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User-friendly, High-throughput, and Fully Automated Data Acquisition Software for Single-particle Cryo-electron Microscopy

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TITLE:

User-friendly, High-throughput, and Fully Automated Data Acquisition Software for Single-particle Cryo-electron Microscopy

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SUMMARY:

Single-particle cryo-electron microscopy demands a suitable software package and user-friendly pipeline for high-throughput automatic data acquisition. Here, we present the application of a fully automated image acquisition software package, Latitude-S, and a practical pipeline for data collection of vitrified biomolecules under low-dose conditions.

ABSTRACT:

In the past several years, technological and methodological advancements in single-particle cryo-electron microscopy (cryo-EM) have paved a new avenue for the high-resolution structure determination of biological macromolecules. Despite the remarkable advances in cryo-EM, there is still scope for improvement in various aspects of the single-particle analysis workflow. Single-particle analysis demands a suitable software package for high-throughput automatic data acquisition. Several automatic data acquisition software packages were developed for automatic imaging for single-particle cryo-EM in the last eight years. This paper presents an application of a fully automated image acquisition pipeline for vitrified biomolecules under low-dose conditions.

It demonstrates a software package, which can collect cryo-EM data fully, automatically, and precisely. Additionally, various microscopic parameters are easily controlled by this software package. This protocol demonstrates the potential of this software package in automated imaging of the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) spike protein with a 200 keV cryo-electron microscope equipped with a direct electron detector (DED). Around 3,000 cryo-EM movie images were acquired in a single session (48 h) of data collection, yielding an atomic-resolution structure of the spike protein of SARS-CoV-2. Furthermore, this structural study indicates that the spike protein adopts two major

conformations, 1-RBD (receptor-binding domain) up open and all RBD down closed conformations.

INTRODUCTION:

Single-particle cryo-EM has become a mainstream structural biology technique for high-resolution structure determination of biological macromolecules[1]. Single-particle reconstruction depends on acquiring a vast number of micrographs of vitrified samples to extract two-dimensional (2D) particle images, which are then used to reconstruct a three-dimensional (3D) structure of a biological macromolecule[2,3]. Before the development of DEDs, the resolution achieved from single-particle reconstruction ranged between 4 and 30 Å[4,5]. Recently, the achievable resolution from single-particle cryo-EM has reached beyond 1.8 Å[6]. DED and automated data acquisition software have been major contributors to this resolution revolution[7], where human intervention for data collection is minimal. Generally, cryo-EM imaging is performed at low electron dose rates (20–100 e/Å^2) to minimize electron beam-induced radiation damage of biological samples, which contributes to the low signal-to-noise ratio (SNR) in the image. This low SNR impedes the characterization of the high-resolution structures of biological macromolecules using single-particle analysis.

The new generation electron detectors are CMOS (complementary metal-oxide-semiconductor)-based detectors, which can overcome these low SNR-related obstacles. These direct detection CMOS cameras allow fast readout of the signal, due to which the camera contributes better point spread function, suitable SNR, and excellent detective quantum efficiency (DQE) for biological macromolecules. Direct detection cameras offer high SNR[8] and low noise in the recorded images, resulting in a quantitative increase in the detective quantum efficiency (DQE)—a measure of how much noise a detector adds to an image. These cameras also record movies at the speed of hundreds of frames per second, which enables fast data acquisition[9,10]. All these characteristics make fast direct detection cameras suitable for low-dose applications.

Motion-corrected stack images are used for data processing to calculate 2D classification and reconstruct a 3D density map of macromolecules by using various software packages such as RELION[11], FREALIGN[12], cryoSPARC[13], cisTEM[14], and EMAN2[15]. However, for single-particle analysis, an enormous dataset is required to achieve a high-resolution structure. Therefore, automatic data acquisition tolls are highly essential for data collection. To record large cryo-EM data sets, several software packages have been used over the past decade. Dedicated software packages, such as AutoEM[16], AutoEMation[17], Leginon[18], SerialEM[19], UCSF-Image4[20], TOM$_2$[21], SAM[22], JAMES[23], JADAS[24], EM-TOOLS, and EPU, have been developed for automated data acquisition.

These software packages use routine tasks to find hole positions automatically by correlating the low-magnification images to high-magnification images, which assists in identifying holes with vitreous ice of appropriative ice thickness for image acquisition under low-dose conditions. These software packages have reduced the number of repetitive tasks and increased the throughput of the cryo-EM data collection by acquiring a vast amount of good-quality data for several days continuously, without any interruption and the physical presence of the operator. Latitude-S is a similar software package, which is used for automatic data

acquisition for single-particle analysis. However, this software package is only suitable for K2/K3 DEDs and is provided with these detectors.

This protocol demonstrates the potential of Latitude-S in the automated image acquisition of SARS-CoV-2 spike protein with a direct electron detector equipped with a 200 keV cryo-EM (see the **Table of Materials**). Using this data collection tool, 3,000 movie files of SARS-CoV-2 spike protein are automatically acquired, and further data processing is carried out to obtain a 3.9–4.4 Å resolution spike protein structure.

PROTOCOL:

NOTE: Three important steps are required for cryo-EM data collection: 1. cryo-EM grid preparation, 2. calibration and alignment of the microscope, 3. automatic data collection (**Figure 1**). Furthermore, automated data collection is subdivided into a. suitable area selection, b. optimization of Latitude-S, c. start automatic hole selection, and d. start automatic data acquisition (**Figure 1**).

1. Cryo-EM grid preparation and sample loading for automatic data acquisition

1.1. Clean the grids using a glow discharger and vary the glow discharge parameters based on experimental requirements (here, 60 s at 20 mA).

1.2. Add a freshly prepared protein sample (3 µL) to the glow-discharged grid and incubate for 10 s.

1.3. Blot the grids for 3–5 s at 100% humidity and quickly plunge them into liquid ethane using a cryo-plunger.

1.4. Clamp the grids manually into a clip ring to form the cartridge using a flexible C-clip ring.

1.5. Load the frozen cartridge-mounted grids into the autoloader cassette and transfer the cassette by the nano cap to the precooled autoloader of the microscope for data collection.

2. Microscope tuning and basic alignment before automatic data acquisition

2.1. Beam shift

2.1.1. Click on **Beam shift** from the **Direct Alignment** tab.

2.1.2. Reduce the magnification, and center the beam to the optical axis using the **Multifunction X and Y** knob.

2.2. Pivot point alignment

2.2.1. Click on the **Beam tilt** option in **Direct Alignment pp X** from the **Direct Alignment** tab.

2.2.2. Condense the beam to a spot and minimize the movement by using the **Multifunction X and Y** knob.

2.3. C2 aperture centering

2.3.1. Select the condenser aperture from the **Alignment** tab.

2.3.2. Condense the beam to a spot, center the beam to the optical axis, and then expand the beam to cover the circle evenly.

2.3.3. Repeat these steps until the Condenser 2 aperture is adjusted.

2.4. Coma-free alignment

2.4.1. Click on **Coma-free Alignment X** from the **Direct Alignment** tab to align the beam to the optical axis.

2.4.2. Use the **Multifunction** knob to minimize the shape and the movement of the FFT (ensure it is stable).

2.4.3. Repeat the same procedure for **Coma–free alignment Y**.

2.5. Set parallel illumination before data collection in the cryo-EM because of the C twin lens.

2.5.1. Insert the objective aperture (70 µm) in diffraction mode.

2.5.2. Focus the objective aperture on the front focal plane of the diffraction lens by controlling the defocus-intensity knob (objective lens and C2 lens current).

2.5.3. Ensure that the crisp edge of the objective aperture is seen after proper defocusing.

2.5.4. Insert the beam stopper and spread the intensity until the gold powder diffraction rings are minimized.

NOTE: If the beam is spread properly, a clear diffraction ring of the gold powder is visible on the screen, which indicates that the beam is parallel.

2.5.5. Retract the beam stopper after setting the parallel illumination and change the microscope mode to **Nano probe**.

NOTE: Check the microscope tuning before starting data collection to ensure the optimum performance of the microscope. All these settings would be performed in the microscope **Direct Alignment GUI** Tab. All the microscope tuning is performed using a test grid before data collection.

3. Data acquisition with Latitude-S

3.1. Start Latitude-S automated data acquisition software.

NOTE: Latitude-S installation also requires microscope calibration, which will be performed before data collection, and the settings will be stored permanently. Five different states for data collection are calibrated with four different magnifications (**Figure 1** and **Figure 2)**. Atlas state and grid state are in two different magnifications in LM mode (low-magnification ranges). The hole state is in SA mode (high-magnification ranges) but with a moderate magnification. Focus and data state use high-magnification SA mode.

3.1.1. Click on **DigitalMicrograph** from the **Start** menu, or double-click on the **DigitalMicrograph** icon on the desktop.

3.1.2. Select the **Technique manager** icon from **DigitalMicrograph**.

NOTE: This system will show **TEM** and **Latitude-S** icons (**Figure 2** and **Supplemental Figure S1**).

3.1.3. Select the **Latitude-S** icon for single-particle automated data collection.

NOTE: The K2 camera operates in three modes: linear/integrated, counted, and super-resolution. The user could select any mode in the interface of **DigitalMicrograph**. Data images could be saved as either dose-fractionated image stacks or as summed images in MRC, TIF, or .dm4 files with different bit depths. Furthermore, data could be saved as motion-corrected images for the K3 camera. On a K2 camera, an unprocessed image stack could be saved as 4-bit MRC, 8-bit TIF, or 8-bit .dm4 files.

3.2. Create a new session based on the settings from a previous session.

3.2.1. Check the **Based on prior session** checkbox in the palette.

3.2.2. Select the **New** button.

3.2.3. Choose the folder containing the session on which the new session's settings are based. Go to the previous session directory to create the new session. Choose the folder to save the new session and associated data.

3.2.4. Select and choose the folder where the new session and associated data will be saved.

NOTE: Each state and its underlying settings (magnification, illumination conditions, image, or projector) and beam shift and camera parameters (total exposure, single-frame exposure, and binning) will be exported from the existing session to the new session. The path of the folder is shown as a text string at the bottom of the palette. Each of the states and configuration palettes has an asterisk (*) appended to the title to show that it has already been set up and is ready to use.

3.3. Continue an existing session.

3.3.1. Press the **Continue** button in the palette to continue an existing session.

NOTE: The atlas montage cannot be modified.

