Common Tundra Standard Operating Procedure version 2.0

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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:

2.1. A Semi-Automated Loading system is a robotic system for loading cryo-EM grids into the microscope. The user inserts autogrids into a cassette within the CLS, which is then loaded into the microscope via the TD using a retractable arm.
2.2. 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.
2.3. An Autogrid is a Cryo-EM grid secured into a C-clip using clip rings (and prepared by the user through "grid clipping").
2.4. Liquid Nitrogen (LN_2) is a cryogenic liquid stored under pressure.
2.5. Definition of terms for tools/equipment can be found in Figure 1.

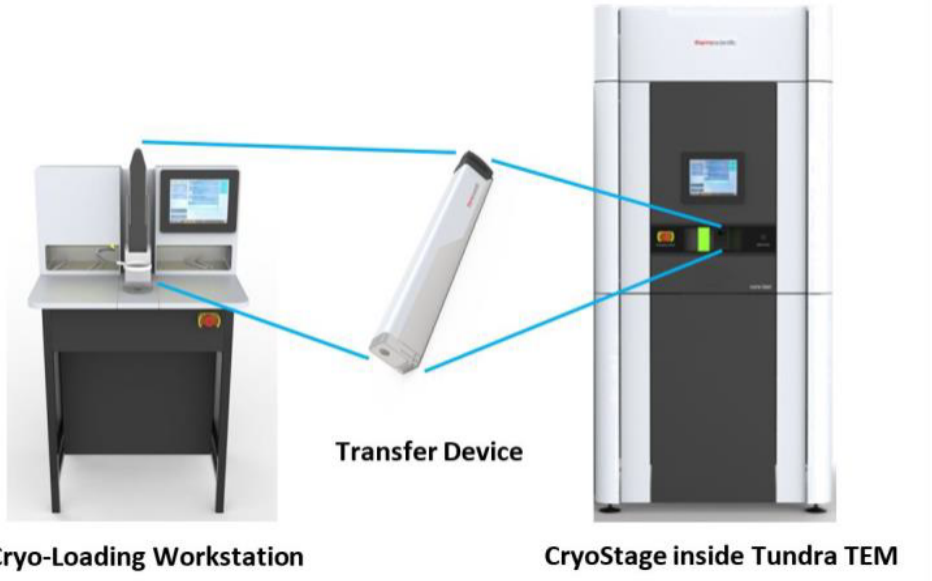

Figure 1. The main components of the Tundra.

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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 (~2 hours).

4.1.1. Cool down the TEM column dewar.

4.1.1.1. Fill 2 to 3 LD4 dewars 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.

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

4.1.1.4. Access the column dewar by opening the back panel of the Tundra. A 2 step ladder must be used to get to the column dewar.

4.1.1.5. Remove the LN_2 sensor rod from dewar and place it on a hook located on the TEM frame. Insert metal funnel into the column dewar.

4.1.1.6. From an LD4, decant LN_2 into a 1 L polypropylene pitcher. Then, pour the decanted LN_2 into the column dewar. Repeat this until a whole LD4 has been poured into the dewar. Reinsert the LN_2 sensor rod back into the dewar and close the TEM enclosure.

4.1.1.7. Wait for the system to recognize the dewar level. The main touchscreen will return to the default state automatically. In total, wait ~30 min for the TEM to fully cool down and for the temperature to equilibrate at -170°C.

4.1.2. Once the microscope temperature has fully equilibrated, the LN_2 level will likely be very low, so add another LD4 of LN_2.

4.1.2.1. 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".

4.1.2.2. The *Nitrogen status* levels of the TEM are "Empty", "Critically Low", "Low", "OK" and "Full".

4.1.3. If the system dewar level is too low, then on the TEM *Actions* tab on the touchscreen select "Refill liquid nitrogen" and repeat sections 4.1.1.4. - 4.1.1.6

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4.2. Prepare for user alignments: If there is a grid in the TEM, unload it.

4.2.1. The user alignments must be performed with no grid on the stage. There may be a grid left in the microscope from the previous cryocycle. If so, unload it using the TD, with the CLS at room temperature, since the unloading/loading processes are much faster than under cryo conditions.

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

4.2.2.1. The cassette inventory may be accessed through the *Cassette* tab. But the casette should be visually verified as well. In the CLS *Actions* tab, select "Load/unload samples from cassette".

4.2.2.2. Despite there being 12 slots, only the first five slots are used by the TEM. Make sure the CLS inventory matches the grids in the cassette.

4.2.3. Unload the grid from the TEM.

4.2.3.1. On the CLS touchscreen *Actions* tab, select "Unload sample from TEM" (Figure 2A).

4.2.3.2. In the first stage "*Preparation*", the CLS will pump down the TD and indicate when ready to be detached from the docking station (~1 min) (Figure 2B).

4.2.3.3. 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 (Figure 2C).

4.2.3.4. It takes ~3 mins to pump down and unload the Autogrid from the TEM (Figure 2D).

4.2.3.5. When the TEM indicates (Figure 2E), 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.

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

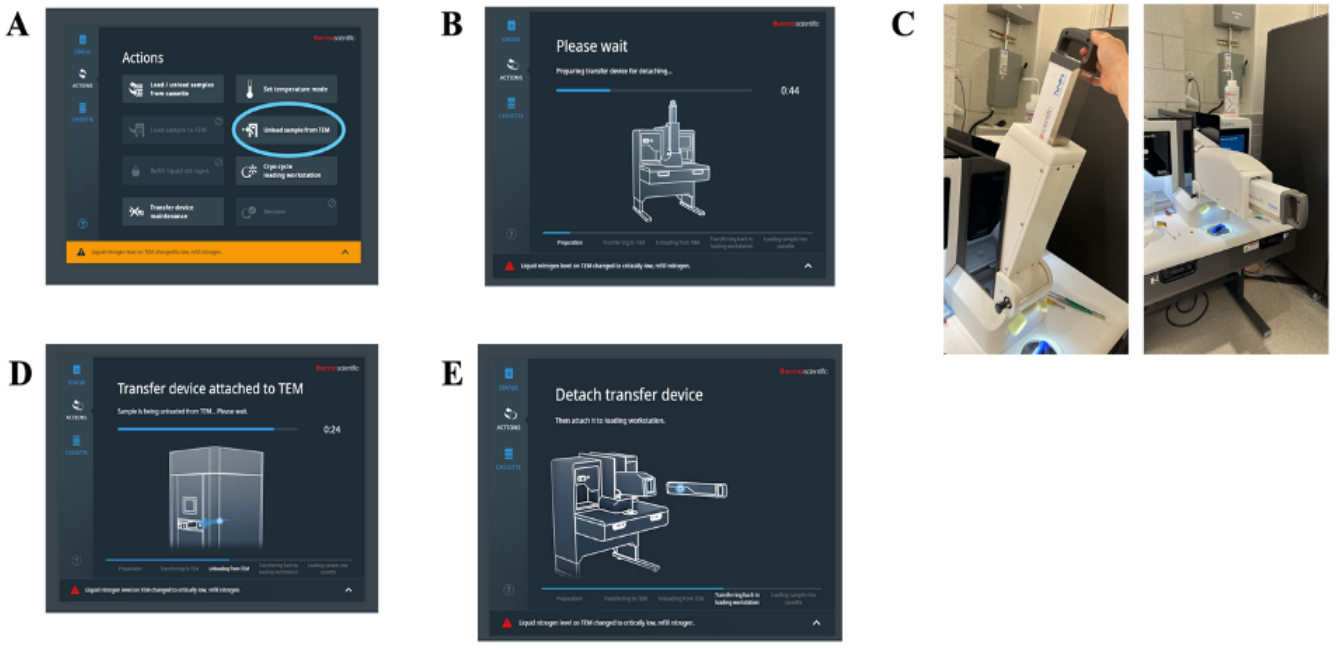

Figure 2. Unloading a grid from the Tundra.

