Alignments and Calibration

1. Fill a dewar with LN2. Start cooling cold trap. As you are working the LN2 will evaporate, check this roughly every 1.5 hours and top it off. When working with cryo samples, you may want to do this more frequently.
2. Logout of emuser. Top computer will be in this account for Cryo cycling. Log out, but don't close the terminal Serial EM connection
3. Log in to your own account and Open software in following order
    i. peoui.exe (microscope interphase)
    ii. Gatan Filter control
    iii. Digital micrograph
    iv. TIA (for communicating the lower computer)

    If the microscope interphase fails to connect to the microscope check the following: Local Area Connection 7 (connected), TEM Server in partial SIM mode (pink icon)
4. Log in to the bottom computer as well. Your Serial EM settings for the camera are saved on your account. Press Ctrl+Alt+Insert to unlock lower machine.
5. In the microscope interphase in Setup -> vacuum check the vacuum levels. Numbers should be around 1, 6, 22, 35, 52.
    a. If camera vacuum is disabled try restarting vacuum by pressing vacuum off and on the panel
    b. FEG, Pow, HT should be yellow
    c. If Pow/FEG greyed out contact Berith
    d. If HT grey, check vacuum. If that is okay check HT in software and turn on if necessary
    e. Extraction should be at 3950. Sometime it's necessary to click twice on operate to set it back to that value.
6. In Search -> Stage open flap and press *Reset XY*
7. Check CryoBox Position. Make sure the CryoBox is at *IN* position. When it is *IN*, the "aperture" indicator points to position "1", close to horizontal direction. This is absolutely needed for a serious cryo session.
8. Insert your sample.
    a. Make sure the Col_Valves are closed before inserting
    b. In Setup -> Vaccum open the flap and press *Prepump Airlock.* This step is crucial during cryo sample insertions
    c. Insert sample:
        i. Insert holder with pin aligned to screw (pos 1: 2 O'clock)
        ii. Turn CW to Position 2(4 O'clock) and isert a little further while turning. At this point the airlock will be pumped down. There should be a timer that says how long for this step. Remember to choose "singlel tilt" or "cryo" at the bottom of the screen. Otherwise, the red light on the port will not turn off.
        iii. Once airlock is done pumping (red light on port turned off), turn holder CCW until pin is aligned "close" position, insert the holder fully and tap.

    d. **IF THE VACUUM CRASHES: wait for column vacuum to come down. When the options become available:**
        i. **In Setup -> HT click on the *HT* button. It should turn yellow.**
        ii. **In Setup -> FEG click on the *FEG* button. If the extraction starts falling click and unclick the "Operate" button to reset it to 3950**
9. Wait for the column vacuum to reach 15.
10. Open the column valves. **You will not be able to see a beam, unless the holder is inserted.**
11. Make sure you're doing all your work on the clockwise side of the crossover. Beam intensity in SerialEM has only been calibrated for that side.
12. In Align -> Alignments open the flap and load a settings file, either yours or those of a trusted user. **Make sure not to overwrite someone else's setting file when saving them later.**
13. Align eucentricity. Important for minimizing specimen shift when tilting, proper alignments later, and more accurate magnifications.
    a. In Search -> Stage click on *Wobbler*. You will need a sample inserted for this step. Zoom in on a grid square with something on it and, using the microscope touch pad, adjust the Z until the sample is not moving side to side.
    Proper Z height should be between -100 and 100 um. If that's not the case, the grid could be bent or torn.
14. Manually focus the sample by eye (either using the small screen in the viewing chamber or with the CCD camera).
15. Focus using autofocus in SerialEM. Do this at a magnification of at least 20KX.
    a. In Camera [-> CCD/TV select CCD. You should hear a noise of it being inserted if it was not selected. If not, you may need to switch back and forth with the Falcon for the system to recognize the switch.
    b. On the lower computer open up Serial EM. In the Camera menu click *Setup* and select GIF as the camera.
    c. Click *View* to take an image. Make sure that there is an object in your field of view.
    d. If you are close to focus, use the autofocus feature.
    e. Your objective lens strength should now be in the range of 88.7-88.9. If it isn't, that means either the focus didn't work properly, or your grid is bent.
16. Retract the holder far enough so that the sample is out of the beam (you can use a pen to hold the holder in place) and take out the objective aperture.
17. Center the condenser aperture. This procedure will center the condenser aperture around the optical axis. The proper alignment of this aperture ensures that changing the strength of condenser lens (intensity) does not change beam position.
    Changing the intensity, check that the beam stays centered while going through cross over (i.e the beam does not "swing"). If the center of the beam moves when going through crossover, the condenser aperture needs to be centered. Do this by using the small knob on the aperture and the small knob on the right side of the column. Continue checking the intensity while doing this.