3.3.2. Choose and navigate to the folder that contains the session that needs to be continued.

3.4. Start an entirely new session.

3.4.1. Click on **New tab** in the palette. Choose the folder that contains the session to be continued. Select a folder to save the data.

NOTE: The default folder name is built by using the date and time.

3.4.2. Click on the **Setting** icon. In the **Manage state explore** box that appears, add state, set the TEM condition, the camera condition, and image/stack, and then name the state.

NOTE: The automated data acquisition workflow uses 5 different states for automated data collection. These states are configured and stored in their respective state palettes. The state summary is given in **Table 1**.

3.5. Configure the atlas state.

3.5.1. Click on **Atlas state palette**.

3.5.2. Configure the atlas state with the following parameters: magnification 115x LM mode in nano probe, illumination conditions—spot size 8 and brightness 934400, binning: 1 and camera exposure time: 1.0 s for imaging at low magnification. Refer to the state summary given in **Table 1**.

3.5.3. Click on **Next** to move to the next state.

NOTE: Atlas state is the lowest magnification state, which provides the survey of the entire grid (**Supplemental Figure S2**). Generally, this state helps us visualize the entire grids at low magnification and judge the ice thickness, flatness, and broken square of the grids. It is recommended to generate the atlas at different areas of the grid to observe the optimal ice thickness and suboptimal ice thickness of the grids (**Supplemental Figure S3**). The mentioned parameters could be varied according to the user's needs.

3.6. Configure the grid state.

3.6.1. Click on the **Grid state palette**.

3.6.2. Configure the Grid state with the following microscope imaging optics (magnification 380x LM mode in Nano probe), illumination conditions (spot size: 8 and brightness 626,200), binning: 1 and camera exposure time: 1.0 s.

3.6.3. See the Latitude-S state summary provided in **Table 1**.

3.6.4. Click on **Next** to move to the next state.

NOTE: The grid state is set at a magnification higher than the atlas state such that the field of view is one grid square (**Figure 2**). In this particular magnification, one grid square is observed. Therefore, holes are observed correctly in this magnification, which helps check the ice thickness of the holes (**Supplemental Figure S4**). A simple bandpass filter is used in the grid state to locate the holes in the patent grid. The mentioned parameters could be varied according to the user's needs.

3.7. Configure the hole state.

3.7.1. Click on the **hole palette**.

3.7.2. Configure the Hole state with the following microscope settings: imaging optics (magnification 4500x SM mode in Nano probe), illumination conditions (spot size: 7 and Beam diameter 8.81 µm), binning: 1 and camera exposure time: 1.0 s.

3.7.3. Change the parameters if required based on the grid type. See the state summary provided in **Table 1**.

3.7.4. Click on **Next** to move to the next state.

NOTE: The SA mode indicates a high-magnification range in the electron microscope. The hole state is in the SA magnification range with a field of view of a few micrometers (10–20 µm) (**Figure 2** and **Supplemental Figure S4**). This magnification range is higher than the Atlas or Grid state but much smaller than Focus/Data state. In this magnification, individual holes will be visible. The hole size is appropriate to observe high degrees of contaminations, empty holes, and the proper ice thickness of the holes. The holes for imaging are selected based on these assumptions. Two filters are used in the hole state: one for cross-correlating a hole reference image with a new hole image and another for adjusting the stage height to the eucentric height.

3.8. Configure the focus state.

3.8.1. Click on **Focus palette**.

3.8.2. Configure the focus state with the following microscope settings: imaging optics (magnification 45,000x SA mode in nano probe), illumination conditions (spot size: 8 and brightness 934400), binning: 1 and camera exposure time: 1.0 s.

3.8.3. Focus on the amorphous carbon area near the hole. Refer to the Latitude-S state summary provided in **Table 1**.

3.8.4. Click on **Next** to move to the next state.

NOTE: The SA mode indicates a high-magnification range in the electron microscope. The Focus state is the higher SA range magnification. In focus mode, the beam is shifted to a nearby carbon area of the target hole and performs focus automatically to collect the data in the data state. A bandpass filter combined with a hanning or soft rectangular filter is used in the focus state to measure the offset between two focus state images of the same area (**Figure 2**). The mentioned parameters could be varied according to the user's needs.

3.9. Configure the data state.

3.9.1. Click on **Data palette**.

3.9.2. Configure the data state with the following microscope settings: imaging optics (e.g., magnification 28,000x, 45,000x, 54,000x at SA mode in nano probe), illumination conditions (spot size: 8 and brightness 934400), binning: 1 and camera exposure time: 1.0 s.

3.9.3. Refer to the Latitude-S state summary given in **Table 1**.

3.9.4. Click on **Next** to move to the next state.

NOTE: Data state is the highest magnification selected based on pixel size requirements and target resolution (**Figure 2**). Generally, after focusing, the beam is automatically shifted to the target area to collect the data. The above-mentioned parameters could be changed based on the user's requirements.

4. Focus configuration

4.1. Click on **Focus configuration palette**. Specify the range of defocus values and the step size in the given tab.

4.2. Press **Next** button to move to the next step.

NOTE: Lower defocus values can be used for high-resolution data acquisition. Generally, -0.5 to -3.0 µm defocus values with 0.25 or 0.5 defocus step sizes are used for image acquisition. Users can skip the focus setup step if they only want to screen the sample. Simply press the **Next** button on the palette to skip the focus configuration step.

5. Fine alignment

5.1. Focus on some features on the grid (e.g., ice contamination hexagonal ice); see **Figure 3**.

NOTE: Features should not be too big or too small. They should be visible at both Atlas state magnification 115x (LA mode) and data magnification.

5.2. Click on the **Capture** button. Position the red cross mark on the same feature on each image of different states.

5.3. Start with focus, data, and hole states because their field of view is much bigger than the atlas and grid states. Zoom on atlas and grid states to position the red cross mark on the same feature in the atlas and grid states.

5.4. Click on the **Calculate** button to calculate the positions of five different states, which will calculate the offsets between each of the states and reflect these to the output window.

NOTE: The offset values are integrated into the states for further use (**Figure 3**). Fine alignment is performed to provide high accuracy of the position of each state (**Figure 3**). This fine alignment helps pinpoint the exact position in all the five states. Fine Alignment is critical for single-particle data acquisition. Therefore, it is highly recommended to perform **Fine Alignment** before imaging.

6. Data acquisition procedure using Latitude-S

6.1. Click on the **Capture palette**.

NOTE: Generally, atlas data is collected at low magnification (115x) to visualize most of the grid squares.

6.2. Choose the size of the atlas to cover the entire grid or part of the grid based on the requirement (e.g., 6 x 6, 8 x 8, 12 x 12, 6 x 8, 8 x 6, 12 x 8, or 8 x 12).

NOTE: 16 by 16 atlas size covers the entire grid.

6.3. Click on the **Capture** button to capture the atlas.

NOTE: The main Latitude-S navigation window opens and fills the available space in DigitalMicrograph (**Supplemental Figure S5**). Three picture panes in the main navigation window show images of the system states at three different magnifications. The overall atlas is currently displayed in its current state of acquisition in the leftmost pane. Tiles in the atlas will fill up as each capture occurs.

6.4. Select the grid square based on the ice thickness by navigating on the atlas (**Supplemental Figure S5**). Once the desired grid squares are selected, click on the **Schedule** button and observe the tiles in the grid square fill up as each grid square is captured.

6.5. Click on the **Schedule** button once the grid squares are selected.

6.6. Select a representative hole in the grid square by adding its position. Once a hole image is acquired, define the data and focus positions and save the layout as a template (**Supplemental Figure S6**).

6.7. Click on **Auto find**, give the hole size (e.g., R1.2/1.3), and click on the **Find** button in the program, which will cause the **Find** program to automatically find the holes based on the hole diameter. Next, click on the **Mark** button to add the template (**Figure 4**) and add red circle marks in all the holes in one grid or partial grid square.

6.8. Set up the intensity to remove the holes from the grid square and ice contamination (**Figure 4**).

NOTE: Finally, the selected holes will be marked in yellow for scheduling the data collection.

6.9. Click on the **Schedule** button in **Latitude tasks** after adding the holes through **Auto find**.

NOTE: Before scheduling the automated data collection, ensure that the liquid nitrogen tank level is sufficient, the autoloader turbo pump is off, and the RAID drive space is free. Latitude-S task manager shows the number of atlas, grid square, hole, and data states scheduled for data collection (**Figure 5**). In Latitude-S GUI, various color schemes will be visible, and the meaning of the different color schemes will be displayed: 1. Yellow indicates unscheduled; 2. Green indicates scheduled; 3. Blue indicates Acquired; 4. Red indicates failed.

7. Cryo-EM data processing

NOTE: Cryo-EM image processing of spike protein is described in detail in recent literature[25].

7.1. Perform image processing of spike protein of SARS-CoV2 using RELION 3.0[11].

7.2. Screen the movie images collected using Latitude-S manually, and perform beam-induced motion correction of the individual movies using MotionCor2 software[9]. Perform the initial screening of the motion-corrected micrographs manually with the help of cisTEM software package[14].

NOTE: Almost 85% of the automatically acquired micrographs were of good quality, and data had signal within 3.7–5.2 Å, which is calculated using cisTEM software[14] (**Supplemental Figure S7A,B**).

7.3. Process the data using the RELION 3.0 software package[11].

7.3.1. Pick spike particles manually and subject them to 2D class classification (**Supplemental Figure S7C**). Use the best 2D class as a reference to autopick 3,99,842 single-spike particles from the micrographs using the RELION autopick tool[11].

NOTE: Three rounds of 2D classification were carried out before subjecting the particles to 3D classification (**Supplemental Figure S8)**. Approximately 2,55,982 single particles were selected for 3D classification, and the data set was classified into six classes. The final 3D auto-refinement was performed with the best class; 85,227 spike particles were obtained from the 3D classification.

7.3.2. After auto refinement, perform per particle defocus refinement with proper beam tilt parameters for resolution improvement. Next, subject the particles to Bayesian polishing using the RELION 3.0 software package[11]. Finally, use the polished particle set for another round of 3D auto-refinement using RELION 3.0[11].

REPRESENTATIVE RESULTS:

In the current pandemic situation, cryo-EM plays a key role in characterizing the structures of various proteins from SARS-CoV-2[26–29], which may help develop vaccines and drugs against the virus. There is an urgent need for fast-paced research efforts with limited human resources to combat the coronavirus disease of 2019. Data acquisition in single-particle cryo-EM is a time-consuming but crucial step in the structure determination of macromolecules. Recent developments in cryo-EM automatic data acquisition have enabled limited human interference in data collection. The Latitude-S software is an important automatic data acquisition software package used here for the automated data collection of purified SARS-CoV2 spike protein.