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4.3. Prepare EPU presets.

4.3.1. EPU and Microscope Companion (MiCo) should be opened on the microscope computer. EPU is used for data collection and MiCo is the main interface for determining microscope status.

4.3.2. Check the Atlas, GridSquare, Hole/EucentricHeight and Data Acquisition presets. Make sure the magnifications, spot sizes, aperture sizes, intensities are appropriate for your grid type. You want these decided pre-alignment because if you change them after, you may have to re-align that preset.

You can load the TFS-supplied preferences (Medium resolution is recommended for screening) or load one from the Saved Preferences section.

Table 1: Example recommended presets

	Magnification	C2 aperture	Spot size	Intensity
Atlas	155	150	4	1.1
GridSquare	940	50	4	0.8-1.0
Hole/EucentricHeight	5100	50	4	0.7-0.9
Data Acquisition	110,000	50	6	0.440

4.3.3. Link the *Data Acquisition* preset with the automation presets (Autofocus, Drift Measurement, Thon Rings). In the *Data Acquisition* preset press *Link Settings*. If this is greyed out, then go to the *Autofocus, Drift Measurement* and *Thon Rings* presets and press *Link Settings* on each of them.

4.4. Perform the User alignments

4.4.1. The User Alignments are direct beam alignments and aperture alignments. The various stages have different time limits shown in MiCo. However, the recommendation is to repeat these alignments every other day to avoid misalignments.

4.4.2. 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.

4.4.3. In EPU, select *Microscope State* tab and select "Recover". Check all tundra components are marked green.

4.4.4. In MiCo -> *Alignments* -> User Alignments, make sure all checkboxes are selected. Press the play button (Figure 3). Wait for the tasks to run and for each task to be marked as completed with a green check (Figure 4).

4.4.5. If any task alignments fails, repeat the alignments at only that preset. If the beam becomes misaligned later (e.g. beam edge is visible), then move over vacuum and repeat the alignments at that preset.

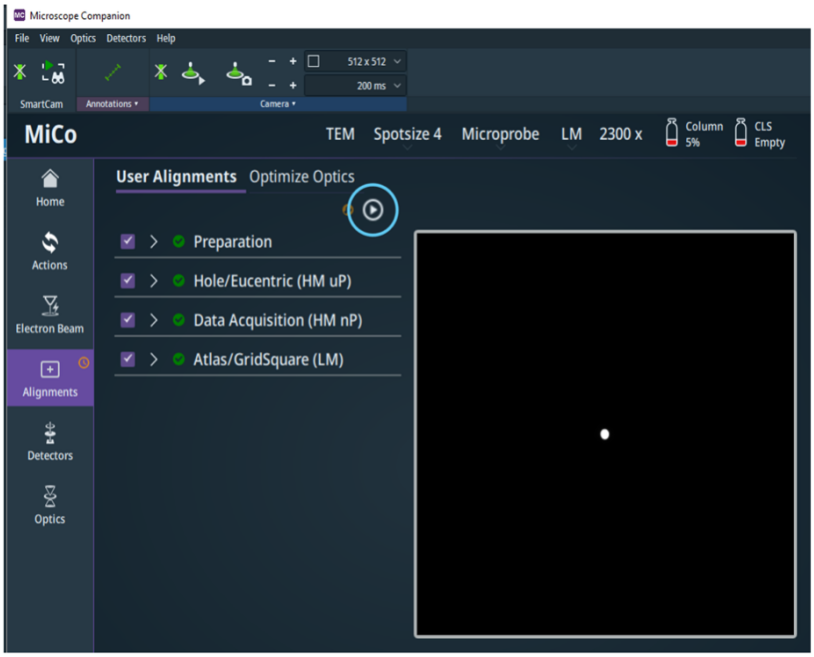

Figure 3: Start the user alignments using the start button, highlighted with the blue circle.

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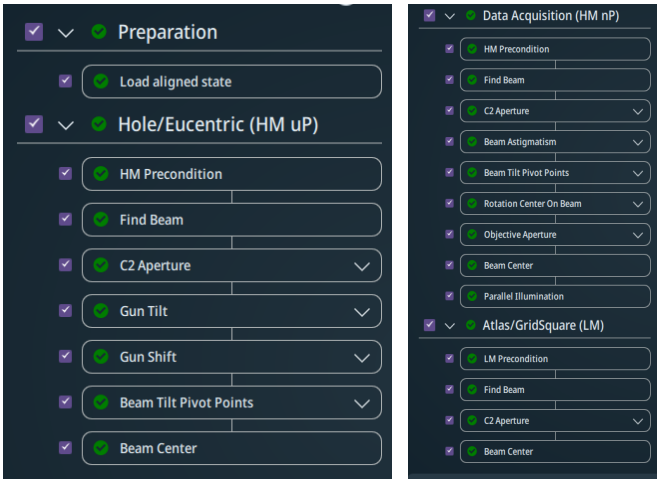

Figure 4: The steps that comprise the User Alignments

4.5. Measure the electron dose.

4.5.1. The electron dose must be measured over vacuum. It must be measured for your Data Acquisition preset once the magnification, spot size, C2 aperture and intensity have been fixed. If you change any of these during the collection, make sure to move over vacuum and re-measure the dose.

4.5.2. Go to *Preparation -> Presets -> Data Acquisition* and press *Preview.* The image should be random noise and should contain no fringes. If there are fringes, change the C2 aperture, **don't alter the intensity from 0.44** as the beam will move out of the parallel range for the beam.

4.5.3. The dose rate measurement should have updated but press *Measure* again to make sure. The dose rate should be in the green range. To get the dose higher or lower, lower or raise the spot size, respectively (Figure 5A).

4.5.4. Once the dose rate is appropriate, select your desired total dose in *Exposure Settings -> Dose.* 50 e/A^2 is recommended as a good starting point.

4.5.5. Selecting the desired total dose changes the exposure time to fit that total dose. It also changes the *current leading parameter* arrow to move it from dose to exposure time. **Before moving on, click the arrow so that it goes from exposure time to dose.** This fixes the exposure time despite the varying dose measured during the collection.

