax the stage mechanical stress. You can also ask to move backwards a different distance by adding 2nd argument to the command, like below.

```
ResetImageShift 2 50
```

This will move 50nm, rather than 25nm as default.

Moving stage with backlash imposed takes extra time itself. Therefore, we don’t want to move stage always using this way, but the final movement to the target. Here is a portion of a function called “AlignToBuffer” I wrote.

```
## align
Loop $iter ind
  $shot
  # still need crop, for Camera which doesn't do flexible sub-size like FEI cameras
  ImageProperties A
  XA = $reportedValue1
  YA = $reportedValue2
  If $XA > $XP OR $YA > $YP
    echo CallFunction MyFuncc::CropImageAToBuffer $buffer
    CallFunction MyFuncs::CropImageAToBuffer $buffer
  Endif
  AlignTo $buffer
  If $ind == $iter
    # last round of loop, relax stage
    ResetImageShift 2
  Else
    ResetImageShift
  Endif
EndLoop
```
Here, I asked stage to relax only at final round of iteration. If you use this function, you should update it to include this nice feature.

Alternatively, we can also directly move stage backwards after ResetImageShift. The idea is if we know the align shift in last image that is to be cleared out by ResetImageShift command, we know the directions of stage movement and we just stage backwards a little. This again demonstrates the powerfulness of flexibility of SerialEM scripting!

```
AlignTo $buffer       # comment out this line if last action is RealignToNavItem
ResetImageShift

## relax
# report shift in buffer A from last round of Align
# move stage 0.025um in opposite directions
ReportAlignShift
shiftX = $repVal5
shiftY = $repVal6

# just in case it got a blank image so no shift found
If $shiftX == 0 OR $shiftY == 0
  signX = 0
  signY = 0
Else
  signX = $shiftX / ABS ( $shiftX )
  signY = $shiftY / ABS ( $shiftY )
Endif

moveX = -1 * $signX * 0.025
moveY = -1 * $signY * 0.025
echo Relaxing ...
MoveStage $moveX $moveY
```

This relaxing portion can be put into a function so the script can be neater.

```
AlignTo $buffer       # comment out this line if last action is RealignToNavItem
ResetImageShift
CallFunction Relax

Function Relax 0 0
## relax
# report shift in buffer A from last round of Align
# move stage 0.025um in opposite directions
ReportAlignShift
shiftX = $repVal5
shiftY = $repVal6

# just in case it got a blank image so no shift found
If $shiftX == 0 OR $shiftY == 0
  signX = 0
  signY = 0
Else
  signX = $shiftX / ABS ( $shiftX )
  signY = $shiftY / ABS ( $shiftY )
Endif

moveX = -1 * $signX * 0.025
moveY = -1 * $signY * 0.025
echo Relaxing ...
MoveStage $moveX $moveY
```

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And if you allow final position with a little Image Shift (very OK with coma compensation in place), then this part positioning can be accurate and simple:

```
AlignTo $buffer  # comment out this line if last action is RealignToNavItem
resetImageShift
CallFunction Relax
AlignTo $buffer  # final round of align to buffer, so position is accurate
```

### 4.6.5 Using Compression on K2 Data

Most people collect single particle data with K2 camera using Super-resolution mode. One of the “hidden” advantages is that the Super-res raw frame data is in 4-bit unsigned integer type, and there are lot of zero’s there. Such data can be compressed very efficiently and losslessly using mature compression algorithms. Unfortunately, MRC is not a file format that can directly use those algorithm libraries for compression. TIFF is.

SerialEM implemented this compression feature in. It gives options not to apply gain reference before saving and to use compressed TIFF as saved data format. This might not sound a big deal, but the minimal size of lossless compressed raw dataset makes huge difference for a facility that runs constantly. The small dataset file size is not only beneficial for long term storage, but also makes it a lot faster to transfer and copy off. Network behaves very differently for a lot of 400MB datasets from a lot of 10GB datasets.

Personally, I recommend to use compressed TIFF and without gain normalization applied for data saving format.

### 4.6.6 Using Local HDD or SSD

It is usually fine to save the frame data directly onto a large size data storage network system. In our systems, a CIFS mount initiates a network drive on K2 computer so that we can directly save to that. However, in the case that the storage system is busy doing some other tasks such as transferring data to customers, being used by local image processing programs etc., directly saving to network drive could take extra time than saving onto local SSD drive on K2 computer.

In our experience, it is best to save raw data on local SSD or HDD first, and then align frames using framewatcher (IMOD program) on-the-fly and let the `framewatcher` move the processed raw frames and aligned output average to network drive. This way, not only the local SSD drive will never be filled, but also the network activities on the LAN are spread out more evenly. Data collection won’t slow down at all due to network performance.

### 4.6.7 Using Multishot

Multi-shot method is perhaps the most efficient way for single particle data collection. It can speed up quite a bit. Please refer a separate note - Tackle the Coma.