Cryo-EM data acquisition of SARS-CoV-2 spike protein was performed with a 200 keV cryo-EM equipped with a K2 Summit DED. The locations for data acquisition on the grid with desirable ice thickness and particle distribution were marked manually. The positions were marked in parallel during data acquisition occurring in the background. At the marked positions, Latitude-S software carried out automated data acquisition at a nominal magnification of 42,000x at the pixel size of 1.17 Å at the specimen level. The configuration for data collection at 42,000x magnification was pre-set and tested already. A total of 40 frames were recorded for 8 s with the electron dose of 2 $e^{-}/Å^2$ per frame; thus, a total dose of 80 $e^{-}/Å^2$ was used for data collection (**Supplemental Figure S9**). The data were acquired at a defocus range of −0.75 µm and −2.25 µm, with 3,000 movie files collected in two days. Every 4 h, periodic checks and adjustments were performed by the software to ensure that all the movie files collected over 48 h were of good quality and there was no beam shift or alignment shift. The data were collected independently without any human intervention. Additionally, Latitude-S automatically stops imaging at the time of liquid nitrogen filling, which reduces unnecessary vibration or mechanical drift in the images.

As mentioned in the protocol section, the initial screening of motion-corrected micrographs was performed manually using cisTEM software[14]. Based on the screening, most of the data were found to be within the signal range of 3.7–5.2 Å (**Supplemental Figure S7A**). This suggests that the automatic data collection using Latitude-S is good, and most of the data are suitable for high-resolution 3D reconstruction. Additionally, images were collected at defocus range (0.75–2.25 µm), and various defocus ranges were manually checked by cisTEM[14]. The acquired data were very close to the setup defocus range in Latitude-S (**Supplemental Figure S7A,B**).

Data processing was performed using the RELION 3.0 software package[11]. Spike particles were manually picked to calculate the 2D class averages. Various structural details (helix and β-sheet) are visible in the 2D class averages (**Supplemental Figure S7C**), which strongly suggests that high-resolution structural characterization is possible using this data set. However, 3D classification also indicates that spike protein has 1-receptor-binding domain (RBD) up open and all RBD down close conformation (**Supplemental Figure S8**). The 3D classification indicates that class-1 has the maximum number of particles, which appear as a 1-RBD up open conformation. Furthermore, class-3 and class-4 have a similar number of particles, and both models appeared to have all RBD down close conformation. However, class-5 shows an intermediate conformation, where 1-RBD is in an intermediate position. However, the 1-RBD up open conformations of the spike protein were reconstructed using C1 symmetry, and the

overall resolution is 4.4 Å (**Figure 6** and **Supplemental Figure S10**). Similarly, all RBD down close conformation (class-3 and class-4) were refined together with C3 symmetry, and the overall resolution at 0.143 FSC is ~3.9 Å (**Figure 7**).

The overall image processing indicates that the spike protein adopts all RBDs down close and 1-RBD up open conformation. Additionally, identify an intermediate conformation of the spike protein was identified. The high-resolution cryo-EM structure of the S2 subdomain of the spike protein indicates the side chains of individual amino acid residues (**Figure 6B** and **Figure 7C**). All the 3D reconstructions and cryo-EM results are highly similar to findings in recently published literature[25]. However, the high-resolution cryo-EM structure of the spike protein was characterized within 15 days, which is only possible because of automatic cryo-EM data acquisition protocols and automatic particle-picking software. Therefore, automatic data acquisition software packages, including Latitude-S, can significantly contribute to the characterization of several high-resolution cryo-EM structures of biological macromolecules.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of automated data acquisition using Latitude-S: General steps to be followed before data collection (grid preparation, sample loading, and microscope tuning). Data acquisition is the main part of this manuscript, and the pipeline to be followed during data acquisition is highlighted. Abbreviations: cryo-EM = cryo-electron microscopy.

Figure 2: **Setup o**f **different states for single-particle data collection using Latitude-S GUI.** (**A**) Latitude-S software package for data acquisition in DM3 software suit. (**B**) Workflow of the data acquisition procedure. (**C**) Expansion of each panel. Abbreviation: GUI = graphical user interface.

Figure 3: Fine alignment to set up high accuracy for focusing and image acquisition. Fine alignment is performed on five states a. Atlas, b. Hole, c. Data, d. Grid, e. Focus. Focus each state by placing the red mark on the same position. It is highly recommended to perform **Fine Alignment** before starting any new session, which will help perform imaging in a particular position. Image acquisition in a particular position (without any major shift) completely depends on the accuracy of the fine alignment.

Figure 4: Automatic hole selection using Latitude-S. Auto find of holes for data acquisition is carried out automatically based on hole size. (**A**) Shows the position of the hole-finding vector for auto-finding the holes. Scale bar = 20 µm. (**B**) Shows the marking of holes using the hole finding vector and adjusting the intensity to remove the marker from the border area and ice contamination. Scale bars = 20 µm (left) and 10 µm (middle). (**C**) Shows the automatic adding of the holes for imaging (yellow). Scale bar = 20 µm.

Figure 5: Live view of data acquisition. (**A**) The positions are marked in yellow, green, blue, and red based on the status of data acquisition in each position. (**B**) Color code for monitoring the status of data acquisition. Green: Scheduled, Yellow: Unscheduled, Blue: Acquire, Red: Failed. The left panel shows several holes colored green (scheduled) and a few holes marked blue (acquired); scale bar = 10 µm. The middle panel shows 4,300x magnification of an individual hole. In this image of a hole (middle panel), the blue square box shows the focus

area, and the green square box shows the imaging region; scale bar = 1000 nm. The right panel shows the acquired image. The extreme right panel shows the scheduled image numbers, total time required for imaging, and how many images are scheduled for imaging.

Figure 6: Spike 3D mode with 1RBD open. (**A**) Auto-refined and sharpened spike protein map of 1-RBD up open is represented in side, top, and bottom views. (**B**) EM map is fitted with crystal structure for better visualization of the side chains. The highlighted regions in the map have densities for side chains. Abbreviations: 3D = three-dimensional; RBD = receptor-binding domain; EM = electron microscopy; NTD = N- terminal domain; S1 = subunit 1; S2 = subunit 2.

Figure 7: All RBD down close conformation of spike protein. (**A**) Auto-refined and sharpened spike protein map of all RBD down close conformation represented in side and top views. (**B**) EM map is fitted with crystal structure for better visualization of the side chains. The arrows show that the regions in the map have densities for side chains (**C**). Abbreviations: RBD = receptor-binding domain; EM = electron microscopy; NTD = N- terminal domain; S1 = subunit 1; S2 = subunit 2.

Supplemental Figure S1: Latitude-S image acquisition GUI: Various microscope controllers (e.g., column valve open/close, screen insert/retract are controlled by the Latitude-S GUI. Column valve, camera, screen status, and liquid nitrogen filling could be controlled in the left panel. At the bottom of this panel, various calibration parameters appear green (e.g., Magnification, beam tilt, objective focus). If any parameter appears black, it indicates that the parameter is not optimized correctly. Therefore, all parameters should be optimized before starting any new session. Abbreviation: GUI = graphical user interface.

Supplemental Figure S2: Representation of Atlas state. Different Atlas states showing grid squares and the type of ice formed. (**A–F**) Various Atlas sizes are also highlighted. (**A, B, D, F**) A thick ice pattern is highlighted. (**C, D**) Broken squares are highlighted. Thick ice and broken regions (marked in the figure) are not suitable for imaging. (**E, F**) Good grid squares for imaging; **A, B, C, D,** and **F** show the grid squares suitable for high-resolution imaging. However, thick ice grid squares and broken grid squares must be excluded. Scale bars = 100 µm (**A–C**), 50 µm (**D, E**), and 25 µm (**F**).

Supplemental Figure S3: Representation of Grid state and Hole state. Grid state and corresponding Hole state are shown in the image. (**A, E**) Empty hole, (**F, G**) thick ice, (**E**) ice contamination, and (**A, B, C, D, E,** and **G**) suitable ice thickness is marked in the image. Suitable ice thickness holes are selected for the image acquisition (**A, B, C, D, E**, and **G**). Scale bars = 10 µm (**A, E, F, G**), 2 µm (**B**), 50 µm (**C**), 5 µm (**D**).

Supplemental Figure S4: Hole reference for automatic imaging. Hole image (QUANTIFOIL-Holey Carbon grid QUANTIFOIL R 1.2/1.3) is captured and saved for future reference. The size of the hole reference could be varied based on the different types of grids. It is recommended to always capture the hole reference before starting any new session.

Supplemental Figure S5: Image-capturing panel at various magnifications and hole selection. (**A**) The capture panel shows the settings for data acquisition. (**B**) The Latitude-S main navigation window shows three consecutive magnifications. In Atlas state (150x), the

grid squares are selected for data acquisition (left-hand panel, scale bar = 50 µm). In higher magnification (380x), a single square is focused (middle panel, scale bar = 20 µm). Further higher magnification (4,300x), holes inside each square are focused (right-hand panel, scale bar = 5 µm). However, these magnifications would change according to the size and shape of the grids.

Supplemental Figure S6: Creating a template for hole selection and imaging. Template generation is performed by adding a position on the hole for data, and the focus is positioned adjacent to the hole on the carbon surface. The focus should position on the carbon area so that the beam diameter should not touch any adjacent hole. Scale bars = 20 µm (left panel), 10 µm (middle panel), 1000 nm (right panel).

Supplemental Figure S7: Cryo-EM imaging of SARS-CoV2 using Latitude-S and image screening. (**A**) Screening of acquired micrographs: 1D CTF fit, 2D CTF fit, and CTF parameters; estimation of the SARS-CoV2 spike protein data using cisTEM. 1D CTF fit and Thon ring shows that the overall signal is 4.8 Å. (**B**) Micrographs at two different defocus value of the spike protein are acquired using Latitude-S. Scale bars = 50 nm. (**C**) Final 2D class average. 2D class average shows top, bottom, and side views of the spike protein. All the high-resolution details are visible in 2D class averages. Abbreviations: cryo-EM = cryo-electron microscopy; 1D = one-dimensional; 2D = two-dimensional; CTF = contrast transfer function; SARS-CoV2 = severe acute respiratory syndrome coronavirus 2.

