Common Tundra Standard Operating Procedure   version 1.0

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1. Purpose:

    1.1. To set up the TFS Tundra for daily microscope use.

    1.2. To transfer clipped autogrids into a Tundra (also referred to as the TEM in this SOP) by using the Cryo Loading Station (CLS) and Transfer Device (TD).

    1.3. Day-to-day user care and maintenance of the Tundra, CLS and TD.

2. Definitions:

- A Semi-Automated Loading (SAL) system is a robotic system for loading cryo-EM grids into the microscope. It has two parts:

  1) A Cryo Loading Station (CLS) where autogrids are loaded into a cassette

  2) A Transfer Device (TD) that retrieves grids from the cassette and which is then loads them into the microscope stage via a retractable arm.

- The TD needs to be manually moved and should not be held more than 1 minute between the CLS (also known as the cryo-Loading Workstation) and the TEM. The TD will facilitate the transfer of the auto grid from the cassette reservoir to the microscope stage. The TD is primarily docked in the CLS and during grid transfer is pumped down to reduce the potential of vacuum crashes and contamination on the sample.

- An Autogrid is a Cryo-EM grid secured into a C-clip using clip rings (and prepared by the user through "grid clipping").

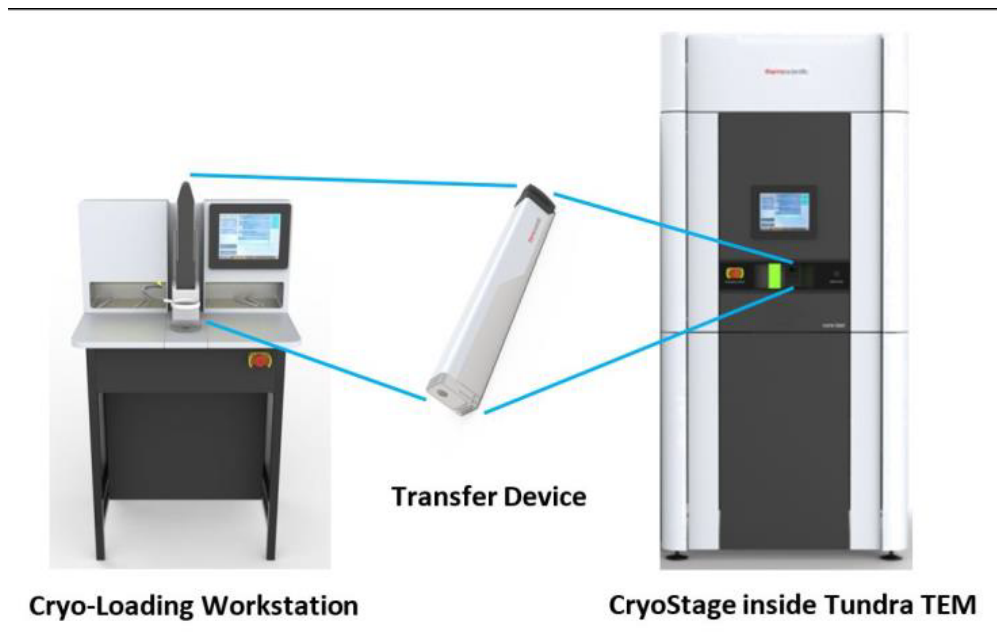- Liquid Nitrogen (LN_2) is a cryogenic liquid stored under pressure.

Transfer Device

Cryo-Loading Workstation   **CryoStage inside Tundra TEM**

Figure 1: The main components of the Tundra.

3. Supplies & Equipment:

- ❏ PPE (BSL-1)
    - ❏ Laboratory Coat
    - ❏ Nitrile Gloves
    - ❏ Goggles / Safety Glasses
    - ❏ Cryogenic Gloves
    - ❏ Face Mask
- ❏ Chemicals/Reagents
    - ❏ Liquid Nitrogen
- ❏ Metal funnel
- ❏ Dewar and Lid
- ❏ Autogrid Cassette
- ❏ Autogrid Tweezers
- ❏ Autogrid box(es)
    - ❏ Clipped holey carbon grid(s) containing vitrified samples
- ❏ Autogrid Box Opening Tool
- ❏ Large Forceps
- ❏ Hair dryer or heat block (to dry tools if needed)

4. Procedure:

4.1. Prepare the Microscope for daily use

4.1.1. The Tundra has a column dewar that should be cooled down before use.

4.1.1.1. To cool down the TEM, 3 to 4 LD4 dewars (4 liters each) should be filled with LN_2.

4.1.1.2. Before starting, check the status of the "Loading workstation", "Transfer Device" and "TEM" on the main touch screen. A green checkmark should appear above each icon to indicate the microscope is ready to use.