### 4.7 SerialEM Note: Tackle the Coma

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**Date-created**  2018-3-11

**Last-updated**  2018-3-21
Abstract. For high resolution data, coma is always a concern or something we don’t want to miss or ignore. With image-beam shift, even on a carefully aligned, coma-free scope, there might always be some coma induced by the shift. On the other hand, if we can collect CryoEM data with some image shift, that would increase the efficiency a lot. The question is how much worse the data becomes with certain mount of image shift in the shots. A more important question is if we can have a way to correct coma that is induced by the image-beam shift.

In this note, I try to explain how to assess coma induced by the shift, more or less quantitatively and how to correct the coma using currently available functions in SerialEM.

This is very fresh, work in progress, both in SerialEM and this document.

4.7.1 Background

The coma we discuss here is axial coma. It is how much the incident direction of electron beam is off from perfect optical axis. This small angle makes electron beam hitting on specimen not perpendicularly. The effect might not be easily seen visually and directly from a typical single particle electron image, but it is very real. If you look at inorganic material lattice image in electron microscope, a tiny alpha angle change will make the lattice image no longer even and symmetric. For single particle image, this introduces a phase error because we assume all the images are taken with perpendicular incident beam. It is hard or almost impossible to correct this error from all the images taken with coma, at least for single particle images. Therefore, if we can eliminate this error experimentally, that would be a good thing to do.

Align scope to minimize coma

There are tools from microscope operating software interface. For example, in FEI Tecnai/Talos/Titan interface, there are Rotation Center and Coma-Free alignment you can use to minimize this instrument parameter. You might call this manual alignment.

There are also separate tools (programs) to align the scope for coma-free purpose in more automated fashion. FEI has Auto-CTF, Legion uses Zemlin plateau method to correct coma; SerialEM also has its own built-in functions to do coma-free alignment. In SerialEM, the two functions are called BTID Coma-free Alignment and Coma-free alignment by CTF. One uses beam tilt induced displacement (BTID) and other uses fitted CTF information. CTF method is quick and accurate, but it does require clear Thon rings to fit. It gives options to use full 9-piece panel (Zemlin plateau) or 5-piece method. They work fairly well to my eyes.

4.7.2 Linearity relationship between Image Shift and Induced Beam Tilt

With the built-in tools to correct and measure coma, it is possible to study the behavior of beam tilt induced by image shift. On a well aligned scope, image shift still introduces extra beam tilt, because the beam is no longer on axis anymore, and alignment for beam shift pivot points perhaps is never accurate enough. This is known, but the relationship between the them was not clear.

With the lastest version, we can run following SerialEM script (this is modified from the David Mastronade’s original one) to learn the behaviors.

```
$ScriptName BTvsIS
extent = { 0.5 1.0 1.5 2.0 2.5 3.0 }
FixComaByCTF
Loop $#extent ind
    ReportImageShift xbase ybase
    SetImageShift $xbase + $extent[$ind] $ybase
    FixComaByCTF 1 1
    ReportComaTiltNeeded xpxplus ypxplus
```

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SetImageShift $xbase - $extent[$ind] $ybase
FixComaByCTF 1 1
ReportComaTiltNeeded xpminus ypminus
SetImageShift $xbase $ybase + $extent[$ind]
FixComaByCTF 1 1
ReportComaTiltNeeded xpyplus ypyplus
SetImageShift $xbase $ybase - $extent[$ind]
FixComaByCTF 1 1
ReportComaTiltNeeded xpyminus ypyminus
SetImageShift $xbase $ybase
xpx = ($xpxplus - $xpminus) / (2 * $extent[$ind])
ypx = ($ypxplus - $ypminus) / (2 * $extent[$ind])
jsonp = ($xyminus - $xminus) / (2 * $extent[$ind])
ypyminus = ($ypyplus - $ypminus) / (2 * $extent[$ind])
echo extent = $extent[$ind] matrix = $xpx $ypx $xpy $ypy
EndLoop

The results obtained indicate linearity relationship between extra beam tilt (coma needed to be corrected) and image shift amount. And we also found that for fixed condition, especially beam size, the bi-linear matrix remained remarkably consistent. This provides a base for automatic correction of coma induced by image shift.

Note: The linear matrix depends on scope alignment, specially Beam-Shift pivot points. We also found that it is sensitive to beam size.

On FEI microscope, image shift and beam shift are linked. The “action” of image shift results in image shifting below Obj lens AND beam shift above obj lens.

### 4.7.3 Procedure to correct the coma induced by image-beam shift in SerialEM

1. decide LD image conditions specially beam size (C2% or IA).
2. perform coma-free correction routine, SerialEM - Focus/Tune - Coma-free alignment by CTF
3. calibrate the linear matrix for current image condition, SerialEM - Calibration - Coma vs. Image Shift.
4. save the SerialEM setting file. (yes, this calibrated matrix is saved in setting file.)
5. Setup multi-shot condition from SerialEM - Navigator - Montaging & Grids - Set Multi-shot Parameters..., and make sure the check box “adjust beam tilt to compensate...” is checked, as shown below.

