National Center for CryoEM Access and Training

Standard Operating Procedures
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Purpose: Basic guide for Krios operators to set up sessions for users
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1. **Purpose:**
   1.1. To setup the Krios system and Leginon ahead of users starting a daily session.

2. **Scope:**
   2.1. Checklist that should be reviewed by microscope operator ahead of data collection.
3. **Definitions**
   3.1. Leginon is a system designed for automated collection of images from a transmission electron microscope; it includes the python-side programs written in python and c, the MySQL database and server, and the mainly php-based image and data viewers on a web server.

4. **Responsibilities:**
   4.1. **Setup a Leginon session**
      4.1.1. Load a cross-grating for alignments (usually slot 1 in a cassette)
      4.1.2. Make sure "turbo auto off" : On Krios2 computer → autoloader tab → options → *turbo auto off* = true
      4.1.3. Make sure lens normalizations on objective only: Normalizations tab → TEM mag change → *objective = true*
      4.1.4. Start up Leginon clients on microscope and K3 computers
      4.1.5. Start up Leginon session Leginon workstation
         4.1.5.1. If user isn't here: /gpfs/sw/bin/change_user.sh <username>; then *betaleginon or nccatleginon*
         4.1.5.2. Holder = cassette identifier
         4.1.5.3. Clients = titan###, Krios#-K3
         4.1.5.4. C2 = 100 µm (check Apertures tab on microscope PC to confirm)
         4.1.5.5. Application = Krios3 MSI-T Nano 3.3 or EF Krios MSI-T
         4.1.5.6. In PresetManager node, load recent presets with desired settings
         4.1.5.7. Check "Cycle presets" = false and "Optimize preset cycling" = false
         4.1.5.8. In exposure node settings: correct image shift coma effect=false
   4.2. **Eucentric height/focus**
      4.2.1. Send low-mag preset to scope, such as gr and an intact square
      4.2.2. Get eucentric height: simulate Z-height in Leginon
      4.2.3. Get eucentric focus: simulate focus in Leginon, use MF button to verify
   4.3. **Beam tilt pivot point and rotation centering**
      4.3.1. Green screen down, send exposure preset to scopeIn direct alignments: *beam tilt PP X ,* minimize beam movement with MF X & Y
      4.3.2. Repeat for *beam tilt PP Y*
      4.3.3. In direct alignments: *rotation centering*, minimize beam movement with MF X&Y
      4.3.4. In direct alignments: *beam shift,* recenter the beam with MF X&Y
   4.4. **Objective aperture centering (if planning to use it, not always asked for)**
      4.4.1. In apertures tab on Krios PC choose 100 µm for objective aperture, click *adjust*
      4.4.2. On Leginon PC Send exposure preset to scope
      4.4.3. Go into diffraction mode and adjust objective aperture centering with MF X&Y
      4.4.4. Exit diffraction mode
   4.5. **Cs corrector**
      4.5.1. Send enn preset to scope