4.1.2. Cool the TEM column dewar with LN_2 (~1 hr).

4.1.2.1. On the TEM touchscreen *Actions* tab, select "Cool down TEM".

4.1.2.2. Access the column dewar by opening the back panel of the Tundra.

4.1.2.3. A 2 step ladder must be used to get to the column dewar.

4.1.2.4. Remove the LN_2 sensor rod from the dewar and place it on the hook located on the TEM frame.

4.1.2.5. Insert metal funnel into the column dewar.

4.1.2.6. From an LD4, decant LN_2 into a 1 L polypropylene pitcher. Then, pour the decanted LN_2 into the column dewar.

4.1.2.7. When the column dewar is full, reinsert the LN_2 sensor rod back into the dewar and close the TEM enclosure.

4.1.2.8. The TEM dewar will take a while to fully cool down (~45 min) and it is recommended to pause between the second and the third LD4 to make sure the temperature has stabilized before proceeding.

4.1.3. Wait for the system to recognize the dewar level. The main touchscreen will return to the default state automatically.

4.1.3.1. A full dewar lasts up to 72 hours. For a 1 day session, make sure the dewar level is one-third full. At 20% the system will give a warning that the *Nitrogen* level is "Low".

4.1.3.2. The *Nitrogen status* levels of the TEM are "Empty", "Critically Low", "Low", "OK" and "Full". The resting temperature will be around -196°C.

4.1.4. If the system dewar level is too low, then on the TEM *Actions* tab on the touchscreen select "Refill liquid nitrogen" and repeat sections 4.1.2 and 4.1.3.

4.2. Prepare the CLS.

    4.2.1. The CLS may be operated in 2 conditions: *Liquid nitrogen temperature* for Vitrified samples and *Negative stain* for Room temperature (RT) samples and cooled down TEM.

    4.2.2. If the sample should remain at RT, then check this is selected. This setting can be used initially when loading/unloading test grids for alignment.

        4.2.2.1.   On the CLS interface, go to the *Actions* tab and select "Set temperature mode".

    4.2.3. If the sample is at cryogenic temperatures, then prepare the CLS for their loading.

        4.2.3.1.   Remove the clear lid and white center cover.

        4.2.3.2.   On the CLS interface, select *liquid nitrogen temperatures* and press *continue*.

    4.2.4. Cool the transfer station with LN_2.

        4.2.4.1.   Initial fill should be performed from the LN2 cylinder's blue valve (Figure 4), looking through the opening in the window lid for a visual.

        4.2.4.2.   Monitor the CLS interface and once the LN2 reaches the line marked *Sample transfer* (Figure 5, close the LN2 valve on the dewar.

        4.2.4.3.   Do not fill higher than the top of the metal block.

        4.2.4.4.   Remove and put back white center cover with each fill.

        4.2.4.5.   Once the LN_2 has finished bubbling, the temperature has stabilized. However, wait a further 30 mins after first cooling the CLS to avoid the motor freezing. This is not part of the interactive process.

        4.2.4.6.   Keep the LN2 topped up in the CLS by regularly topping up via a LD4 dewar.

Figure 2: The cryo loading station. A) Screen during initial filling with liquid nitrogen. B) The window to the grid loading area.. C) The tool storage area.

4.3. If an Autogrid is in the Microscope: Unload sample from TEM

    4.3.1. The standard state of the microscope during a cryo cycle is to not have an Autogrid on the stage. If there is a sample in the TEM, unload the sample with the TD. This may be done with the CLS at RT or cryo conditions.

    4.3.2. Verify there is room in the cassette for the grid to be unloaded from the TEM.

        4.3.2.1.   The inventory recognized by the system may be accessed through the *Cassette* tab.

        4.3.2.2.   To visually verify the cassette, on the CLS touchscreen, go to the *Actions* tab and select "Load/unload samples from cassette"

4.3.2.3. Despite there being 12 slots, only the first five slots are used by the TEM.

4.3.3. Unload the grid from the TEM

4.3.3.1. On the CLS touchscreen *Actions* tab, select "Unload sample from TEM".

4.3.3.2. In the first stage "*Preparation*", the CLS will pump down the TD and indicate when ready to be detached from the docking station (~8 mins).