Fig.1 Setup Multi-shot and Beam Tilt Compensation
Note: There is a script command to retrieve the calibrated Coma vs ImageShift Matrix

```
reportComaVsISmatrix xpx xpy ypx ypy
```

It is very safe to change beam tilt this way, as beam tilt will always get restored to its original value after being corrected for this specific image shift. The exception is SerialEM program crash during the multi-shot routine finishes. If that happens, which is very rare, then one only needs to perform SerialEM - Focus/Tune - Coma-free alignment by CTF after restarting SerialEM. The matrix saved in the setting file should be still good as long as your beam size remains the same.

As always, for details please read the helpfile related sections. Two of them are:

- Coma vs. Image Shift command (Calibration - Focus & Tuning sub-menu)
- Multiple Record Setup Dialog

### 4.8 SerialEM Note: Utilize JPG File format

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**Created**  2018-04-29  
**Updated**  2018-05-03

**Abstract**  SerialEM supports MRC and TIF file formats. Although SerialEM doesn’t directly save JPG file from graphic interface as default, it does support JPG file format.

JPG file format can be very convenient for sharing and viewing due to its small file size. When we send screening results to users via DropBox, we also upload all the JPG files for LMM, MMM maps and single record shots. These JPG files take very little disc space and can be directly viewed on the web browsers. People love this feature.
In this doc, two scripts or functions were introduced to conveniently save maps and single shots to JPG format. It is nice to be able to do it in-the-fly with SerialEM imaging.

### 4.8.1 Save Map Overview to JPG

The function to save maps to JPG is below.

```plaintext
Function MapToJPG 0 0

# SerialEM Script to convert map overview to a jpg image.
# it works on currently selected map item and should work for "Acquire at points...".
#
# Chen Xu <chen.xu@umassmed.edu>
# Created: 2018-04-27
# Updated: 2018-04-27
#
# skip non-map item
ReportNavItem
If $RepVal5 != 2       # if not a map item
   Echo -> Not a map item, exit!
   Exit
EndIf
EndFunction
```

One of tricks here is to load map into a buffer **unbinned** before saving to JPG. When we make montage maps using script to open montage files, the default binning for overview display is usually not 1. To take advantage of full resolution of the map, we load it unbinned. The other trick is to define a temporary loading (Read-in) buffer so the read-in buffer setup in **Buffer Control** panel becomes irrelevant.

This function can be used by inserting a line right below line `NewMap` in your script like here:

```plaintext
NewMap
CallFunction MyFuncs::MapToJPG
```

It can also be used standalone as a script for multiple maps using “Acquire at points...”.

```plaintext
ScriptName MapToJPG
CallFunction MyFuncs::MapToJPG
```

This function and its script work on **current** item of map. However, if you run on a point item and create a map from this, the above function or script won’t work on this in-the-fly situation because **current** item is the very point item instead of newly created map. For this, one needs to use a slight different one as below.

```plaintext
Function NewMapToJPG 0 0

# SerialEM Script to convert last item - map overview to a jpg image.
```

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# It uses Note string as part of jpg filename.
# it works on an item which creates a map and should work for "Acquire at points..."
# as "Run Script after".
#
# Chen Xu <chen.xu@umassmed.edu>
# Created: 2018-04-27
# Updated: 2018-04-30
#
#
# skip non-map item
ReportOtherItem -1  # last item - supposedly the newly created map.
If $RepVal5 != 2  # if not a map item
  Echo -> Not a map item, exit ...  
  Exit
EndIf

# load map overview into Q unbinned
SetUserSetting BufferToReadInto 16  # Q is 16th in alphabet, if A is 0.
SetUserSetting LoadMapsUnbinned 1
LoadOtherMap -1  # last item on the nav list

# make a jpeg image
ReduceImage Q 2  # assuming loading buffer is Q, and reduce 2 to make JPG
  --image density range more pleasant
SaveToOtherFile A JPG JPG $navNote.jpg
EndFunction

The trick here is to Report and Load the last item in the nav list which is the newly created map.

### 4.8.2 Save Single Shots to JPG

We can also save every single shot to JPG format along with MRC images. The MRC file is required to be opened. The JPG filename contains root name of the MRC file and section numbers.

```
Function AToJPG 0 0
  # SerialEM Script to save image in buffer A to a jpg image.
  # It reduces image in A by 2 for comfortable JPG density range. It
  # takes current filename and Z into jpg filename. Therefore, MRC file
  # is required to be opened.
  #
  # Chen Xu <chen.xu@umassmed.edu>
  # Created: 2018-04-29
  # Updated: 2018-04-29

  ReportCurrentFilename 1
  root = $RepVal1
  ReportFileName Z
  z = $RepVal1

  ReduceImage A 2
  SaveToOtherFile A JPG JPG $root-$z.jpg
EndFunction
```

It can be used after saving MRC image for each exposure, like below:
4.9 SerialEM Note: Setup Email on K2 Computer

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Created 2018-05-05
Updated 2018-05-05

Abstract SerialEM can send email; this feature has been there for a long time. I did not feel this was a must have so did not pay much attention to it. Now, we run scope 24/7 and like to get notification when something is wrong so we won’t lose too much time.