Supplemental Figure S8: Data processing of SARS-CoV2 spike protein data acquired using Latitude-S software. The image shows the workflow followed for processing the cryo-EM data of spike protein. 3D classification of spike protein is performed using Relion 3.0. Class-1 shows the 1-RBD up open conformation. Class-3 and Class-4 show all RBD down close conformation of spike-protein. Class-5 shows the intermediate conformation of the spike protein. Abbreviations: cryo-EM = cryo-electron microscopy; 3D = three-dimensional; RBD = receptor-binding domain; SARS-CoV2 = severe acute respiratory syndrome coronavirus 2.

Supplemental Figure S9: Dose-fractionated image captured as a reference for final image acquisition. Dose fractionation images are captured with an 8.0 s exposure and 0.2 s/frame exposure. Click on the **Auto save** button near the camera to save the movie files automatically. After image acquisition, click on the image and save the dose-fractionated image parameters using the **image updated** button in the data collection state.

Supplemental Figure S10: **Angular distribution and Fourier shell correlation of the generated SARS-CoV-2 1 RBD up open conformation spike protein map.** (**A**) Angular distribution of the final 3D model of 1-RBD up open conformation of the spike protein. Blue represents lower values, and red represents higher values of the normalized particle distribution. (**B**) Fourier shell correlation curve showing 4.4 Å resolution of the 1-RBD up open conformation of spike protein, estimated at the cut-off. Abbreviations: 3D = three-dimensional; RBD = receptor-binding domain; SARS-CoV2 = severe acute respiratory syndrome coronavirus 2; FSC = Fourier shell correlation.

Table 1: Latitude-S state setup summary.

Table 2: Camera specifications.

Table 3: Microscope configuration.

DISCUSSION:

Latitude-S is an intuitive user interface, which provides an environment to automatically set up and collect thousands of high-resolution micrographs or movie files in two days. It provides easy navigation across the grids and maintains the position of the microscope stage while it moves from low magnification to high magnification. Each step of data acquisition with Latitude-S is time-efficient, with features such as a simple user interface, fast streaming of data at up to 4.5 GB/s speed, and simultaneous display of data during acquisition. Additionally, precalibrated dose fractionation, dose rate, focus, and magnification parameters were easily reloaded to start a new session of automatic data acquisition and save time.

Acquiring data automatically in the absence of constant monitoring and without compromising the quality of the data set is a challenging and time-consuming task. Automated data collection using Latitude-S software is convenient when time and resources are major constraints, particularly during this pandemic. However, several cryo-EM structures of various proteins of SARS-CoV-2 have been resolved in the past few months, which will help pharmaceutical companies develop vaccines. Different laboratories use different types of automatic data collection software packages to collect data. We used Latitude-S with a K2 Summit DED for cryo-EM automatic data collection on holey carbon grids or homemade GO coated grids[30].

That study was performed using the above-mentioned parameters in the protocol section, which provides strong evidence that Latitude-S is a suitable and ideal software package for automatic data collection for single-particle cryo-EM. However, it is highly recommended to follow certain protocols before starting the imaging using a K2 camera. The K2 direct detection camera requires basic maintenance routines to achieve the highest performance. Regular annealing of the camera to 50° for 24 h helps the sensor perform optimally by reducing the background noise and contamination at the surface level. However, after camera annealing, updating the gain and dark references of the camera is a mandatory step (takes ~45 min) before performing any image acquisition.

Although Latitude-S is a stable and user-friendly software package for cryo-EM automatic data collection, it is essential to optimize various parameters (magnifications, spot size, brightness, and dose rate) in 5 different states of Latitude-S at the beginning. The magnification of holes or grid states depends on the hole sizes of the holey grids or holey grid types (e.g., R2/2 or R1.2/1.3 or R 0.6/1). For example, the R0.6/1 type grid hole size is 0.6 µm, and the hole size of the R2/2 grid is 2 µm. Thus, two different types of magnification are required to visualize the holes properly for grid types R0.6/1 and R2/2 in the grid and hole states in Latitude-S.

Therefore, the magnification settings for various types of grids in 5 different states will be variable. The spot size and the brightness highly depend on the magnification. Hence, these values may change at different magnification values. Therefore, it is recommended to optimize the various parameters of the 5 different states of Latitude-S using different types of cryo-EM grids before starting automatic data acquisition. However, once all the parameters

are optimized and saved, it is easy to reload all the parameters based on the user's requirement and use Latitude-S at different magnifications or grid types.

An important benefit of using K2 with Latitude-S is that users can easily regulate the open/close the beam valve, insert/retract the camera, insert/retract the phosphor screen of the microscope, and regulate liquid nitrogen filling using the GUI of Latitude-S. However, any other options (such as gun tilt, gun shift, beam shift, pivot points, C2 aperture centering, rotation center, coma-free alignment) are not accessible through the K2 Latitude-S GUI Tab (**Supplemental Figure S1** and **Figure 2)**. During the long hours of data collection, the position of the beam may shift.

Latitude-S can execute automated periodic checks and corrections to keep track of the system's stability throughout the data acquisition period. The stability of the system is maintained by centering the beam and updating the dark references. The constant check ensures the high quality of the acquired data. In Latitude-S, eucentric height (Z-height) is corrected only once before data collection, and the eucentric height is calculated automatically by Latitude-S when it changes the grid square. The focus is automatically measured and adjusted based on the user-defined focus range. The program will reset the stage Z position if it exceeds the given threshold value. This stability is controlled through the System Stability palette. However, like other automatic data acquisition packages, Latitude-S also has some limitations.

Latitude-S cannot calculate the eucentric height (Z-height) if the grid is uneven. In this scenario, it cannot collect any data, or the defocus values will be completely out of range. Therefore, users should be extremely cautious to prepare their grids without any bend and image only flat-surface grids using Latitude-S. Furthermore, unlike Leginon, SerialEM, and UCSF-Image, Latitude-S is not a freely available software package. Latitude-S is compatible with Gatan cameras, including filtered or standalone K2, K3, and K3 base direct detection cameras, as well as Rio and OneView cameras. Another important disadvantage for users is that it is not compatible with other popular DEDs such as Falcon DED. However, this is also true for EPU, another automatic data acquisition software package, which is available with cryo microscopes and only compatible with the Falcon camera. However, EPU is also functional with K2/K3 with an energy filter (BioQuantum K3 Imaging Filter) but not with a standalone K2/K3 camera.

Latitude-S is quite similar to EPU, SerialEM, AutoEM, AutoEMation, and Leginon, which are software packages used for automatic data acquisition for single-particle cryo-EM. However, Latitude-S is only compatible with K2 DED, K3 DED, or BioQuantum K3 Imaging Filter. Additionally, continuous technical support is provided by the company for Latitude-S users. This technical support is beneficial for small user groups, who need to use the K2 DED, K3 DED, or BioQuantum K3 Imaging Filter devices for data acquisition and have no prior knowledge on how to set up or use free software packages such as SerialEM and Leginon.

There are many other features, such as microcrystal electron diffraction (microED), tomography, and energy-dispersive X-ray spectrometry (EDS), which are available in various other versions of Latitude. Therefore, users can use the same software package for data collection in other modes. To our knowledge, data collection for microED, tomography, and

EDS is not available in EPU or any other software packages. Therefore, this Latitude software package could be useful for different purposes in addition to automatic data acquisition in single-particle cryoEM. However, SerialEM and Leginon, both free software packages, are suitable for Falcon or K2/K3 cameras and are extremely useful for new users. However, Latitude-S is not freely available, which might be a disadvantage of this software package.

In summary, the Latitude-S automatic data acquisition tool is as good as other automatic data acquisition software packages (e.g., EPU, Leginon, SerialEM, UCSF-Image). Latitude-S is an extremely stable and user-friendly data acquisition software package, which is available with filtered or standalone K2, K3, and K3 base direct detection cameras, as well as Rio and OneView cameras.

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DISCLOSURES:

The authors have no competing or financial conflicts of interest to declare.