4.3.3.3. For "Transferring to TEM", remove the TD from the CLS and attach to the TEM. The Transfer Device **must always be moved with two hands**. When the TD is in transit between the CLS and TEM, make sure the front port is kept clean and free from obstructions. Make sure to securely insert the TD into the TEM and gently maintain some pressure on the TD in the microscope until the roughing pump engages, then let go of the TD. It takes ~3 mins to pump down and unload the Autogrid from the TEM.

4.3.3.4. Remove the TD from the TEM and place it in the dock of the CLS.The grid from the TEM will then be loaded into the cassette.

4.3.3.5. Press load/unload sample from cassette. Transfer the grid into the box for storage or disposal.

4.4. <u>User alignments</u>

4.4.1. EPU and Microscope Companion (MiCo) should be opened on the microscope computer.

4.4.2. EPU is used for data collection and several of the automated features for the Tundra. MiCo is the main interface for determining microscope status and for opening and closing column valves.

4.4.3. The User Alignments are direct beam alignments and aperture alignments. The various stages have different time limits and EPU has a timer that reminds the user if each alignment is past its limit. However, the recommendation is to repeat these alignments each day as the microscope is prone to misalignments.

4.4.4. There are three stages corresponding to alignments at the Hole/Eucentric, Data Acquisition and Atlas/GridSquare presets. These should be performed in full for each session. But, if the beam is misaligned at a particular preset during a session, then that preset can be aligned on its own by unclicking the other presets.

4.4.5. User alignments are performed over vacuum. When the Atlas preset is aligned at the start of a session, there must be no grid in the column. Later alignments of only the HE or DA presets can be performed over a broken grid square, removing the potential need to unload a grid from the microscope.

4.4.6. In EPU, select *Microscope State* tab and select "Recover".

4.4.7. In MiCo, select *Alignments* tab, go to the "User Alignments" tab, select all 3 main alignments "High Magnification uP", "Data Acquisition (HM nP)", "Atlas (LM uP)" and press the play button (Figure 4).

4.4.8. If the edge of the beam is visible even after alignments, then try repeating the alignment at that particular preset. For Atlas, Grid
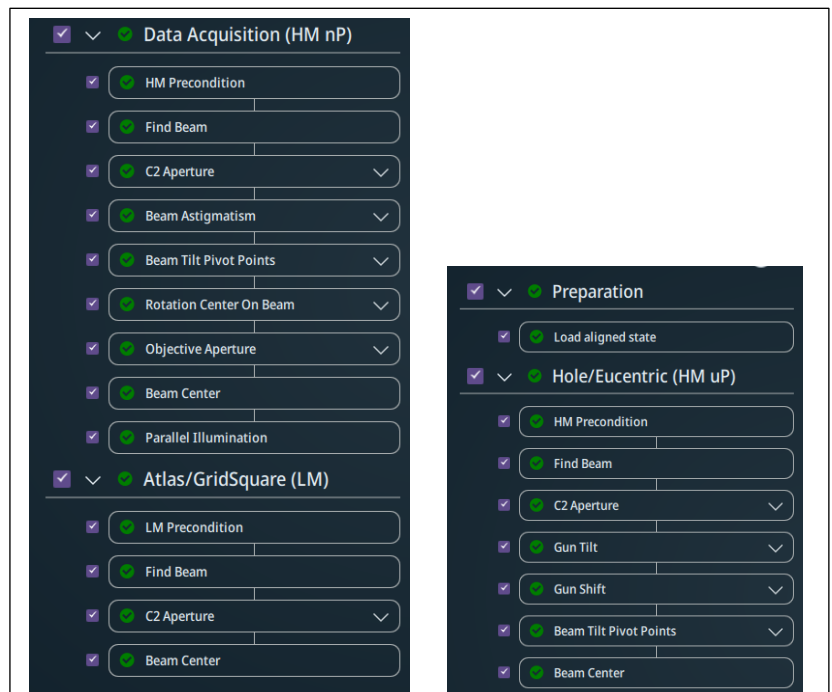

Figure 4: The steps that form the User Alignments

Square or Hole Eucentric presets, the beam can be made wider to cover the sensor by increasing the intensity.