Although setting up a standalone email server might not be the easiest thing, setting up a SMTP server and relay service on K2 computer is not that hard. This is partially because K2 computer is running a server operating system - Windows 2008 R2.

In this doc, I list what I have done to setup email notification on our SerialEM system.

4.9.1 Setup SMTP and Relay Service

I followed instructions in an on-line article Setup and Configure SMTP Server on Windows Server 2008 R2. It has images for each step and very easy to follow.

4.9.2 On SerialEM Part

There are a few steps we have to do on SerialEM side.

1. add two property lines in property file:

```plaintext
# SMTPserver
SMTPServer 127.0.0.1
SendMailFrom admin@talos-k2.cryoem.umassmed.edu
```

2. define email address to receive the notification. This from Tilt Series Menu - Set Email Address.

3. insert a command line in main collection script for single particle data collection. Location of this line doesn’t matter.

```plaintext
ErrorBoxSendEmail Script Stopped!
```

4. check “Send email at end” in Acquire at points … dialog window.

4.10 SerialEM Note: K3 is installed on Talos

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Date_Created 2018-10-25
Last_Updated 2019-02-27

Abstract We have upgraded from K2 to K3 on Talos Arctica. Overall, we have had more positive experience than negative one. We are now collecting the first benchmark data, just one week after K3 installation was completed. Of course, we are using SerialEM on it.

I wanted to share our experience here. Hope it helps people to prepare for their own installation.

4.10.1 Installation Went Smooth

The installation was fairly smooth. The engineers had installed K3 at other sites before so they have quite some experience already. Total installation only took three full days. We had two K3 systems in crates - one for Talos and one for Krios. Therefore, one unique advantage we had was an extra set of identical hardware components to swap test when needed.

There were two failed hardware components in the original package for Talos, one was processing board and the other was MIB box and cable. Once these two faulty hardware components were replaced with ones in Krios K3 crates, the system came up nicely.

One can imagine, without spare hardware parts to trial and test, the installation could have taken a lot longer. We were lucky.

4.10.2 SerialEM Control

It was pretty easy to get good control of K3 camera based on previous K2 camera setup. There are only a few things we needed to redo for SerialEM calibration.

2. Image Shift calibration for all the mags to be used.
3. Pixelsizes at mag index 4, 16 and 17. They are 46X(LM), 2300X(LM) and 1250X (M). Note: This is only to get SerialEM going, more precise measurement of pixel size at final image mags will need to be done carefully using different methods, for high resolution image processing.
4. Stage Shift calibration for mag index 4, 16 and 17. They are 46X(LM), 2300X(LM) and 1250X (M). Double check tilting axis angles from this step too.

The K3 camera section of properties is below:

| CameraProperties | 1 |
| Name              | K3 |
| K2Type            | 3 |
| DMGainReferenceName | K3-18140113 Gain Ref. x1.m0.dm4 |
| # THESE 5 WILL NEED CHANGING IF CAMERA ORIENTATION CHANGES |
| CameraSizeX       | 5760 |
| CameraSizeY       | 4092 |
| SizeCheckSwapped  | 1 |
| RotationAndFlip   | 0 # accedently 1 before |
| DRMRotationAndFlip| 0 |
| #UsableArea       | 0 0 3712 3840 # top left bottom right! |
| UseSocket         | 0 |
| MakesUnsignedImages| 1 |
| XMustBeMultipleOf | 4 |
| YMustBeMultipleOf | 2 |
| FourPortReadout   | 0 |

(continues on next page)
4.10.3 Shutter Control

There are a number of things one should pay attention to, in my opinion. The shutter control is the top 1 on the list.

**Shutter control.** This is perhaps the most important thing you do not want to miss. If shutter control is not working properly, you might have sample burned without notice. Normally, if shutter control is not working, you will have hard time preparing gain reference. So you might notice it. However, since we are not required to prepare gain reference often in daily bases, if it stops working, you might or might not notice it promptly. You might still get image, but your sample might not be protected as it should be.

With properly working shutter, the beam will get blanked if following conditions are all met:

1. Hardware components are communicating with each other normally.
2. DM is running and K3 camera is in inserted position.
3. Software configuration in DM interface - Camera Configuration has set properly as idle state for shutter one “Pre-specimen” to be closed. There is normally only single shutter cable from Gatan MIB box - shutter 1 connecting to FEI shutter router “CSU” box at one of the channels. This is a BNC connector. In our case, it connects to Channel C - Blanker. Make sure it is the blanker, as the other one on CSU channel “shutter” means below specimen.
4. large screen of scope is in raised position (large screen is a switch to trigger sending or retracting 5V signal through the shutter cable).

5. In FEI scope “CCD/TV Camera” interface, make sure the fake camera name assigned for K2/K3 (Falcon in our case) is selected from the list and “insert” button is in yellow color. Click on it if this is not. This is to tell FEI CSU shutter router to let Channel C take control electronically, not to mechanically insert K3 camera, as K3 is not fully integrated into FEI TIA system. This is a standalone camera in that sense. In fact, newer version of FEI software no longer requires to add a fake camera onto camera list. Instead, there is a large button “Standalone Camera” to be clicked to do the same.