- 4.5.2. Exit EFtem mode, camera=**CETA**, magnification=120kx (due to pixel size and C1A1 value), spot size 2, beam diameter ~800 nm
- 4.5.3. Make sure zero objective stigmator and image A1 applied:
  Stigmator → “objective” & “image A1” = 0
- 4.5.4. Acquire **test** image (1 s integration, binning 1, readout full; confirm you see focused carbon on TIA)
- 4.5.5. Open “Image corrector user interface” program
- 4.5.6. Measurement → C1A1 tab → start
  - 4.5.6.1. Using C1 measurements and focus knob or zheight, get to -1.38 µm defocus **(half of C1A1 value, which is 2.76 µm at 120K mag)**
  - 4.5.6.2. Should see ~5000 counts
  - 4.5.6.3. Click “A1 coarse” 100% to correct astigmatism until below 10nm
- 4.5.7. Tableau tab → Standard and outer tilt = 21 mrad
  - 4.5.7.1. Click start (standard, which is more exhaustive taking 16 measurements)
  - 4.5.7.2. Should see A1 (astigmatism) < 10 nm
  - 4.5.7.3. Accept - this moves you to correction page
- 4.5.8. Start by correcting B2 or A2 coarse with +50% or +75%
- 4.5.9. Redo the tableau, correct other parameters
- 4.5.10. Final desired values: A = Astigmation; B = Coma; C = Defocus; S = Star Aberration
  - 4.5.10.1. **A1 < 10 nm**
  - 4.5.10.2. **B2 < 50 nm**
  - 4.5.10.3. A2 < 100 nm
  - 4.5.10.4. C3 < 10 µm
  - 4.5.10.5. A3 < 2 µm
  - 4.5.10.6. **S3 < 0.5 µm**
- 4.5.11. If Cs-corrector alignments do not work, try:
  - 4.5.11.1. to move to a nearby area of carbon with more lighter area - more carbon - as opposed to darker, gold-rich areas of a cross-grating
  - 4.5.11.2. Move to another square (needs new Z-height done manually)
  - 4.5.11.3. Touch up pivot points and rotation centering
  - 4.5.11.4. Make sure that Objective astigmatism and A1 are zero
- 4.5.12. When done, retract CETA and insert EF-CCD
- 4.5.13. Turn on EF-TEM mode

4.6. Beam tilt image (if not using Cs corrector)

4.7. **After annealing cycle only: GMS gains**

- 4.7.1. In GMS on K3 computer go to Camera → prepare gain reference
- 4.7.2. Set the bright beam first: 64kx mag, spot size 1, intensity ~3.4 µm, adjust to 280 counts
  - 4.7.2.1. 15 electron/pixel/sec
- 4.7.3. Collect linear gain with default settings
- 4.7.4. Select “yes” when prompted to acquire counting gain, select “yes” again to update hardware dark
- 4.7.5. Collect counting gain with default settings

4.8. **Energy filter tuning (mag dependent)**

- 4.8.1. Setup bright beam for energy filter alignment: send EFtune64k preset to scope
- 4.8.2. Set magnification to 64kx, spot size should be 1, beam intensity <2 µm well centered over the GIF
- 4.8.3. Aperture mode = mask
  - 4.8.3.1. Streaks means beam is too dim**
  - 4.8.3.2. Change intensity until the streaks disappear
- 4.8.4. On K3 computer in GMS switch to Linear mode and view to verify that the beam has ~1000-2000 counts per second and no edge/fringes
- 4.8.5. Put in mask and double check if you see streaks^

- 4.8.6. Tune GIF → center ZLP → Tune GIF → Full Tune
- 4.8.7. Or quick tune (two settings at a time until complete)
- 4.8.8. You will see orange if energy filter is tuned well
- 4.8.9. In ZLP node in Leginon (camera icon) add reference then set St.deviation to 0 and check every 3600 s
- 4.8.10. If no reference, bypass reference target

4.9. **Hardware dark & Leginon gain reference**

- 4.9.1. Send Gain preset
- 4.9.2. Confirm that beam is centered over the camera (no beam edge/fringe)
- 4.9.3. Confirm dose rate of 30 electrons/pix/sec via DigitalMicrograph (GMS) 1x view counted
  - 4.9.3.1. If not change and save intensity
- 4.9.4. In K1_correction node in Leginon:
  - 4.9.4.1. TEM = KriosEF
  - 4.9.4.2. Camera = GatanK3
  - 4.9.4.3. Camera configuration = 5760 x 4092, bin 2x2
  - 4.9.4.4. 2500 ms exposure, 50 ms exposure per frame, average 5 images to combine
- 4.9.5. Acquire bright in both channels (HW dark will automatically collect via DM)
- 4.9.6. Take “corrected” image in both channels (watch should be flat and blank) - Channel 0 and 1