4.4.9. If any of the alignments fail, individual alignments should be selected and re-run through MiCo.

4.4.10. The column valves should be automatically closed after alignments, but go to the *Vacuum* tab in MiCo and close column valves.

4.5. Load a test grid into the TEM
- 4.5.1. After *User alignments*, *Optimize optic*s must be performed, in which there is further beam alignment and correction of beam astigmatism and coma.
- 4.5.2. *Optimize optic*s is performed over a layer of carbon as carbon generates a strong signal. Any carbon grid may be used, but TFS applications recommends using a holey carbon grid. If a dry test grid is used, the CLS can be at room temperature (RT) and doing this will actually speed up the loading and unloading processes.
- 4.5.3. Load a clipped, holey carbon grid into the cassette in the CLS.
  - 4.5.3.1. The grid can be a dry test grid or a grid of vitrified sample.
- 4.5.4. The inventory recognized by the system may be accessed through the *Cassette* tab.
- 4.5.5. To visually verify the inventory on the CLS touchscreen *Actions* tab, select "Load / unload samples from cassette".
- 4.5.6. The cassette only uses the first five slots. Leave slots 6-12 empty.
- 4.5.7. Transfer grids to/from the cassette slots as required.
- 4.5.8. Update the inventory on the CLS interface.

> ⚠️ **Avoid contamination:** Always wear gloves when handling anything that enters the microscope vacuum.

4.6. Transfer grid into TEM.
- 4.6.1. Press *Load sample into TEM*
- 4.6.2. Select one grid for transfer to TEM.
- 4.6.3. There are 4 main stages in this process: "*Loading to transfer device*", "*Transferring to TEM*", "*Loading to TEM*", and "*Transferring back to loading workstation*".
- 4.6.4. The CLS will pump down the TD and indicate when the TD is ready to be detached from the docking station. Make sure to use two hands when moving the TD.
- 4.6.5. When the TD is in transit, make sure the front port is kept clean and free from obstructions.
- 4.6.6. Remove the TD from the CLS and attach it to the TEM. Make sure to securely insert (leaving a hand on handle) the TD into the TEM as the roughing pump engages.
- 4.6.7. The TD takes ~3 mins to pump down and an additional 3 mins to load the grid into the TEM.
- 4.6.8. Remove TD from the TEM and place it in the docking station of the CLS.

4.7. Optimize Optics
- 4.7.1. EPU and MiCo should be opened on the microscope computer.
- 4.7.2. Optimize optics should be performed after User alignments. EPU has a timer that reminds the user if alignments are older than 24 hours, but either way these should be done at the beginning of any new session.
  - 4.7.2.1. These alignments may be done either in EPU or MiCo, but it is recommended to start them through EPU as it ensures they are performed over carbon and the stage is at eucentric height.
  - 4.7.2.2. In EPU, select *Auto Functions* tab, then "Alignments", "Optimize Optics" and press start.
  - 4.7.2.3. Use the context menus to move the stage to a carbon area and press resume to start the beam optimization.
- 4.7.3. Data acquisition alignments
  - 4.7.3.1. Load aligned state
  - 4.7.3.2. Optimize Beam Preconditions

4.7.3.3.　　Find Beam
4.7.3.4.　　Set Magnification For C2
4.7.3.5.　　Parallel Illumination
4.7.3.6.　　Beam Center
4.7.3.7.　　Restore Magnification
4.7.3.8.　　Image Astigmatism
4.7.3.9.　　Image Coma
4.7.3.10.　　Image Astigmatism
　4.7.4. Column valves should be closed automatically after Optimize optics has finished, but make sure they are closed via MiCo -> Home -> Vacuum.