In our case, when all above conditions are met, the green LED “shutter” indicator on K3 power supply unit should be on. The “Blanker” orange color LED indicator on Channel C will be lit when idle. It blinks when a shot is taken from DM or SerialEM. If you take an exposure for 3 seconds, the LED will disappear for 3 seconds. The two images below show Gatan Power Supply unit and FEI CSU unit:

**Fig.1 Gatan K3 Camera Power Supply Unit** (click for full size image)

![Gatan K3 Camera Power Supply Unit](image1)

**Fig.2 FEI Shutter Router Unit (CSU)** (click for full size image)

![FEI Shutter Router Unit (CSU)](image2)
Please note: at least in our case, during an exposure, there is nothing change to reflect shutter status from either CCD/TV camera interface or FEI’s Jave program “Shutter Blanker Monitor”. This is probably due to Gatan camera being an “external” camera.

To make absolutely sure the shutter is working properly, it is better to check it with burn marker method. You lift large screen and wait for sometime and take an image of ice sample or plastic sample in a lower mag, and you check if you see any sign of burn marker. If no burn marker seen, that would indicate the beam is blanked without a shot is taken.

4.10.4 Other things to Watch

I listed a few more other things here that I also paid attention to.

1. Camera mounting orientation. This is not critical but can give you an easier life later. Our camera is mounted in the way that camera insertion is toward autoloader. Then there is no need to configure camera rotation and flip in DM configuration.

2. There is no existing fiber NIC available (like the Spare port on K2 computer) for us to use. However, there is a Ethernet NIC on the motherboard you can use. I prefer to have fiber NIC for faster data transfer so I added one PCI-E 8X 10GbE network card into the main computer. It sits in the very first PCI-E slot from the top. I literally get ~1GB/s real data transfer speed, from SSD Raid X drive to my storage via CIFS. Reverse direction - from storage to local SSD is about 600+ MB/s.

3. I pre-ordered extended 40 meter long data cable bundle, that includes 5 fiber bundles and one Cat6 cable. It also needs a long USB cable to connect to FEI computer for COM port communication for remoteTEM running on FEI scope for scope function calls. This one is easy to miss. I am using remote KVM system for the USB signal. The one we bought is this one, it does support 4k resolution, but refreshing frequency drops to 30Hz.

4. Only at starting computer, we hear huge jet engine kind of loud sounds. After it is running, it is still noisy not too bad. I heard some lab were testing to use soundproof rack to host the computer. If this is no concern for vibration, then it would be better to locate the K3 computer and soundproof rack in the scope room. I would like that a lot. Not sure how much more heat load this one gives compared to previously K2 computer plus its processors though. I have a feeling that this soundproof server rack should work - https://www.rackmountsolutions.net/12u-ucoustic-soundproof-server-rack/, but haven’t tested anything myself yet. Hope to hear from people about their experience.

5. There is Nvidia card K2200 for monitor display. That one doesn’t have HDMI port, only two DisplayPort ports.
If you need to buy KVM for remote AV/USB purpose, make sure to buy the unit that supports DisplayPort directly. DP to HDMI converter might not give 4K resolution that 32 inch Dell 4K monitor offers.

6. You should check water flow and air pressure gauge often for a fresh installation of K3, as they might change a bit in the beginning. We had a startup hiccup when the water is a little too low (~19 GPM). It became fine after it was raised to 24 GPM.

7. If there is any memory test error on any of the processors, one should shutdown and restart computer rather than a software reboot. Power cycle is likely needed to clear out memory errors.

8. K3 outputs more data than K2, one has to deal with storage capacity seriously if you run a scope efficiently. Otherwise, one might find that you quickly run out of data storage space. Saving frame data with compression and without gain applied has clear advantages here!

9. Our K3 system package came with a GP100 Nvidia card. Also there is MotionCor2 utility via DM interface. However, there is no way to access to MotionCor2 outside of DM. Fortunately, we can still utilize the powerful GPU card. If we run framewatcher to align ~30-40 Super-res frames, it can do as fast as ~10 seconds for one stack. This is sufficient at least for our session monitoring purpose. Very nice indeed!

10. Always remember to retract K3 camera first BEFORE you try to insert Ceta camera.

11. Sometimes on our system, when restarting DM, the communication between DM and microscope gets interrupted. A Keyspan USB Serial Adapter is used to establish the communication in our case. Unplugging and replugging the USB connection usually fixes the problem. However, it it almost impossible to do it remotely. We have found a workaround to re-activate the Keyspan USB Serial Adapter. 1) Exit DM first. 2) From Device Manager, find “Kayspan USB Serial Adapter”, in its “Driver” tab, disable and enable it. This will reset the adapter. 3) Restarting DM. After that, communication will be OK.

4.10.5 Additional Info

There is some additional information regarding the K3 camera from UTSW facility that you might find useful. Please find the pdf file.