18. Perform gun and beam alignments. All of these alignmentt should be performed at magnification of 10KX.
    a. In the Align tab do the Gun Shift and Gun Tilt alignments:
       This part of the alignment only needs to be done every few sessions. Once you enter this alignment procedure it resets the settings to a default state. If you decide you don't want to continue doing the alignment you will need to reload your latest settings file again.
        i. Gun tilt. Important for having maximum beam intensity. Adjusts the gun deflection coils so that the electron beam is emitted parallel to the optical axis. This ensures that all the electrons produced can be used for imaging.
           Make sure the beam covers the small screen and maximize exposure time with the multifunction buttons
        ii. Gun shift. Adjusts the gun deflection coils such that the electron beam travels straight down the optical axis through the center of the C1 lens. Center the beam with the multifunction buttons
        iii. Spot size dependent gun shift. So that changing spot size will not cause beam to shift.
           The last step of this alignment centers all spot sizes relative to spot 11. In order to do this correctly, first you need to center spot 11 in relation to the column walls. Do this by finding the middle point between the points where the beam disappears (it hits the column walls). Once you have found that, you can shift the beam to the center of the fluorescent screen with the shift x and shift y buttons. Then proceed to move down in spot sizes and center each spot size on the screen using the multifunction buttons.
           You can also correct for condenser astigmatism during this process by toggling back and forth with the R2 button on the touchpad.Fixing it for the spot sizes at which you will image ensures that the beam will be circular under your imaging conditions.
    b. Alternatively, you can do a simpler version of the above alignment in the tune tab.
       Go through Gun tilt, then Gun shift at spot size 3 and beam shift at spot size 6. Cycle between the two until the beam stays centered in both.
    c. In the Tune tab do the beam tilt. Purpose is to decouple the beam tilt and beam shift operations, meaning that beam tilt will not occur when the beam is shifted and vise versa. Important for high resolution imaging in which a beam tilt would result in a change in rotation center. Also important for when using autofocus. Converge the two bright spots using the multifunction knobs.
    d. If not already done, do the beam shift. To ensure that the beam entering the objective system is parallel to the optical axis. This will minimize aberrations in the final image. Move the beam to the center of the screen using the multifunction knobs.

e. In the Tune tab do Rotation center: (you will need to re-insert the sample for this step): make the expanding circle concentric, i.e. it is uniformly expanding from the center.
f. You are now done with aligning the scope. Make sure to save the parameters to your Alignment file. Do this is under the Align tab. Select your filename and click the Save button.

19. Perform Falcon protection calibration. The Falcon detector is more sensitive to electron-beam damage than scintillated CCD and therefore should not be overexposed with an excessive amount of electron dose. A dose protection system has been implemented in the software which protectors the Falcon detector from potentially damaging situations. In addition, it protects the camera in unsupported modes.
    In Falcon Calibration open flap and choose the file that has OK next to it. If none has an OK it could be due to one of two reasons: the bi-annual falcon calibration has to be done, in which case you should contact Berith. The extraction of the microscope isn't set to the extraction for which the bi-annual calibration was done. Check in the FEG panel that the extraction is at that same number, if not, click and unclick *Operate*, to bring it back to the correct extraction (usually 3950).
    In Falcon Calibration panel press *Beam current*
20. Collect a Gain Reference for the Falcon.
    a. Go to Low Dose mode on the camera software. Make sure the beam is centered, the intensity looks good, and the magnification for Record is around what you will be using. Update Low Dose settings (Click and Unclick Continuous update).Then unclick Low Dose.
    b. Record an image in Serial EM with the Falcon. Make sure that this looks reasonable (has counts ~3000 for negative stain and 10,000 for cryo)
    c. In the CCD/TV Camera Open the flap and select *Falcon Reference Image* Set the conditions to acquire 160 frames with 10 images to average and check the 'Remove noise from image' box
        i. Select *Acquire a single image*
        ii. If intensity meter at 0% then Unblank (should see following colors on buttons: Insert is Yellow; Blank is Grey; Acquire is White)
        iii. Adjust the intensity until the Intensity indicator is in the green region
        iv. Click *Acquire Full Set*
        v. After it is done click the *Load to TIA buttons*.
    d. Check that Record in Serial EM is giving a good image.
21. Reinsert your sample.

Imaging Process

Best practice is to collect an overview of the entire mesh and identify the grid squares that look the best. Once we find those we can go in and look at those squares and find locations of interest.