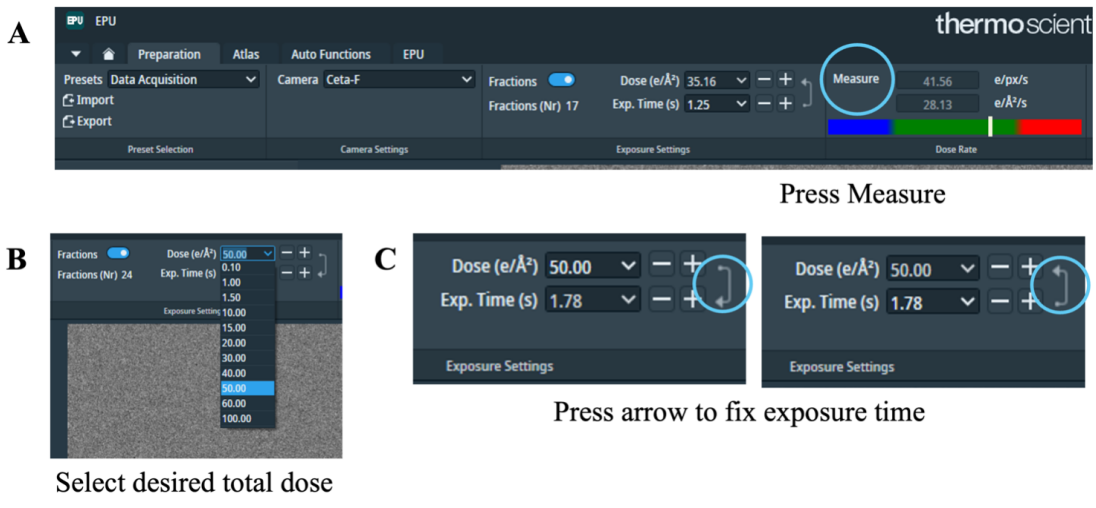

Figure 5: Determining total dose and exposure time.

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4.6. Prepare to optimize optics: Transfer a carbon alignment grid into the TEM.

4.6.1. The *Optimize optics* alignments are 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 speed up the loading and unloading processes.

4.6.2. Load a clipped, holey carbon grid into the cassette in the CLS.

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

4.6.2.2. To visually verify the inventory, on the CLS touchscreen *Actions* tab, select "Load / unload samples from cassette".

4.6.2.3. Transfer grids to/from the cassette slots as required. Update the inventory on the CLS interface.

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

Load grid into TEM

4.6.3. Load grid into TEM

4.6.3.1. On the CLS, press *Load sample into TEM* (Figure 6A).

4.6.3.2. Select one grid for transfer to TEM.

4.6.3.3. There are 4 main stages in this process: "*Loading to transfer device*", "*Transferring to TEM*", "*Loading to TEM*", and "*Transferring back to loading workstation*".

4.6.3.4. The CLS will pump down the TD and indicate when the TD is ready to be detached from the docking station.

4.6.3.5. Detach the TD from the CLS (Figure 6B). Make sure to use two hands when moving the TD.

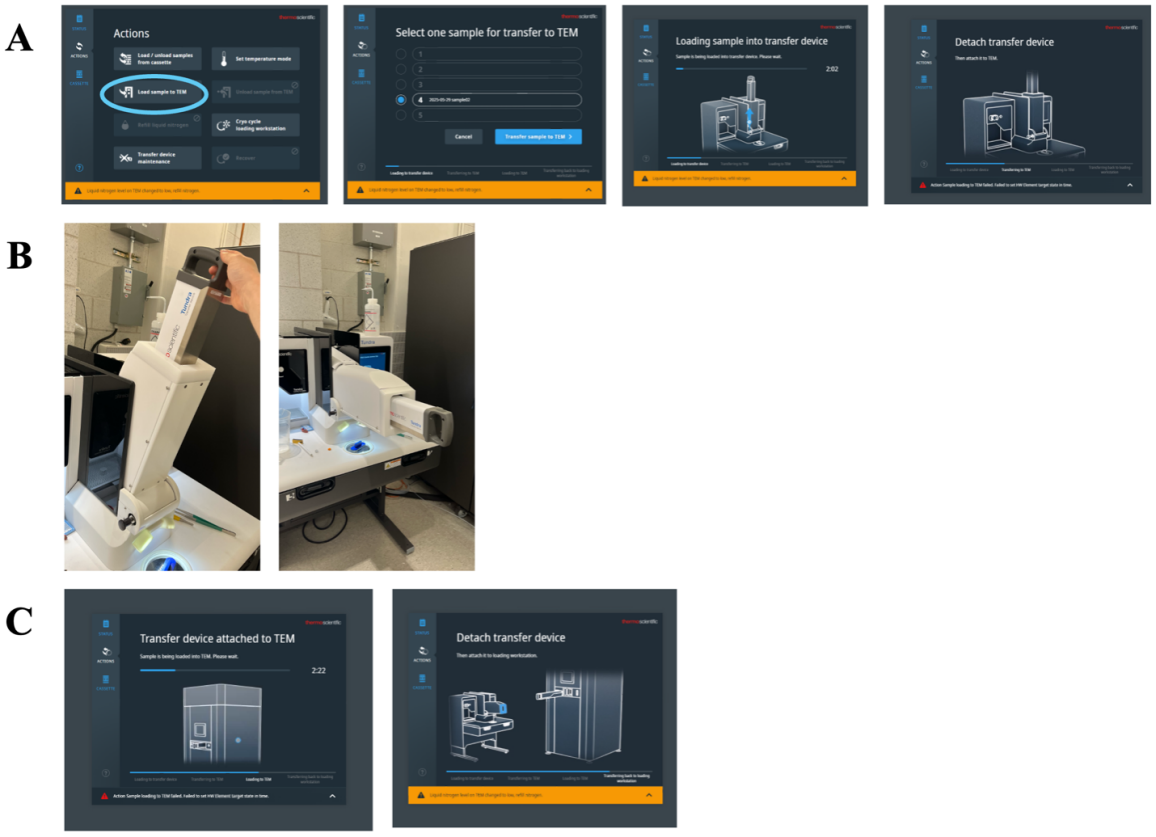

Figure 6: Loading a grid into the tundra. *A) The first processes on the CLS, leading to the detachment of the transfer device (TD). B) Detaching the transfer device. Pull out the black pin and pull down the TD so it is parallel with the floor. C) The TEM screen during grid loading.*

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4.6.3.6. When the TD is in transit, make sure the front port is kept clean and free from obstructions.

4.6.3.7. 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.

4.6.4. Once the grid has been transferred from the TD to the stage and you are prompted to do so, remove TD from the TEM and place it in the docking station of the CLS (Figure 6C).

4.7. Perform the Optimize Optics alignments.

4.7.1. After User alignments, Optimize optics must be performed, in which there is further beam alignment and correction of beam astigmatism and coma. EPU has a timer that reminds the user if alignments are older than 24 hours.

4.7.2. Although these alignments may be done either in EPU or MiCo, it is recommended to start them through EPU as it ensures they are performed over carbon and the stage is at eucentric height.