4.11 SerialEM Note: Adding Point Items by Template Matching

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Last_Updated 2019-07-25

Abstract This article describes a method of automatically picking points in SerialEM maps. Currently SerialEM does not have built-in functionality of adding points by correlation. However, SerialEM is now capable of running external Windows programs via the RunInShell script command, as well as merging in externally defined navigator items. This means that is it possible to use any external program to search for desirable points and add them to the navigator. As an example, I present a graphical tool called semmatch that uses cross-correlation based template matching. Hopefully in the future others will be inspired to implement their own methods and programs to improve the SerialEM user experience.
4.11.1 Install

A SerialEM script called TemplateMatch_GUI at the end of this article is used to launch semmatch. All that is required is to install semmatch in order to use the script. Installation instructions are on the github page, https://github.com/alberttxu/semmatch

4.11.2 Usage

1. First, select a map item from the navigator. You can double click to load the image, or single click to simply highlight.
   
   • Before running the script, you can optionally crop out a template in SerialEM. See TemplateMatch_GUI for details.

2. Run TemplateMatch_GUI
• +/- to zoom in and out.
• To crop, just click and drag.
• Change the threshold and click search until you are satisfied with the result.
• Click Save and Quit to merge the generated points into SerialEM. To quit without saving, close the window from the top.

ScriptName TemplateMatch_GUI

### Before running this script:
# (Optional) Save a template of a hole/pattern as a jpg image.
# 1. Crop a hole using ctrl+shift+drag,
# 3. Using the Edit/Run one Line prompt, run
#   SaveToOtherFile A JPG JPG T.jpg
#
# If something goes wrong, set Debug = 1
Debug = 0  # True = 1 ; False = 0

### semmatch arguments
threshold = 0.8
acquire = 1  # True = 1 ; False = 0
groupOption = 4
  # 0 = no groups
  # 1 = groups based on radius
  # 2 = all points as one group
  # 3 = specify a certain number of groups
  # 4 = specify number of points per group

# names of temporary files used by semmatch
outputNav = semmatch_nav.nav
image = MMM.jpg
template = T.jpg

ReportIfNavOpen
If $reportedValue1 != 2
  Exit
Endif
ReportNavFile 1
navfile = $reportedValue1$reportedValue2
(continues on next page)
navdir = $reportedValue3
SetDirectory $navdir

If $acquire != 1 AND $acquire != 0
    Echo acquire should be either 1 or 0
    Exit
Endif

If $Debug == 1
    debugStr = /k
ElseIf $Debug == 0
    debugStr = /c
Else
    Echo Debug should be either 1 or 0
    Exit
Endif

## load and bin MMM map
ReportNavItem
If $RepVal5 != 2
    # if not a map item
    Echo Not a map item. Select a Map item from the navigator.
    Exit
Endif

MAP = $navLabel
Echo Map Label: $MAP
SetUserSetting BufferToReadInto 16
SetUserSetting LoadMapsUnbinned 1
# uncheck Montage Controls "Align pieces in overview"
ReportUserSetting MontageAlignPieces alignChecked
If $alignChecked == 1
    SetUserSetting MontageAlignPieces 0
Endif
LoadNavMap

# reduce image if larger than 2000x2000
maxdimLimit = 2000
ImageProperties Q width height
maxdim = $width
If $width < $height
    maxdim = $height
Endif

If $maxdim < $maxdimLimit
    Copy Q A
    reduction = 1
Else
    reduction = $maxdim / $maxdimLimit
    ReduceImage Q $reduction
Endif
Show Q

## make a jpeg image
SaveToOtherFile A JPG JPG $image
Echo saved $image

ReportOtherItem -1
newLabel = $navIntLabel + 1

(continues on next page)
4.12 SerialEM Note: Refine ZLP on Au Foil Grids

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Date-Created 2019-08-23

Last-Updated 2020-06-15

Abstract This is to introduce new implemented “Custom Time” script commands to handle refining Zero Loss Peak (ZLP) over Au foil grids.

4.12.1 Background Information

SerialEM has one script command to handle the ZLP refinement over time. Very useful. For example, if we put a line at the end of script LD-group like this:

```
RefineZLP 30
```

It will refine ZLP position if longer than 30 minutes since last refinement. It works very well for us, on a typical carbon based holey grid. Unfortunately, it doesn’t run well on a gold foil based grid which it is very “black” between holes. This is mainly due to large pieces of gold crystals that form the film diffract electron beams away in relatively large angle so they don’t hit the camera. SerialEM’s refineZLP routine works by comparing camera counts at different energy shifts. If there is clear count drop, the slit edge is detected. When with a Au foil grid, if the area but not a hole is used for such refinement, the black nature of the film causes this procedure likely to fail. When we take multi-hole exposure for 4 holes, for example, the stage position is on such “black” area.

It is possible to move to one of the multi-holes after exposure is done, but we prefer not to it for every exposure because ZLP might have been refined recently. Otherwise, time is wasted.