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Figure 1

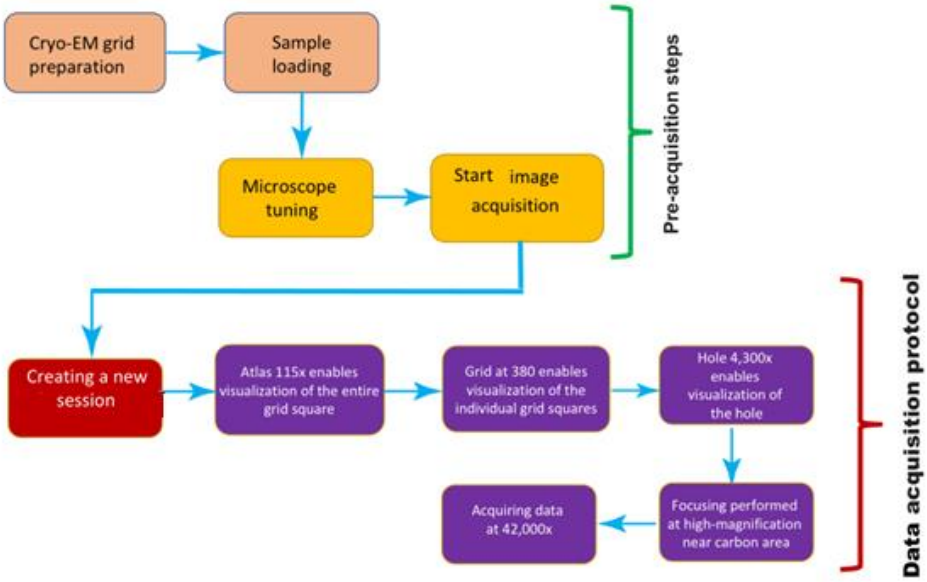


Figure 2

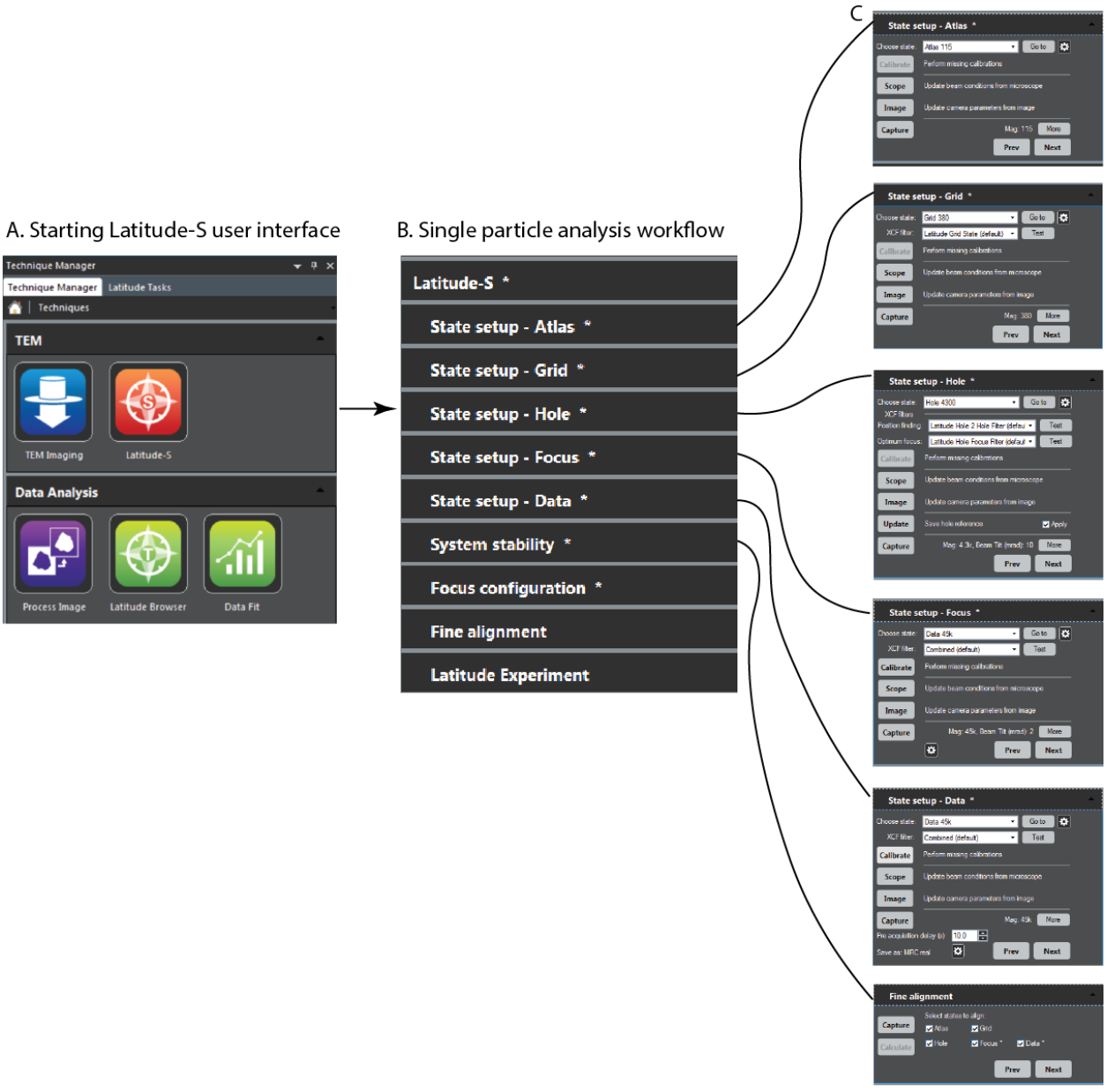


Figure 3

Fine alignment

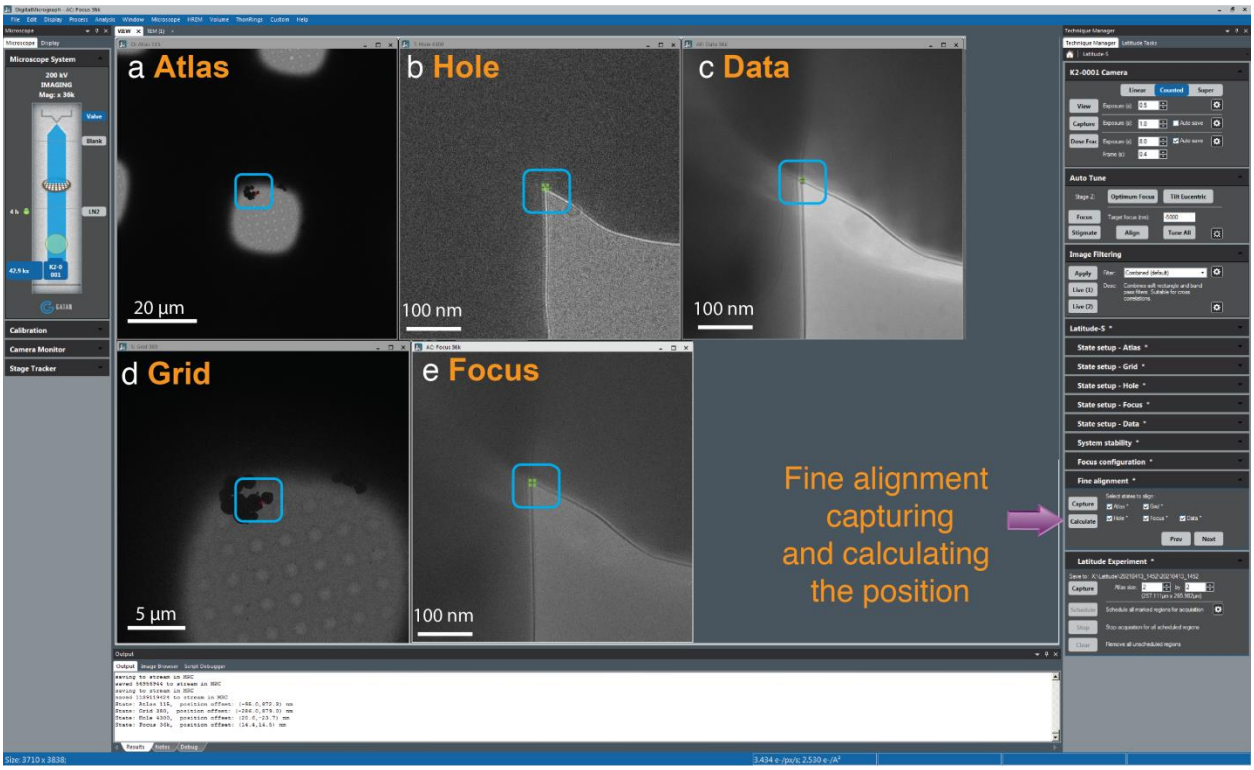
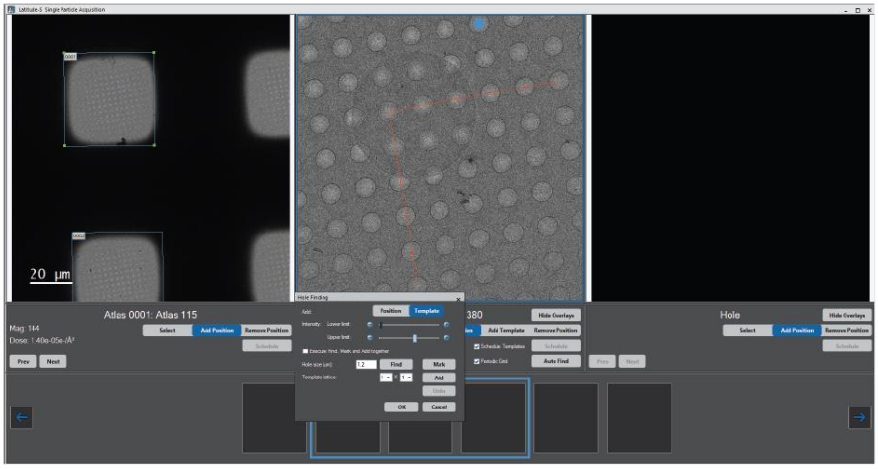
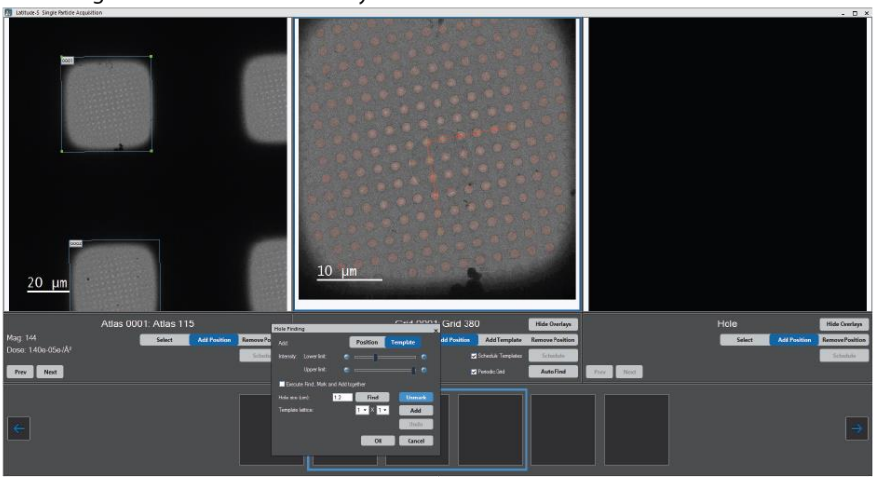