4.8.　Load samples into the TEM
　4.8.1. Place your grid box in the CLS box positions under the LN2. Unscrew the lid and screw it into the metal block to hold down the grid box(s).
　4.8.2. Cool the autogrid tweezers in LN2.
　4.8.3. On the CLS interface, press *load/unload samples from the cassette*.
　4.8.4. If a previous grid is present in the cassette then remove it accordingly.
　4.8.5. Transfer grids, one at a time, correctly into the cassette **(see site specific instructions for correct loading orientation)**. They should be placed between the L and the spring in each position. Place grids in any of the positions from 1-5. Positions 6-12 are not available to the TD.
　4.8.6. If a grid is on the microscope stage, leave one empty position (see **site specific instructions**).
　4.8.7. Check for secure insertion in the cassette by **gently** tapping the top edge of the autogrid with the autogrid tweezers.
　4.8.8. On the CLS interface, edit the name of the sample.
　4.8.9. Transfer grids to TEM as per the instructions in section 4.5.

4.9.　Grid screening
　4.9.1. Now that the microscope is aligned and the grid is loaded, the image shifts should be calibrated.
　　4.9.1.1.　　Find a piece of contamination that is visible in all four presets. You want to be centered on this contamination at Data Acquisition preset.
　　4.9.1.2.　　In the preparation tab go to *Tasks -> Calibrate Image Shifts*.
　　4.9.1.3.　　If eucentric height has not been identified, then press Auto-Eucentric.
　　4.9.1.4.　　Press *Acquire*. Once all four images have been taken, click on the matching feature in each image. Press store calibrations.
　　4.9.1.5.　　To check the calibrations, press *Acquire* again and check the feature is centered.
　4.9.2. Take an atlas of the grid.
　　4.9.2.1.　　In the *Atlas* tab, click the *Session Setup* tab and then *New Session*. Fill in the details and make a note of the name. Later on, use the same name for your EPU session.
　　4.9.2.2.　　Go to the *Screening* tab. To image the full grid, keep the *Number of Tiles* empty, otherwise specify a number (25 is a good option) to reduce the time taken. Press *Start*.
　4.9.3. Look around the different areas of the grid using the *Preparation* tab *-> acquisition and optics settings*. Measure the eucentric height (*Auto Functions -> Auto-eucentric by beam tilt*) and measure the focus for each new grid square (*Auto Functions -> Autofocus*). Find the area of the grid you want to collect on.

4.10.　　Automated data collection
　4.10.1.　Automated data collection is performed using the *EPU* tab
　4.10.2.　Set up a new session using *Session creation* and *Session setup*.
　　4.10.2.1.　　Check grid type matches sample
　　4.10.2.2.　　Check the speed settings.
　4.10.3.　Select squares in which to collect using *Square Selection*.

4.10.4. Select holes in which to collect using *Hole Selection*. Press *Auto-Eucentric* to find eucentric height, then acquire to take an image of the square. '

4.10.5. Press *Measure hole size* to find the holes using the yellow circles, then press *find holes* and check the circles are over the holes. You can select/unselect the holes either automatically via the *smart filter* button or the *filter ice quality* histogram, or manually using the *selection brush*.

4.10.6. You only need to set up one square. You should select the remaining squares but EPU will automatically auto-eucentric, find holes and select the best ones.
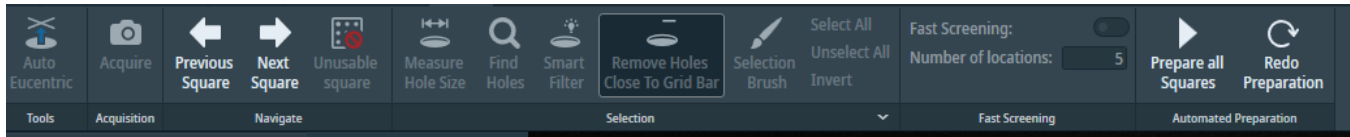

Figure 5: The options available in the Hole Selection tab

4.11. Template definition

4.11.1. In the *Template definition* tab, press acquire, then press *find and center hole*.

4.11.2. Add an *Acquisition area*. The Tundra only allows for one shot per hole. Edit the defocus range for the acquisition area.

4.11.3. Add an *Autofocus area*, place the blue circle over an area of carbon between the holes. For accuracy, use *recurrence* always, for speed use *recurrence* after a certain distance.