Therefore, we need a timer to track time and then perform two tasks in this situation after certain period. 1) move to a nearby hole, and 2) refine ZLP.
4.12.2 Timer Function Commands

This timer function is now available.
The two script commands related to the timer functions are:

```
SetCustomTime name
ReportCustomInterval name
```

Below is how to use them.

4.12.3 Use the Timer Functions

1. set timer to start counting, for example, one can simply run one-line command like this:

```
SetCustomTime ZLP
```

ZLP is the name of this timer. You can set up multiple timers with different names so they won’t confuse.

2. Add this section to the end of your LD-group:

```
SetCustomTime ZLP
ReportCustomInterval ZLP
If $repVal1 >= 30
    MoveToLastMultiHole  # move to a hole (bright area)
    RefineZLP            # refine ZLP now!
    SetCustomTime ZLP    # reset timer
Endif
```

You can use the timer function, similar to the above example, to perform various periodic tasks across the Acquire items conveniently.

4.13 SerialEM Note: Flexible Timer

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**Date-created** 2019-10-09

**Last-updated** 2018-10-09

**Abstract** Note about the flexible timer available for scripting and an example how to use it.

4.13.1 Background information

In SerialEM scripting, there are a few commands with timer built in. For example, command `LongOperation` can take a few specific actions with timer defined. Here is one of the example:

```
LongOperation Da 3
```

This will perform dark reference for K2/K3 camera every 3 hours. This is very handy indeed. Another example is `RefineZLP`.

4.13. SerialEM Note: Flexible Timer 67
RefineZLP 30

This will perform refining ZLP every 30 minutes.

However, if we want to set a timer and to do a multiple and general actions when time is up, a more flexible timer is needed. In the case shown in picture below, which is Au-foil grid, we use multiple hole mechanism to collected images in these 4 holes using beam-image shift while the stage is centered at the middle of the 4 hole pattern. When the procedure is finished, the shift is reset and Record beam would be hitting on the black Au crystals.

**Fig.1 4-hole black Au crystals**

It is known that this kind of black crystal film is bad for refineZLP routine to work properly. It needs to use a hole area to make it work. As you can see, a simple timer built in for a specific function is not sufficient here. Two actions are needed: 1) move to one of the four holes and 2) perform RefineZLP. It requires a more flexible timer to do this.

### 4.13.2 Flexible Timer

A typical timer which could handle multiple actions would be like this:

```c
If time is up
    do task1
    do task2
    ...
EndIf
```

One of the script commands related to timer is SetCustomTime. Below is example code to perform two actions mentioned above.

```c
### move to a hole and refineZLP every 30 minutes
ReportCustomTime ZLP
if $elapsed >= 30
    StageToLastMultiHole
    #ImageShiftToLastMultiHole
    RefineZLP  # refine now
    SetCustomTime ZLP  # reset it
Endif
###
```

### 4.14 SerialEM Note: More About X,Y Positioning

**Author** Chen Xu
Abstract Robust positioning to the target position is critical for high level operation of CryoEM data collection. In this note, I like to share my own version of the latest script to perform X,Y positioning task. And I try to explain every line of the code and ideas behind them as well.

I have spent a lot of time thinking and testing about this. If you have better and different ideas, I love to hear.

4.14.1 The script lines

The script is fairly short, as shown below. It can be inserted in beginning of a single particle data collection script.

```plaintext
buffer = T
RealignmentToNavItem 0
ResetImageShift 2
#Copy A $buffer
AcquireToMatchBuffer $buffer
AlignTo $buffer 0 1
```