Figure 4

A. Auto finder based on grid hole size qauntifoil R1.2/1.3



B. Marking of holes based on intensity



C. Add

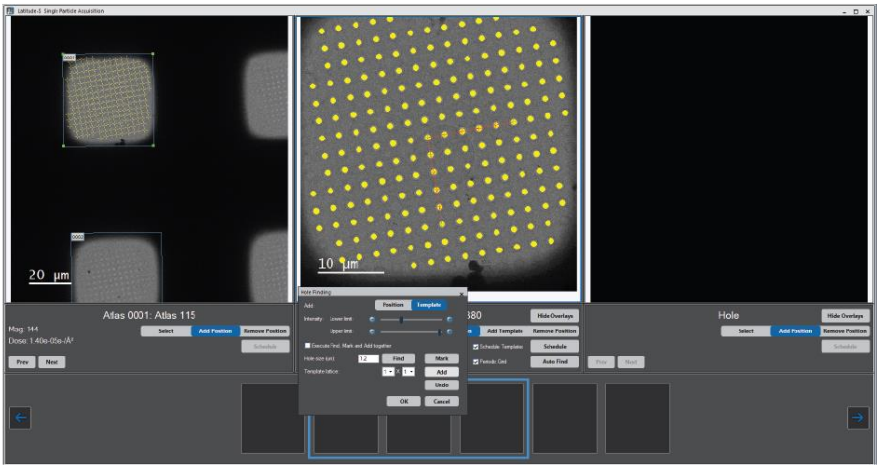
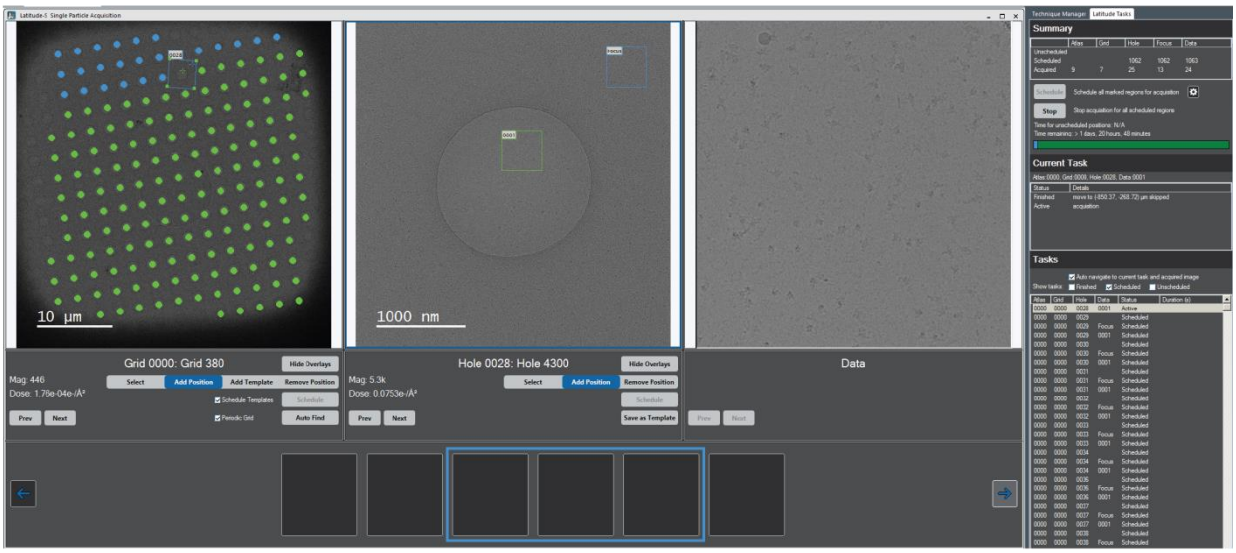


Figure 5

A. Data acquisition, monitoring progress from task manager



B. Colour code for monitoring

- Unscheduled
- Schedule
- Acquire
- Failed

Figure 6

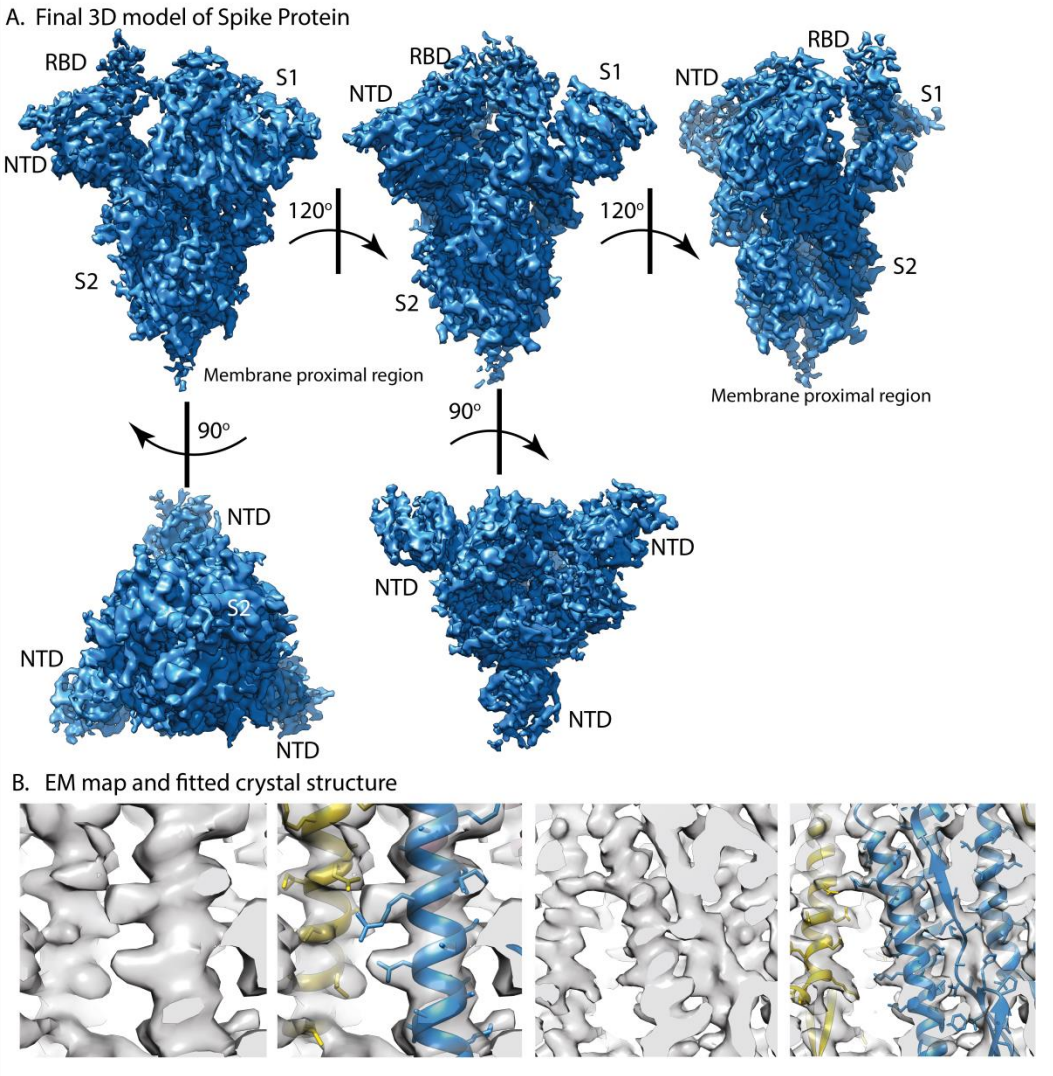


Figure 7

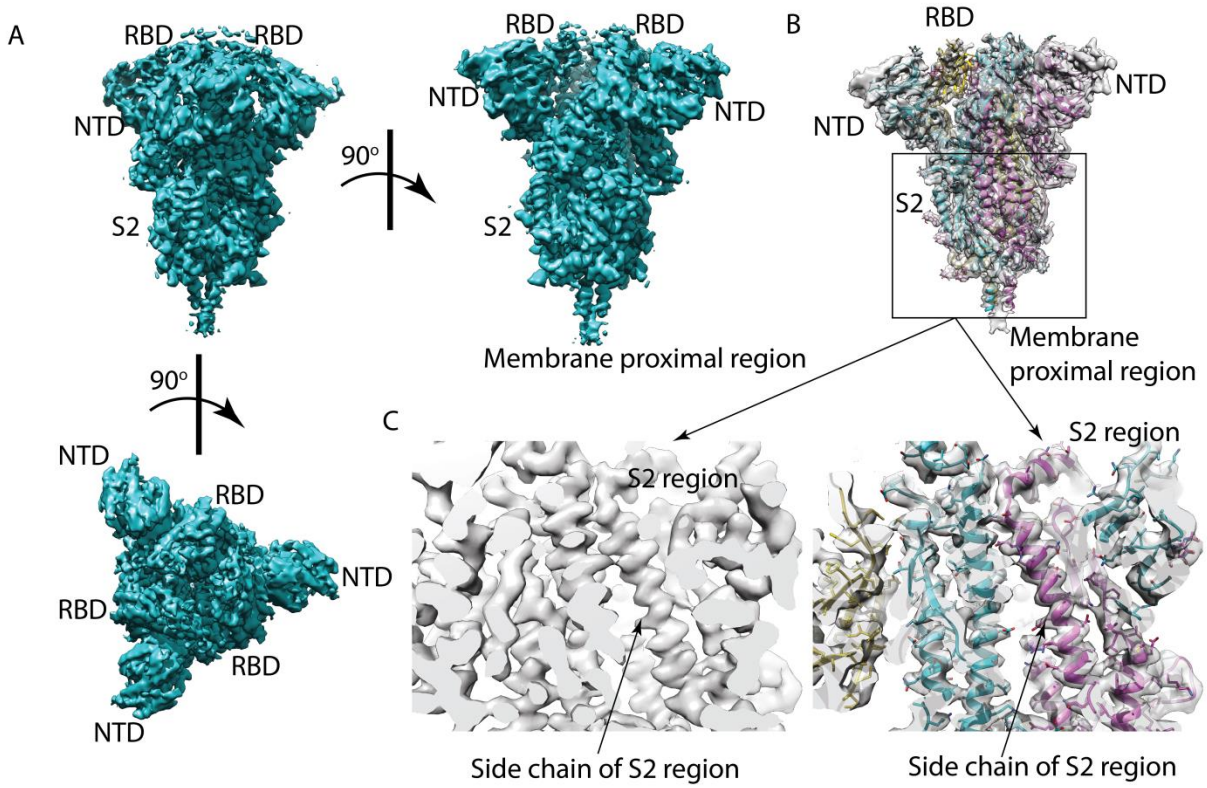


Table 1 Latitude-S State Setup Summary

Atlas Image State	Atlas 115
Magnification	115, Pixel size 34.7 nm
Indicated Defocus	4.38 mm
Spot Size	8
Brightness	934400
Mode	IMAGINGILM
Binning	1
Exposure Time	1.0 s
Grid Survey State	Grid 380
Magnification	380, Pixel size 11.2 nm
Indicated Defocus	2.37 mm
Spot Size	8
Brightness	626200
Mode	IMAGINGILM
Binning	1
Exposure Time	1.0 s
Hole Survey State	Hole 3400
Magnification	3,400, Pixel size 1.20 nm
Indicated Defocus	-0.75 µm
Spot Size	7
Beam Diameter	8.81 µm
Mode	IMAGINGILM, SA, Mh
Binning	1
Exposure Time	1.0 s
Focus State	Focus 45k
Magnification	45,000, Pixel size 0.0924 nm
Indicated Defocus	4.51 µm
Spot Size	6
Beam Diameter	0.716 µm
Mode	IMAGINGILM, SA, Mh
Binning	1
Exposure Time	1.0 s
Acqisition State	Data 45k
Magnification	45,000, Pixel size 0.0924 nm
Defocus Setup	Min: -4,500 nm, Max: -1,500 nm, Steps: 250 nm
Spot Size	6
Beam Diameter	0.752 µm
Mode	IMAGINGILM,SA,Mh
Binning	1
Exposure Time	Total 8 s exposure for 20 frames