4.7.3. Firstly, acquire an Atlas image in *Preparation -> Presets -> Atlas*. Move over an area of carbon by Right Click -> Move stage here (Figure 7A).

4.7.4. In EPU, select *Auto Functions* tab, then "Alignments", "Optimize Optics" and press *Start* (Figure 7B).

4.7.5. Use the context menus to move the stage to a carbon area and press Resume to start the beam optimization (Figure 7C).

4.7.6. Make sure all tasks in MiCo -> Alignments -> Optimize Optic get a green check (Figure 7D).

4.7.7. Column valves should be closed automatically after Optimize optics has finished, but make sure they are closed via MiCo -> Home -> Vacuum.

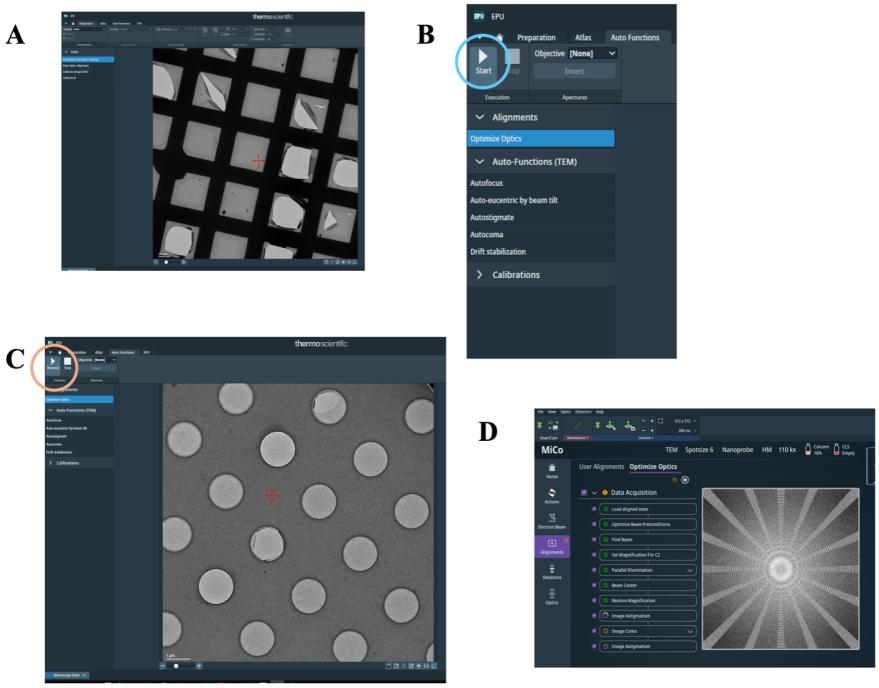

Figure 7: Performing optimize optics alignments. *A) Centering an area with carbon for the alignments. B) Starting the alignments in EPU -> Auto-Functions. C) The stage must be centered on carbon film between the holes. D) Tasks completing in MiCo -> Alignments.*

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4.8. Calibrate the image shifts between presets.

4.8.1. Now that the microscope is aligned you should check that the image shifts are aligned between presets. This means that as you change magnification, you stay centered on the same feature. This is important when targeting in the center of a hole.

4.8.2. Find a piece of contamination that is visible in all four presets (Figure 8A). A good example is shown below of a large ice feature with corners. Broken holes with corners are good for aligning too.

4.8.3. The stage must be centered on this contamination in the Data Acquisition preset (Figure 8B).

4.8.4. In the preparation tab go to *Tasks -> Calibrate Image Shifts* (Figure 8C).

4.8.5. If eucentric height has not been identified, then press Auto-Eucentric.

4.8.6. Press *Acquire*. Once all four images have been taken, click on the matching feature in each image (Figure 8D). Press *Store Calibrations.*

4.8.7. To check the calibrations, press *Acquire* again and check the feature is centered.

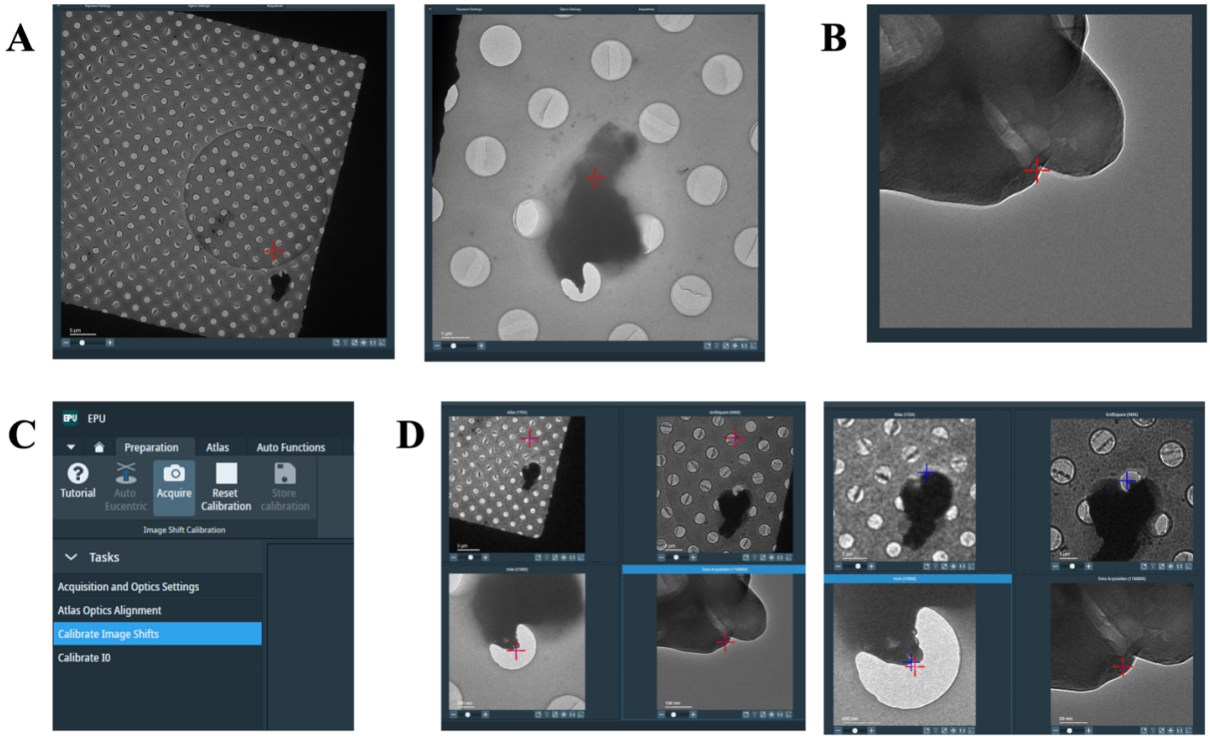

Figure 8: Calibrating image shifts on the Tundra using EPU. *A) An example of a good feature for aligning the image shifts. B) A data acquisition taken with the stage aligned on the fature. C) Starting the image shift calibration in EPU -> Preparation -> Calibrate Image Shifts. D) Performing the calibration process.*

4.9. Unload the alignment grid according to the instructions in 4.2.

4.10. Switch the temperature of the CLS to cryo conditions.

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

4.10.2. On the CLS interface, go to the *Actions* tab and select “Set temperature mode”. Select *liquid nitrogen temperatures* and press *continue*.