4.14.2 Explanations Line by Line

```plaintext
buffer = T

This is to define which buffer will be used to store reference image, a whole image or a cropped area of an image. Buffer after N are all beyond rolling range, thus won’t be pushed out by taking too many images.

RealignmentToNavItem 0

RealignmentToNavItem is one of the most important functions in SerialEM, in my opinion. It will bring the specimen stage to a valid map item. It typically uses combination of stage shift and image shift to get the job done. 0 here means to stays in the conditions from which the map was created. For example, the map was generated using LD View, and the scope currently is at LD R, the scope will switch to the View mag, beam intensity etc.. After realign is done, it stays in View mag. Argument 1 will bring scope back to R, after routine finishes.

This command line will bring the specimen to the picked item position, with some image shift in the last image of the routine takes, in buffer A.

I should point out that this perhaps reflects one of the most fundamental differences between SerialEM and other data collection software - it doesn’t rely on the template at all. As long as an item in a valid map is defined (picked), SerialEM will drive the stage there!

ResetImageShift 2

ResetImageShift is to clear out any image shift existing in the system and use stage shift to compensate. Then, there is no image shift, which means beam is straight down on the axis. However, the intrinsic inaccuracy of stage movement makes target being slightly off, more or less.

The argument 2 here means stage will clear the backlash by moving to opposite direction for 0.025 microns as default. This can be very useful to slow down the stage drifting after moving to a new location. Low drift is a very good thing since there is no way to correct drifts accumulated within a frame. This is particularly true if one has to use long frame time on some camera system.
#Copy A $buffer

If not commented out, this line will copy the last image (after realign) in buffer A to a target buffer (T in this case). If one uses a fix image, for example, a cropped hole as reference image, then it should be manually copy into T and leave this line commented out.

AcquireToMatchBuffer $buffer

This is a new command, available in 3.8 beta Dec 10th, 2019 built and later. It does two things: 1) take a shot using the exact condition of what in the reference buffer for mag, beam condition, binning, exposure time etc.; 2) make the final image the same size as what in the reference buffer, by cropping if necessary. I used to have to do this in a lengthy script using two functions.

AlignTo $buffer 0 1

Simply align the image in buffer A to reference buffer. This would make the target right on again with image shift. The very last argument 1 means no trimming to any of the source image and reference image. This is needed for UltrAuFoil® Holey Gold Films grid which have vary “dark” region of the film.

### 4.14.3 Other thoughts

1. It is helpful to use large defocus offset for map and realigning, as the contrast is significantly better. On our Krios, we use -300um for View offset (in LD).

2. If offset is more than 200um, it most likely needs High-def Mag calibration. With this, system dynamically interpolates the stage shift matrix which is calibrated using near-focus condition. This makes stage movement much more accurate and robust.

3. If possible, use whole image as template instead of sub-area. Using sub-area such as a single hole is a quick workaround for a grid which has periodic feature and 5-point way of picking points might be not very accurate due to local geometry variation.

### 4.15 FastTomo: A Hybrid Approach to Speedup Tomography Data Collection

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**Abstract** Due to the imperfection of stage movement, a tomography target position (X, Y, Z) usually keeps changing with tilting angle. In order to obtain useful data, some kind of engineering control has to be in place to ensure the target postions are within the acceptable range through the entire tilting series. This engineering control might involve complete prediction or/and frequent tracking for X, Y and Z postions. Unfortunately, these extra control actions cost time for each tilting series collection. Here, we propose a hybrid method to make the data collection as fast as possible without sacrificing too much data quality.

We use a precalibration for each and every tilting series for Z, and we use proportional control to constantly compensate X, Y potions. We try to eliminate tracking and auto-focusing shots as much as possible.

We present a SerialEM script using this Hybrid method for all three tilting schemes: uni-directional, bi-directional and dose-symmetric. We slightly modify the oringal Hagen scheme to dose-symmetric with options.
to 1) switch to bi-directional beyond certain defined angle and 2) change exposure time at high angle ranges after switching to bi-directional.

4.15.1 Some Brief background Information

Our tests on stage tilting behaviour mainly show two things. 1) Statistically, it is hard to get specimen height to desirable range of eucentricity. There seems to be always some error to get to eucentric height, large or small. 2) the relationship between tilt angle and X,Y,Z position is not quite linear when the specimen is not close enough to eucentric height; even at very close to eucentric position it is no longer linear at high tilt range such as above +/- 45 degree.

Most of time consuming actions are the multiple tracking shots and auto-focusing procedures.

This makes us to think if we could have a way to eliminate most of, if not completely, the Focus and Tracking Shots, we might save time for tilting series data collection.

4.15.2 FastTomo Script

For Z, we found that a Sine function can describe the Z change fairly well. We only need a few tilt points to pre-calibrate such a Sine curve for each target position. Such pre-calibration does cause some time, but the prediction based on the calibrated Sine curve seems to be very robust and we then completely eliminate Focus shots in the collection step. The Objective lens is used to compensate.

For X,Y, we do not perform any precalibration. Instead, we use the returned Record images to perform the proportional control. Namely, we align the feature from Record image at each tilt to its previous one and we use Image Shift to compensate. We completely eliminate Tracking T shots in the procedure except in dose-symmetric scheme which T is used only in switching angles.

The SerialEM script - FastTomo and its usage can be found from the github.com project page.

4.15.3 A Few Points to Discuss

1. For a reliable correlation using R images, the dose is probably too low and signal is too weak without any binning. Therefore, a proper binning for R image is expected. This is not a problem for K2/K3 camera, as returned image can be binned while the raw frames are saved on the side without any binning. For a non-K2/K3 camera, one can modify the script easily to ReduceImage for R shot after saving and store that in the reference buffer.

2. The same idea and method can be also applied to a FISE collection, provided the frames from each tilt can be grabbed and available for scripting.

3. A single, long exposure with shutter opening and closing during tilting series saves time for startup delay and returning time, compared to conventional way of multiple exposure one after another. But the significant time saving is from minimizing the numbers of F and T shots already! Using our script FastTomo, the total time for bi-directional tilting series from -51 to +51 degree with 3 degree step is about 6 minutes including pre-calibration for Z prediction curve.

4.16 Index

- genindex
Index

A
Abstract, 13, 14, 18, 20, 23, 27, 28, 33, 38, 40, 42, 43, 47, 52, 54, 57, 58, 62, 66, 67, 69, 70

C
Cryo-EM Training - Basic (level I & II), 7

G
Goal, 7, 9

R
Requirement, 7, 9

S
SerialEM Training, 9