1. Open a navigator window. Navigator -> Open

2. Collect a Low Mag montage of the entire grid.
    a. Change the magnification to LM180x or LM120x. Move to roughly the middle of the grid.
    b. Switch Serial EM and TIA over to the GIF. Can't do Low Mag in Low Dose mode so turn that mode off.
    c. Look on screen to make sure the intensity seems appropriate. Take a View image to see that captured images look good. **If you notice that there are hexagonal cells in your image, those are from the CCD. This means your intensity is too high.**
    d. Start a montage: File -> New Montage. Make sure you check "Ask about making Map…", in the save options check all the options. For a mesh try 8x8 as the size of the montage. When saving the montage it's good to have some consistent naming conventions, such as: (DATE)-(SAMPLE_ID)-(MONTAGE_DEPTH)-(POSITION).mrc
    e. After Montage is done click yes to when asked about making a map. This will allow you to click on the map image, save positions and reload it when necessary, using the Navigator.
    In the Navigator window click the Add Points button. Now on the Montage map you can add points to the map list. Choose all grid squares that seem interesting.
3. If you haven't already, set up low dose. For single particle data collection the following are a good starting point: A typical setup like this might work for you too.
    View: mag = 1700x, binning = 4, area = full.
    Focus: mag = 100kX - 200kX, binning = 4, area = half or quarter-wide.
    Trial: mag = The same as Focus (check box "keep Focus and Trial the same"), binning = 4, area = full. The beam size should slightly smaller than CCD area so the edge of the beam can be seen on T image. This is needed for "CenterBeamFromImage" routine to work nicely.
    Record: mag = 59,000x, binning = 1 or 2, area = full
    Set proper offsets for View mag, *first* defocus and *then* IS. The IS offset only works for the specific mags of **R** and **V**. If down the road, you change the mag of **V** or **R**, you need to recheck this
4. We will now go to marked grid squares and create medium mag montages (remember to go to Low Dose after the Low Mag Montage and to re-insert objective aperture)
    a. In the Navigator window click the position that you want to go to and click the *Move to XY.*
    b. Get the new Z-height. You need to refine your Z-position. Go to Tasks tab and perform (i) Eucentric Rough, (ii) Eucentric Fine. This will save the height to the current position. After this it's good to perform an Autofocus (do this every few locations on the square when viewing)
    c. Collect a Medium Mag Montage for a chosen grid square.
        i. Move to the center of the square.
        ii. File -> Close (Closes the current montage)
        iii. File -> New Montage (save a new name that indicates which grid square you are now looking at).

To get good stitching on your montage you need to have good enough contrast on the holes as well as enough overlap of images for the correlation to work. I suggest doing a 5x5 montage at 4700x magnification with 250px overlap with x and y. To get enough contrast you should set the exposure for the View to 0.1 sec (assuming (assuming a dose of 62 e/px/s) and changing the defocus to about -10um.

iv. After getting your Montage save the map and mark positions for which you want to collect high mag images. Remember to flag all of them as "A" - Acquire.

5. Check your main Macro. Make sure the main Macro and all other are loaded in Macro editors. Here is an example:
   Example 3. LD.txt

```
MacroName LD
#main macro for data collecting in LD mode

## positioning - goto a spot
RealignToNavItem 1
Copy A P

## Center beam
T
CenterBeamFromImage
T

## Adjust Z to Eucentricity
Call Z_byG

## refine X Y
Call AlignToP

## now autofocus
G
G

## Drift Control
Call Drift

## Take shot
R
S
#=== end ====
```

Run this macro for one point (highlighted in navigator window). If it works fine, then the hard part is done. Congratulations!

6. Acquire at points. In Navigator -> Acquire at point and define the macro as the main task.

Shutdown Procedure

1. Remove your sample
    a. Pull back on sample till resistance
    b. Turn CW while pulling back. Don't do this too slow or you can crash the vacuum
2. Close down software (reverse order from the start up procedure)
    a. Serial EM
    b. TIA
    c. Digital Micrograph
    d. Filter Control
    e. Scope Software
3. Log out from both the top and bottom computers
4. Initiate the Cryo Cycle
    a. Log in to the top computer with 'emuser'
    b. Take the LN cup off the copper wires and place pitcher underneath
    c. Go to Vacuum tab, click the > arrow, click the Cryo Cycle button. It should start a timer of roughly 3 hours

If you did not place the copper wires of the sample stage in contact with LN you do not need to do a cryo cycle. This will be a rare occasion, but if you are practicing sample insertion or checking the alignment of the scope without doing real imaging work you don't need to cool the sample stage.

Notes from Chen:

Eucentricity, without wobbling the stage if possible.If possible, try not to use procedure that involves stage wobble. The main reason is that wobbling the stage will likely introduce LN2 bubbling again in the dewar of 626 side-entry cryo holder. The quickest way is perhaps to run Z_byVmacro in Low-Dose mode.

Example 1. Z_byV

```
MacroName Z_byV
################################
# Z_byV.txt
# by Chen Xu, Feb 8, 2013
################################
#
# a macro to adjust the eccentric center using beam tilted pairs.
```

```
# It uses Autofocus to measure the focus and adjust Z instead.
#
# assume the Eucentric Focus has been calibrated for the mag you use.

#====================================
# remember the starting focus for V
#====================================
GoToLowDoseArea V
ReportFocus
DEF = $ReportedValue1

#====================
# set objective lens
#====================
SetEucentricFocus
NormalizeLenses 2
Delay 1

#===========
# Adjust Z
#===========
Loop 2
Autofocus -1 1
ReportAutofocus
t = -1 * $reportedValue1
MoveStage 0 0 $t
echo --> Z moved $t micron
EndLoop

#====================================
# set back original defocus of View
#====================================
SetStandardFocus $DEF
```