4.10.2.1. Remove the clear lid and white center cover.

4.10.2.2. Open the LN2 tank’s blue liquid valve. You should hear gas being released into the CLS. This is air in the line being forced out by the liquid nitrogen.

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

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

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

4.10.3. Although the first CLS fill should be performed from the LN2 tank's blue valve, topping up should be done regularly via a LD4 dewar.

4.11. Load a cryo sample into the TEM.

4.11.1. **Cool the autogrid tweezers in LN2.**

4.11.2. 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.

4.11.3. On the CLS interface, press *load/unload samples from the cassette*.

4.11.4. Transfer the grid correctly into the cassette. 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. See site specific instructions for correct loading orientation.

4.11.5. Check for secure insertion in the cassette by gently tapping the top edge of the autogrid with the autogrid tweezers.

4.11.6. On the CLS interface, mark the slot as empty.

4.11.7. Transfer grids to TEM as per the instructions in section 4.5.

4.11.8. **Keep any remaining samples in a full benchtop dewar or in a larger storage dewar.** Do **not** store them in the CLS as the LN2 evaporates rapidly.

4.12. Grid Screening

4.12.1. Find Z focus for the grid.

4.12.1.1. Putting the grid at the correct Z height before taking the atlas will increase the square targeting accuracy, as well as increasing the likelihood that subsequent automated Z height finding succeeds.

4.12.1.2. Move over an intact grid square. Take an atlas image by going to *Preparation -> Acquisition and Optics settings -> Presets -> Atlas*. Right click on the center of a square and click *move stage here*.

4.12.1.3. If no squares are visible, you may be over a thick region of the grid. Search through other regions by right clicking outside the image and selecting *move stage here and acquire.*

4.12.1.4. Once over a grid square, go to *Auto Functions -> Auto-eucentric by beam tilt -> Start.* The auto-eucentric must succeed before moving on. If it fails, use the beam tilt images to figure out the issue. You may need to move the stage to a better area.

4.12.1.5. If the auto-eucentric fails, there are several possible explanations detailed in the 'troubleshooting' section.

4.12.2. Take an atlas of the grid.

4.12.2.1. In the *Atlas* tab, click the *Session Setup* tab and then *New Session* (Figure 9A, blue circle). Fill in the details and put "_Atlas" at the end. Make a note of the name and later, use a similar name for your EPU session.

4.12.2.2. In the *Screening* tab, image the full grid with 6x6 tiles, by keeping *Number of Tiles* empty. Otherwise, specify a number (16 or 25 are good options) to reduce the time taken. Press *Start* (Figure 9B, orange circle).

4.12.2.3. Wait for the atlas to finish taking (Figure 9C).

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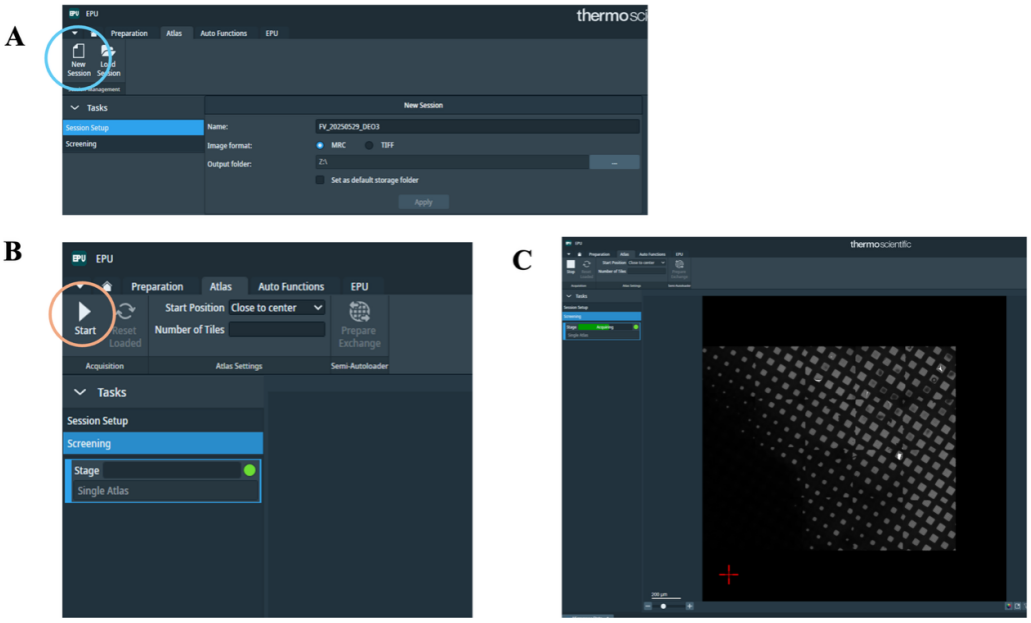

Figure 9: Taking an atlas of a grid. *A) Starting a new session in EPU -> Atlas. B) Starting the acquisition process. C. The atlas being acquired.*

4.12.3. Automated data collection using the *EPU* tab.

4.12.3.1. There are two options for screening grids.

4.12.3.1.1. Quickest - Take images manually through the preparation tab. However, these will not be saved automatically. You should *Right click -> Export image* to save.

4.12.3.1.2. Best – Take images automatically through the EPU tab. This is slower, but it will make sure the images are in focus, automatically save them and allow for subsequent viewing in the Athena image viewer.

4.12.3.1.3. You could do both by first checking particles manually, then taking more images using the EPU tab.

4.12.3.2. Start the EPU session.

4.12.3.2.1. Go to EPU-> *Session creation* and press *New Session* (Figure 10A).

4.12.3.2.2. For the prompt about using the preferences of the current session, select 'Yes' if this grid is the same as the previous, otherwise select 'No' (Figure 10B).

4.12.3.2.3. In *Session setup,* match the grid type to the sample. For *session type* use *Manual* and for *Acquisition Mode* use *Faster* (Figure 10C).

4.12.3.2.4. Image format should be MRC and the output folder should be the Z:\ drive.

4.12.3.3. Set up Athena to save images for later viewing.

4.12.3.3.1. In *Athena Settings*, click *Select.*

4.12.3.3.2. Create a new folder for this box of grids. This can house multiple EPU sessions to make data analysis easier, so its name should not be grid specific but box specific. Make sure you remember to select this folder for subsequent grids.

4.12.3.3.3. Create a new *single particle analysis.* The name of this should be specific to your grid/sample. Create a new Screening run with the same name. If you later decide to collect data on this grid, you can attach it as an Imaging run to the same single particle analysis.