4.10. **New dose for exposure**

- 4.10.1. Send exposure preset to scope (usually enn)
- 4.10.2. Adjust beam intensity to match gain reference intensity (30 e/px/s)
- 4.10.3. Confirm that beam is centered over camera (no beam edge/fringe)
- 4.10.4. Example: SS5, ~1.7 µm intensity, 30 electrons/pix/sec, 2000 msec exposure
- 4.10.5. Make sure enn spot size is the same in fcn and fan
- 4.10.6. Acquire new dose (should be ~32 e/A^2)
- 4.10.7. Check that other presets have dose (sq, hln, fan, fcn and enn)

4.11. **Ice thickness**

- 4.11.1. enn preset manager settings save 8x8 checked off
- 4.11.2. Simulate an exposure - record pixel “mean value” (should be ~65)
- 4.11.3. In the IceT node make sure that measure ice thickness by ALS is checked
- 4.11.4. If needed - check ice thickness by energy filter (slows down your collection!)
- 4.11.5. Enter the mean value into vacuum intensity and use 410 as ALS coefficient
- 4.11.6. Take off 8x8 afterwards (if screening after, IceT values will differ, this is for time efficiency)

4.12. **Preset alignments**

- 4.12.1. Make sure that fan, fcn and enn presets have image and beam shift = 0
- 4.12.2. Use fan or enn preset as reference
- 4.12.3. Using flu-screen find a feature such as a couple of beads or an ice chunk
- 4.12.4. In navigation node turn crosshair on, take an image, remember the feature position or take a screenshot
- 4.12.5. Send hln preset to scope and take an image
- 4.12.6. Select movement “image shift” and click the feature - another image will be taken
- 4.12.7. Verify that crosshair on enn image and hln image are in the same position on a feature. If not - adjust image shift one more time
- 4.12.8. Go to presets manager → hln → preset settings → image shift → click “from scope”
- 4.12.9. Now send the sq preset and repeat the alignment procedure comparing sq and hln images
- 4.12.10. Finally align sq preset to gr preset
- 4.12.11. After gr image is taken switch back to standalone camera on microscope PC: camera tab → flap-out → shutter control → standalone

4.13. **Test images**

- 4.13.1. Set up defocus range (typically -1 to -2 µm)
- 4.13.2. Make sure that:
  - 4.13.2.1. Square node: wait for node = OFF
  - 4.13.2.2. Hole targeting node: allow verification = ON and queue = ON
  - 4.13.2.3. Hole node: wait for node = ON
  - 4.13.2.4. Exposure targeting node: allow verification = ON queue = OFF
  - 4.13.2.5. Exposure node: delay = 1 s, delay before first = 4 s
  - 4.13.2.6. In square node Simulate a square target
- 4.13.3. In square targeting node pick a hole target (green) and a z-focus target (blu) then hit submit (play button) and submit queue (play button in a circle)
- 4.13.4. In hole targeting choose 3 spheres close to the edge of an image and 3 targets away from the spheres closer to the center, then hit submit
- 4.13.5. Start frame alignment and CTF estimation in Appion
  - 4.13.5.1. Go to emgweb.nysbc.org → image viewer and choose current session
  - 4.13.5.2. Click processing (opens a new window)
  - 4.13.5.3. Select frame alignment → MotionCor2 → select preset enn → command
  - 4.13.5.4. ssh ingestion/buffer server x2, paste and run the command using gpuid 0 and 1
  - 4.13.5.5. Select ctf estimation → CTFFIND4 → preset enn-a → command
  - 4.13.5.6. ssh ingestion/buffer x3, paste and add the flag --nproc=5

4.14. **Switch to user's grid and collect an atlas**

- 4.14.1. Check that objective aperture is out
- 4.14.2. Load user's grid
- 4.14.3. Using flu-screen find a good-looking square
- 4.14.4. Simulate Z-height
- 4.14.5. In grid targeting node go to settings and set atlas name and size (usually 0.009)
- 4.14.6. Calculate and collect an atlas (should be 23 targets)