Camera setup	Counted, Gain Normalized, Defect Corrected
Data save setup	MRC

Table 2 Camera Specifications

Specification	K2 Base
TEM operating voltage	200-400 kV
Sensor active area	19.2 mm × 18.6 mm
Sensor size in pixels	3838 × 3710
Physic al pixel size	5 µm
Binning	1–8x
Sensor read-out	Any arbitrary area
Magnification relative to film	1.3–1.5x
Sensor read-out speed	50 full fps
Transfer speed to computer	8 full fps
Image display	8 full fps
DQE performance (300 kV)	>0.30 (peak) >0.25 at 0.5 of physical Nyquist
Software	Gatan Microscopy Suite including DigitalMicrograph

K2 Summit
7676 × 7420 Super-Resolution
400 full fps
40 full fps
10 full fps
>0.7 (peak) >0.50 at 0.5 of physical Nyquist >0.06 at 1.25 of physical Nyquist

Table 3 Microscope Configuration

Configuration Option	Value
Microscope Type	Talos Arctica G2
High Tension	200 kV
Source	XFEG
Lens	Cryo Twin
Vacuum System	Talos TMP IGP
Sample Loader	AutoLoader

आण्विक जैवभौतिकी ऐकक Molecular Biophysics Unit
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To
Vidhya Iyer, Ph.D.
Review Editor,
JoVE,

July 2, 2021

&

Prof. Abigail Lytton-Jean, (Editor)
Journal of Visualized Experiments,
Koch Institute for Integrative Cancer Research at MIT.

Subject: Response Requested: JoVE Submission JoVE62832R1 - [EMID:4ea92d7f424240bb] on dated June 25, 2021

Dear Dr.Vidhya Iyer, Ph.D.,

At the outset, we would like to thank you for giving us an opportunity to resubmit the revised manuscript in your journal. Additionally, we would like to thank the Review Editor (Vidhya Iyer, Ph.D. Review Editor) for the constructive suggestions, comments and for changing the formatting of our manuscript. By addressing these comments, we feel the quality and impact of our manuscript have significantly improved. According to the Editor's suggestions, 10 figures are shifted to supplemental figures and 7 main figures are there. Additionally, we have modified the title of the manuscript. Please find enclosed our revised manuscript entitled, "A User-friendly, High-throughput, and Fully Automated Data Acquisition Software for Single Particle Cryo-electron Microscopy" which we are submitting for review as a video article in the Methods Collection on cryo-EM in your prestigious journal "Journal of Visualized Experiments".

Two figure files "main figures" and "supplemental figures" are attached with this manuscript. We have addressed all the comments of the Editor's and highlighted all the answers in the manuscript using the reply button of the Editor's comments. Additionally, some sentences, which are not required or modified are "strikethrough" but did not delete it from the manuscript. If we delete the sentence, the Editor's comments and Author's answer willalso deleted. Therefore, these lines

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are only "strikethrough", not deleted from the text. Track changes are on to visualize the changes are made during this revision. Also, we highlighted the Protocol (including headings and spacing) that identifies the essential steps {**starting from Protocols Number 3 (3.1.) to Protocol number 6 (6.12)**} of the protocol for the video.

All authors reviewed the manuscript, and Structure Final File Checklist and agreed to the submission of the revised manuscript. We hope the manuscript will be acceptable for publication. Please feel free to contact me if you have any questions.

Sincerely,

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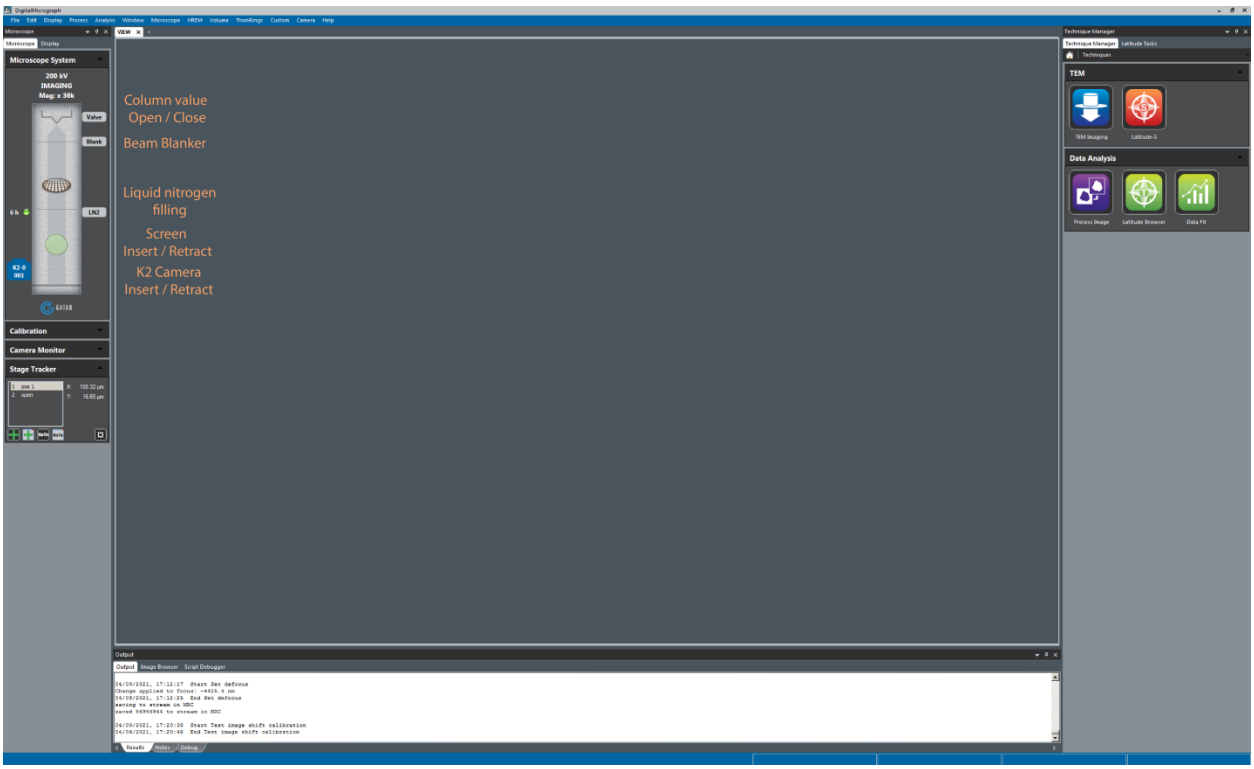
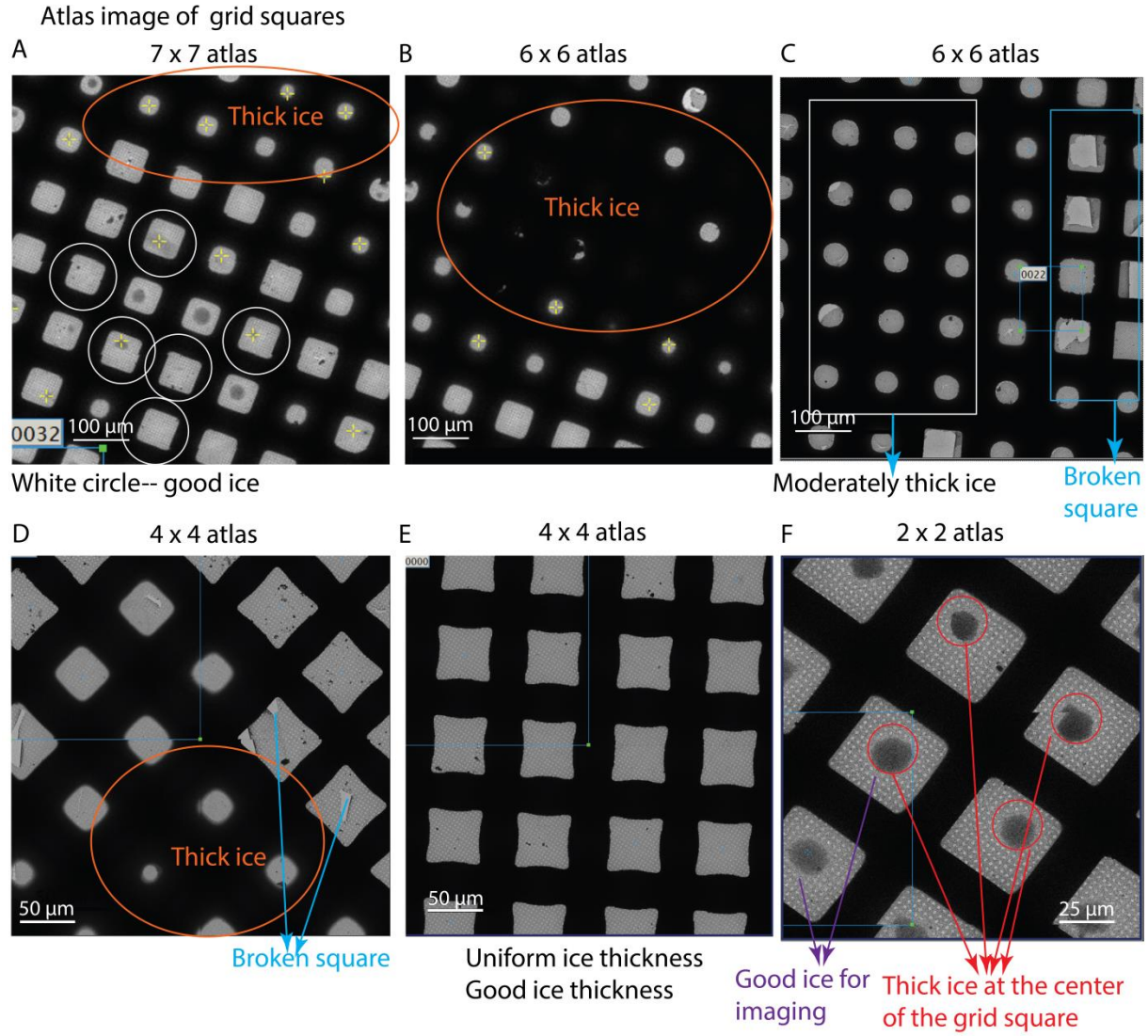
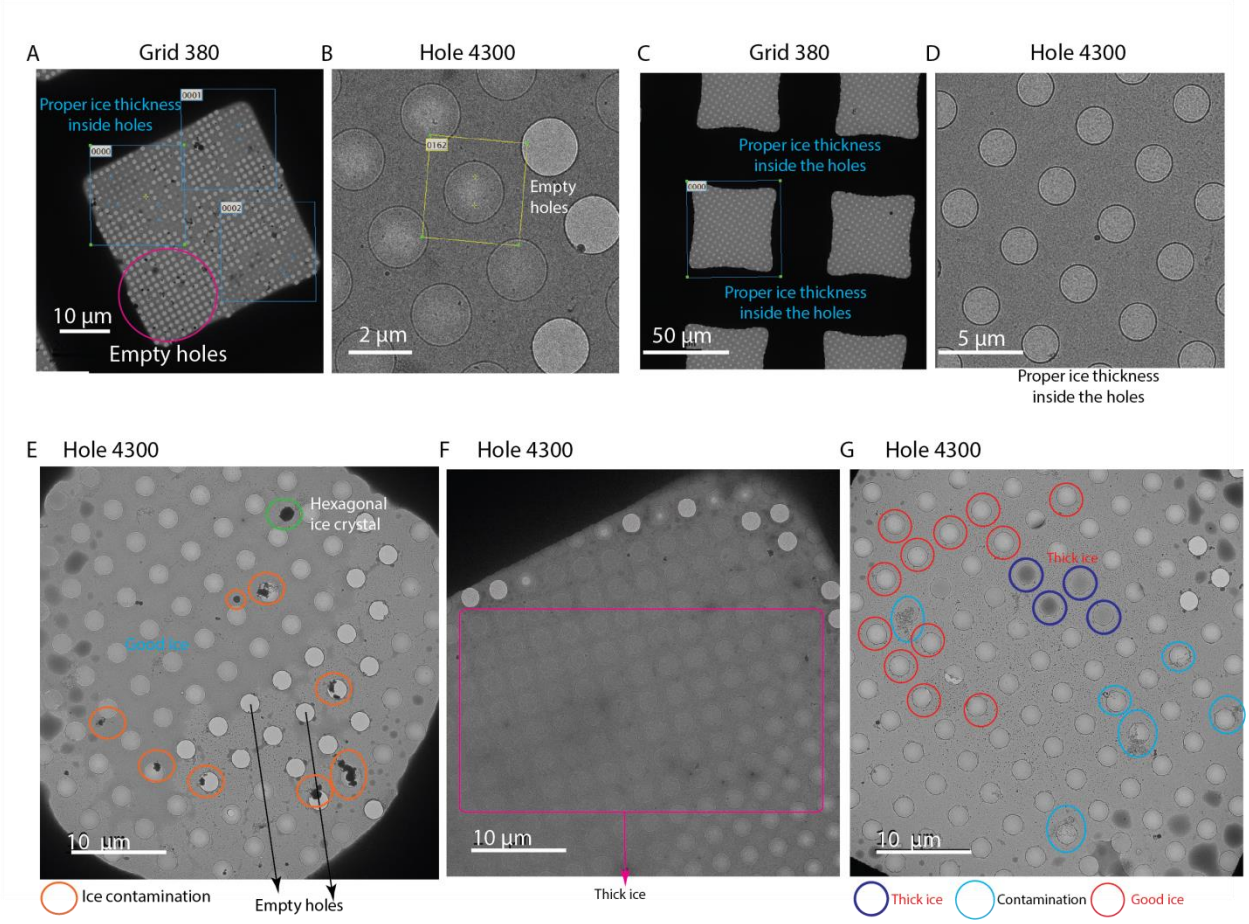
Supplemental Figure S1