4.12.3.3.4. Click apply to start the EPU session.

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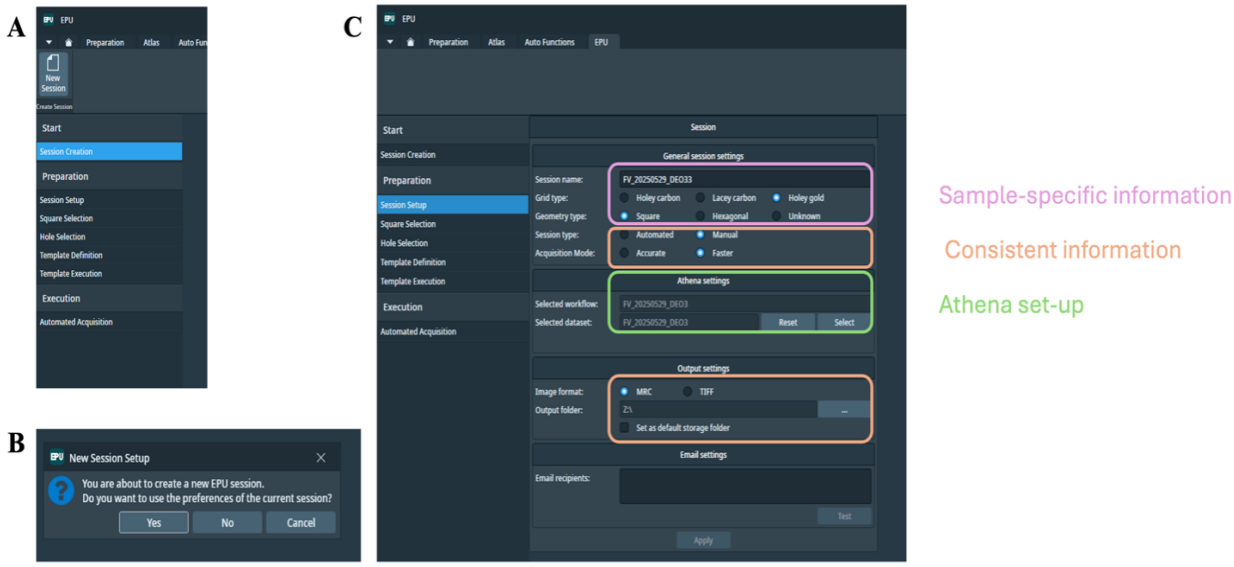

Figure 10: Setting up an EPU session for automated data collection.

4.12.4. Square Selection.

4.12.4.1. *Go to the Square Selection* tab (Figure 11).

4.12.4.2. First, unselect all squares (Blue oval).

4.12.4.3. Then, for squares you'd like to image, *Right Click -> Select* (Pink circle). This will turn the square green (Orange circle). Similar squares to those you selected will be highlighted with a white and green dashed square (Green circle).

4.12.4.4. If the square has not been recognized by the automated square finder, you can still add it manually by *Right Click -> Add new grid square here* (Yellow circle).

4.12.4.5. It is recommended to pick at least three squares of varying sizes and location across the grid.

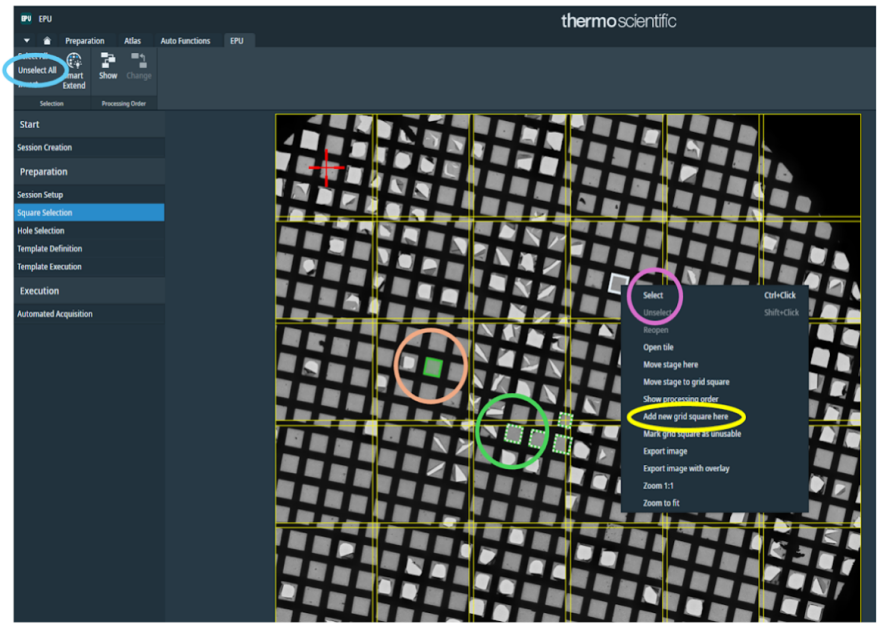

Figure 11: Square selection in EPU.

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4.12.5. Hole selection.

4.12.5.1. Move to the hole selection tab (Figure 12). Go to square 1/x using the *Navigate* buttons.

4.12.5.2. Press *Auto-Eucentric* to find eucentric height (Figure 12, blue circle). The microscope will automatically acquire an image of the square.

4.12.5.3. Press *Measure hole size* to find the holes using the yellow circles, then press *find holes* (Figure 12, pink square). Check the circles are over the holes and if not, take time to get this as accurate as possible.

The hole size needs to be accurate for later on-the-fly hole finding and the spacing is important for hole selection and correct filtering using the Ice Quality Filter.

4.12.5.4. Try to remove hole close to the grid bar as these often have bad ice. Press *Remove Holes Close to Grid Bar.* However, this button often does not work on the Tundra.

4.12.5.5. Use *Filter Ice Quality* to remove holes found outside the grid square, as well as thick hole and empty holes. Take time to tune this well as once the filter is set up, the remaining squares can be automatically filtered.

4.12.5.6. You may also want to try the *smart filter* and then play with parameters.

4.12.5.7. Use *Fast Screening* to randomly pick a subset of the holes for screening (Figure 12, orange square).

4.12.5.8. If in Manual mode, you can use the *Selection Brush* to manually add or remove holes. Hover over the button to find shortcuts for this feature.

4.12.5.9. You only need to manually prepare one square. For the remaining squares, use P*repare All Squares* (Figure 12, green square).

This will automatically go through each remaining square and find eucentric height, acquire a square image, find the holes, filter them and randomly pick a subset for screening.

4.12.5.10. If auto-eucentric fails, check what your Hole/Eucentric height image looks like in the *Preparation* tab. Likely you are over thick ice with little contrast or a huge ice feature.

Back in the *Hole Targeting* tab, click *Acquire* to image the square anyway. Identify a better area for auto-eucentric, then move the center of the square that failed by *Right Click -> Move Stage Here.* Press *Auto Eucentric* again.

4.12.5.11. Check through all squares to make sure you are happy with the automated hole selection and that each square has a Z height shown in green text (Figure 12, yellow circle).