4.11.4. Add a *Drift Measurement area*, place the purple circle over the purple *Autofocus area.*

4.11.5. The purple *Autofocus area* and pink *Drift Measurement area* may overlap with a green Acquisition area circle, but should not overlap with the hole. If they are encroaching on the hole, then reduce the intensity for these by going to the *Preparation* tab -> *Acquisition and Optics Settings* and in the *Presets* tab reduce the intensity slightly (by 0.05-0.1) for the *Autofocus* and *Drift Measurement* presets. Make sure the intensities are the same value.
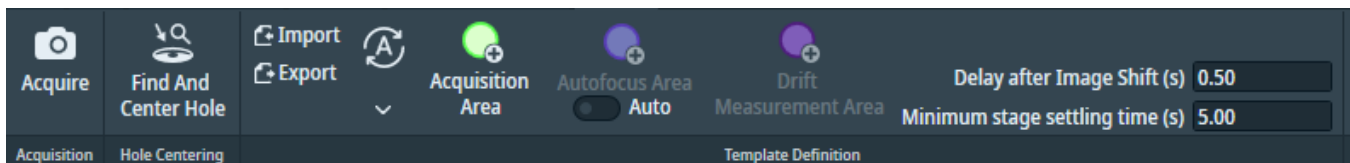

Figure 6: The options available in the Template Definition tab

4.12. Press *Start* in the *Template Execution* tab to check the acquisition.

4.13. In the *Automated Acquisition* tab, make sure the *Close Col. Valves* button is checked and then press *Start*.

4.14. After the collection has finished:

4.14.1. Check the column valves are closed.

4.14.2. Remove the grid from the TEM using the TD following instructions in ???.

4.14.3. Store the grid appropriately.

4.15. Clean up

4.15.1. See **site specific instructions** for clean-up procedures for the center you are working at.

4.15.2. Remove grids and grid boxes from the CLS and store appropriately.

4.15.3. Remove all tools and return to their respective warm-up stations. The CLS has a warming station to the back left.

4.15.4. Cryo cycle the CLS. Leave the clear lid off.

4.15.5. Empty remaining LN2 from dewars and leave them to dry.

4.15.6. If you have finished with the TEM, cryo cycle it by selecting on the touchscreen interface.

5. Troubleshooting

    5.1. If the CLS does not respond, press *recover* on the CLS interface.

    5.2. If there is no response from recovery, then press the white reset button on the left panel.

    5.3. If the reset button fails, call TFS service or SEMC staff.

6. **Additional Figures:** N/A

7. **Chemicals:**
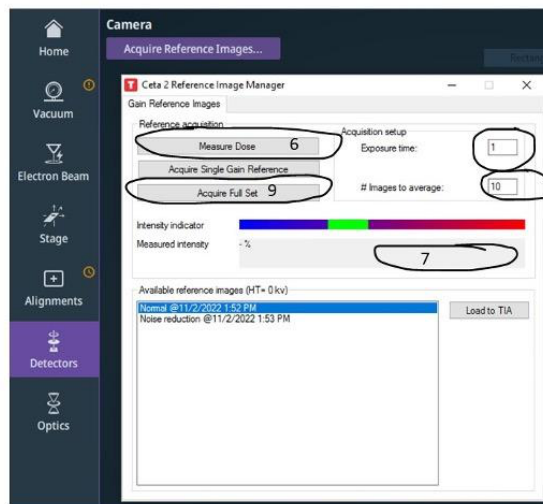
    7.1. Liquid Nitrogen

8. **Waste Disposal:**

    8.1. Follow facility procedure for proper disposal **(see site specific instructions)**.

    8.2. Biohazardous waste will be collected in designated bins lined with red /orange biohazard bags.

    8.3. Chemical hazardous waste will be segregated by hazard class (e.g. flammable, corrosive) and state (e.g. solid, liquid), appropriately labeled, and placed in the laboratory's hazardous waste collection.

9. Appendix: **Gain reference. Perform gain correction if needed.**

    9.1 Perform microscope alignments.

    9.2. Go to your data acquisition preset/optics settings.

    9.3. Go to an empty area or broken area.

    9.4. At the Mico/Apollo window, press the "Detectors" tab Click Acquire Reference Images.

    9.5. This will open a new window "Ceta 2 Reference Image Manager".

    9.6. Click "Measure Dose".

    9.7. This will give the measured intensity (dose). Any dose rate between 10-100 electron/pixel/second.

    9.8. Calculate Exposure time X # Images to average X Measured intensity. The resulting value should be around 3000. (eg 1.25 (Exposure time)X 170 (Images to average) X Measured intensity (14.13)=3002.

    9.9 Click "Acquire Full Set".