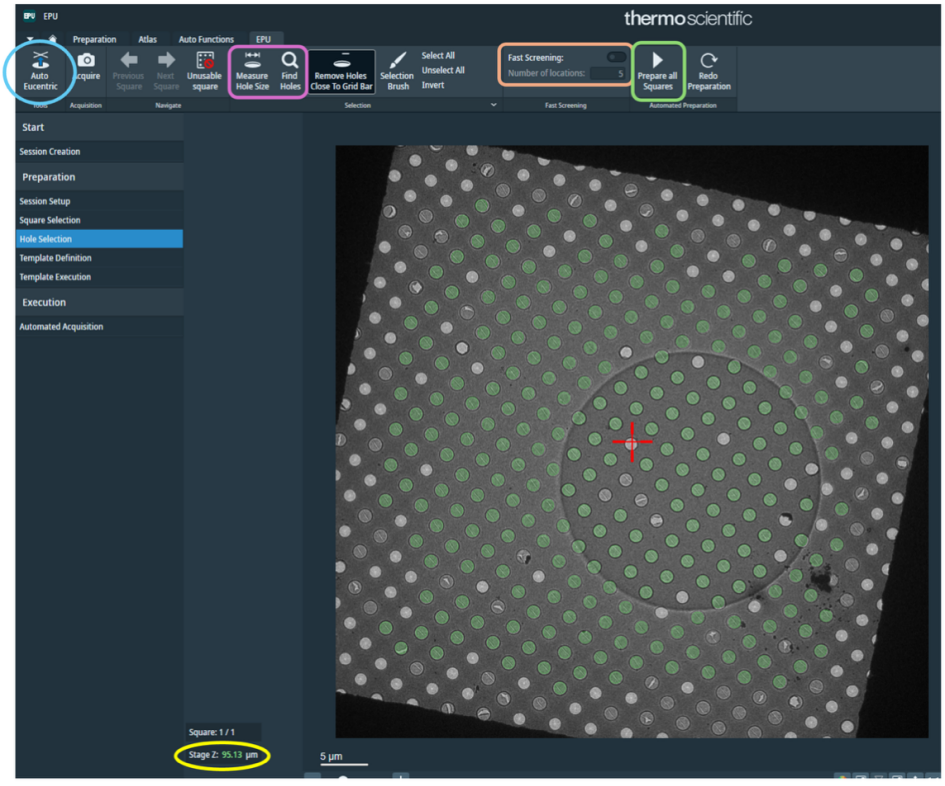

Figure 12. The options available in the Hole Selection tab

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4.12.6. Template definition

4.12.6.1. In the *Template definition* tab, press *acquire*, then *find and center hole* (Figure 13A).

4.12.6.2. If you notice the yellow hole finder is a different size to the actual hole, change the hole size in the hole selection tab and acquire a template definition again to check. You want the hole finder size to match as closely as possible.

4.12.6.3. Add an *Acquisition area*. The large beam diameter on the Tundra only allows for one shot per hole. Edit the defocus range for the acquisition area (Figure 13B). For screening, use a single defocus value of 1-2 µm. For a data collection, use a comma separated series of defocus values.

4.12.6.4. Add an *Autofocus area* and place the blue circle between the holes. *Recurrence* should be *After Centering* (Figure 13C).

4.12.6.5. Do not add a *Drift Measurement Area.* This slows the collection down. If you consistently notice drift in your images, alert a member of staff.

4.12.6.6. The purple *Autofocus area* may overlap with a green *Acquisition area* but should not overlap with the hole. If the autofocus beam would burn the holes, then you should reduce the size of the C2 aperture. Do not alter the intensity as you will take the illuminated area out of the parallel range.

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

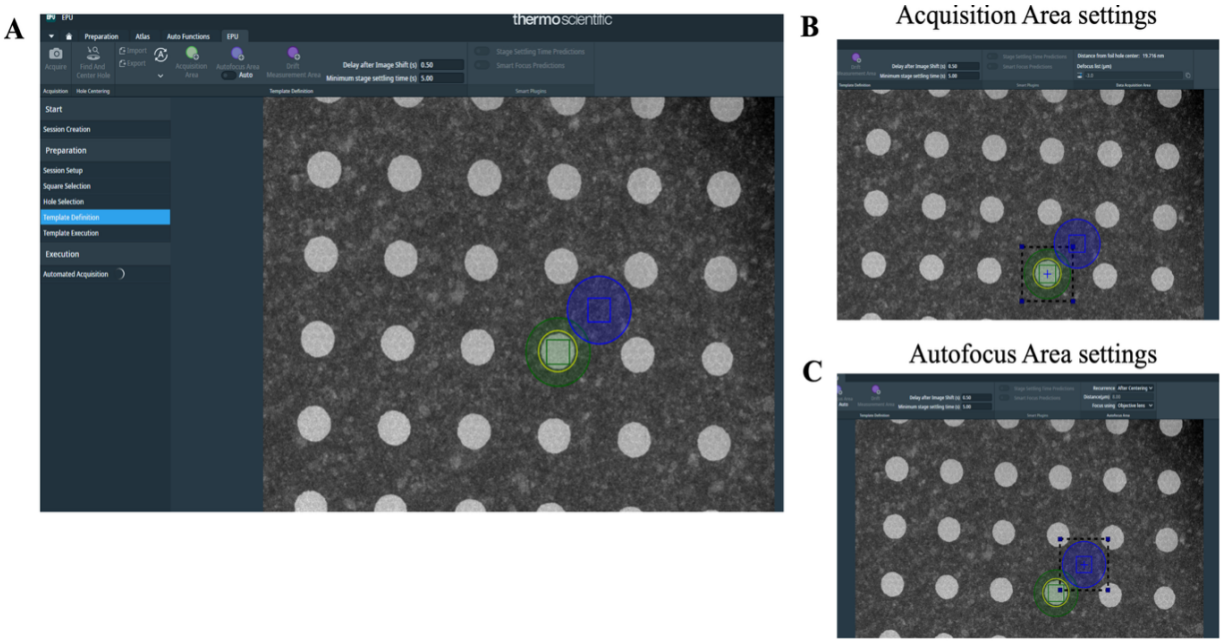

Figure 13: The options available in the Template Definition tab. *A) The template definition taskbar. B) The Acquisition Area settings. C) The Autofocus Area Settings.*

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

4.12.8. After the collection has finished, check the column valves are closed. Remove the grid from the TEM using the TD following instructions in 4. 2. Store the grid appropriately in a dewar. **Do not store them in the CLS as this warms up quickly.**

4.12.9. Repeat the screening process with each grid.

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4.13. Clean up

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

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

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

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

4.13.5. If you have finished with the TEM, cryocycle it by selecting on the touchscreen interface.

5. Troubleshooting

5.1. Unload/load sample is greyed out.

5.1.1. Press '?' button. This will tell you why this process is not accessible.

5.1.2. Often the column valves are still open. Close the column valves in MiCo.

5.1.3. Another common reason is that there is not enough liquid nitrogen in the CLS. Top up the nitrogen using an LD4 dewar until the unload/load button becomes available.

5.2. The automated hole finder is failing, leading to FoilHoles being skipped. The failing is because the yellow hole finder is smaller/larger than the actual holes

5.2.1. Pause the collection at *EPU -> Automated Acquisition -> Pause Run.*

5.2.2. In *Hole Selection*, adjust the size of the yellow hole template.

5.2.3. Test the hole finder in *Template Execution* before starting the EPU run.

5.3. White patches consistently in all images.

5.3.1. These are likely reflections from the gold foil. They can be removed by inserting the objective aperture.

5.3.2. Pause the collection at *EPU -> Automated Acquisition -> Pause Run.*

5.3.3. Insert the 100 µm objective aperture by going to *Auto-Functions -> Optimize Optics -> Objective* and changing [None] to 100.

5.3.4. Re-start the collection.

5.3.5. Taking an atlas image will require retraction of the objective aperture, as it obscures most of the image.

5.3.6. The objective aperture should also be retracted when a grid is loaded or unloaded.White patches consistently in all images.

5.4. EPU crashes.

5.4.1. Make sure EPU is closed. Task manager may have to be used for this.

5.4.2. Open EPU again using the icon on the bottom of the screen.

5.4.3. If EPU will not restart correctly, get a member of staff to restart the TEM server.

5.5. The CLS does not respond.

5.5.1. Press *recover* on the CLS interface.

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

5.5.3. If the reset button fails, call SEMC staff.

5.6. Auto-eucentric fails.

5.6.1. Take an image in Preparation -> Hole/EucentricHeight. Are you over grid bar or a large piece of ice? If so, move over a better area with features it can correlate.

5.6.2. Your current Z height might be too far from the desired Z height. Go to the TEM UI .

5.6.3. Make a guess if you need to move to a positive or negative Z height. In *the Search tab -> Stage -> Set,* type either 200 or -200 in then Z box and press *Go To.*

5.6.4. Try Auto-Eucentric again and see if it succeeds.

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6. Additional Figures:

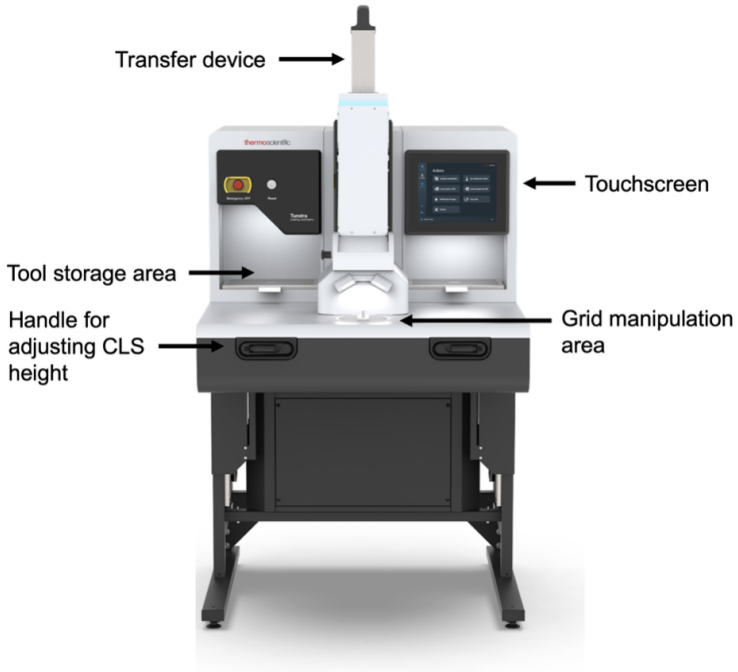

Figure 14: Diagram of the Tundra cryo loading station (CLS).

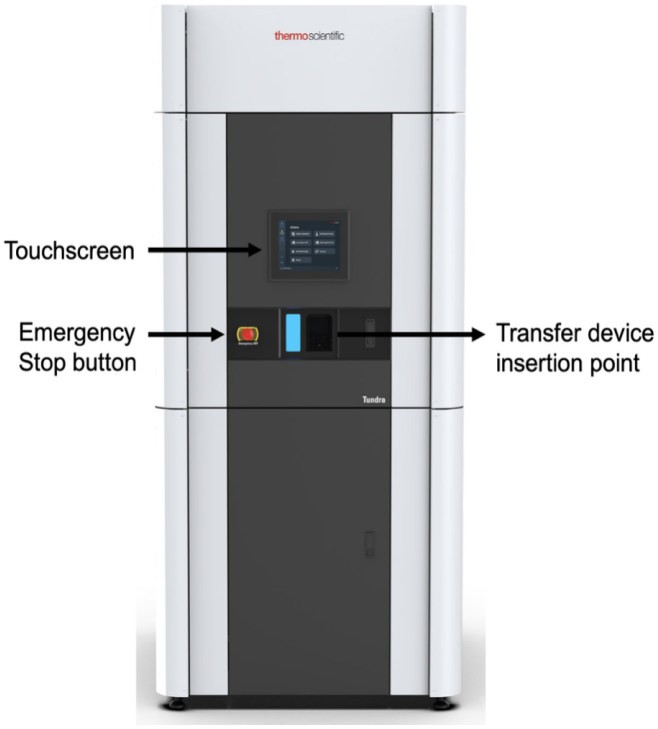

Figure 15: Diagram of the Tundra TEM.

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7. Chemicals:

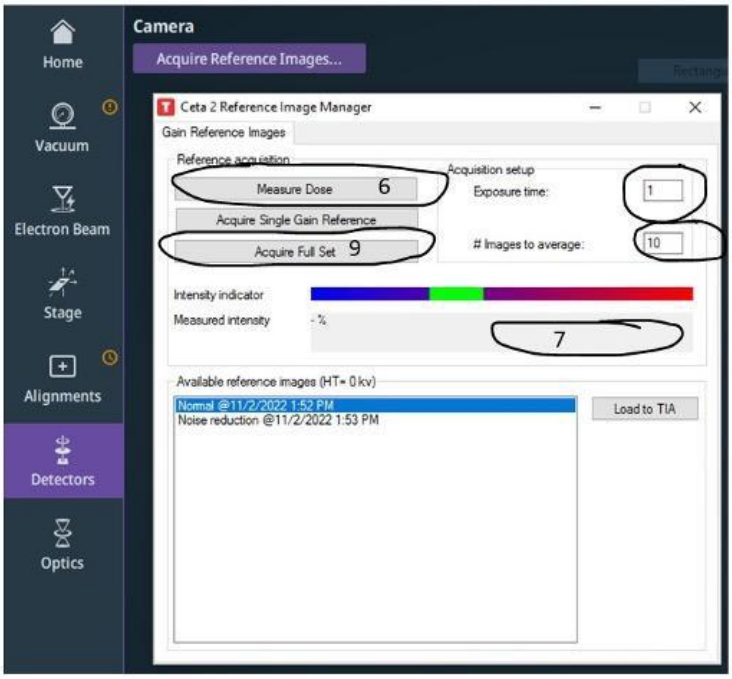
7.1. Liquid Nitrogen

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".



Step 6= Measure dose

Step 7= Give you measure Intensity

-Exposure time and measure intensity should be similar to the one that is shown in EPU. (Adjust the exposure time is needed

-Change the images to average in such a way that the value of the result should be around 3000.

Step 9=acquire Full set

Notes:

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