Supplemental Figure S2

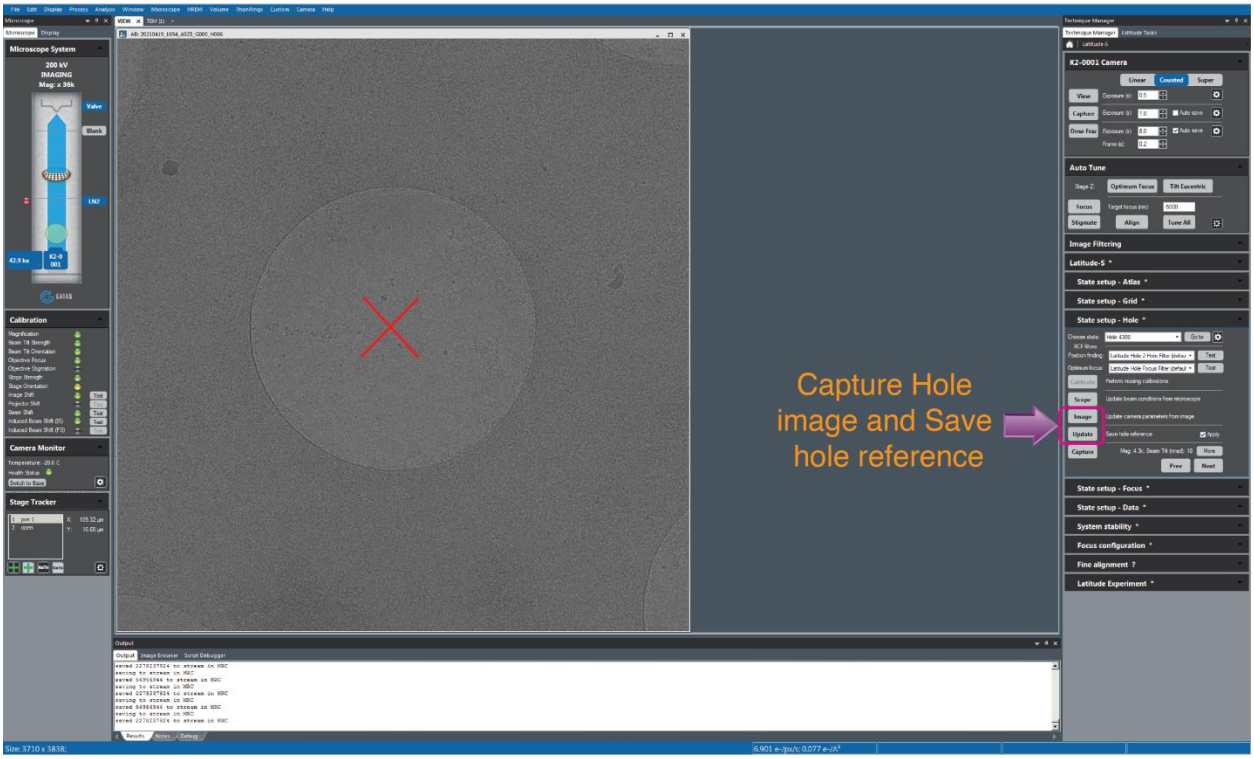


Supplemental Figure S3



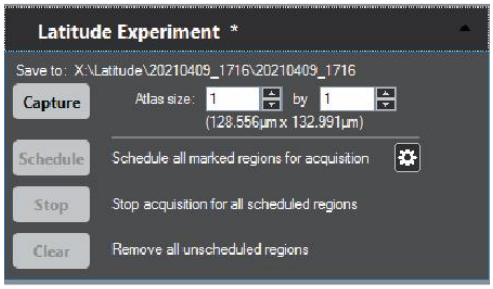
Supplemental Figure S4

Hole Reference update

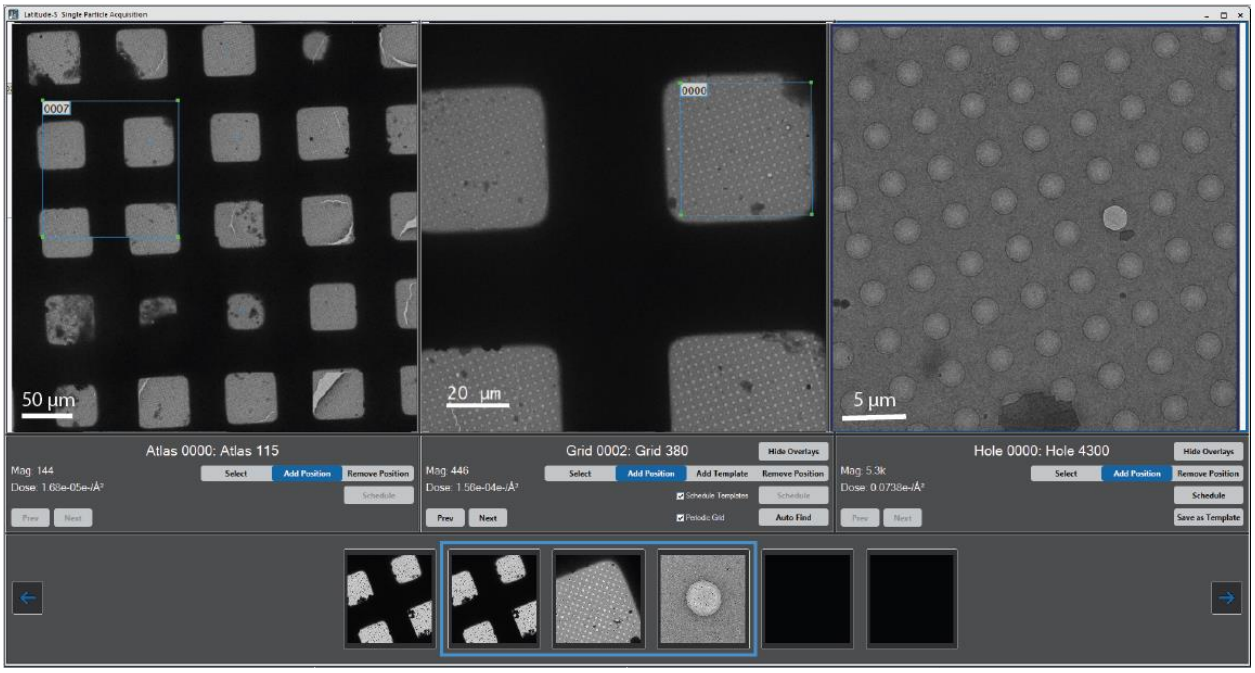


Supplemental Figure S5

A. Capture window

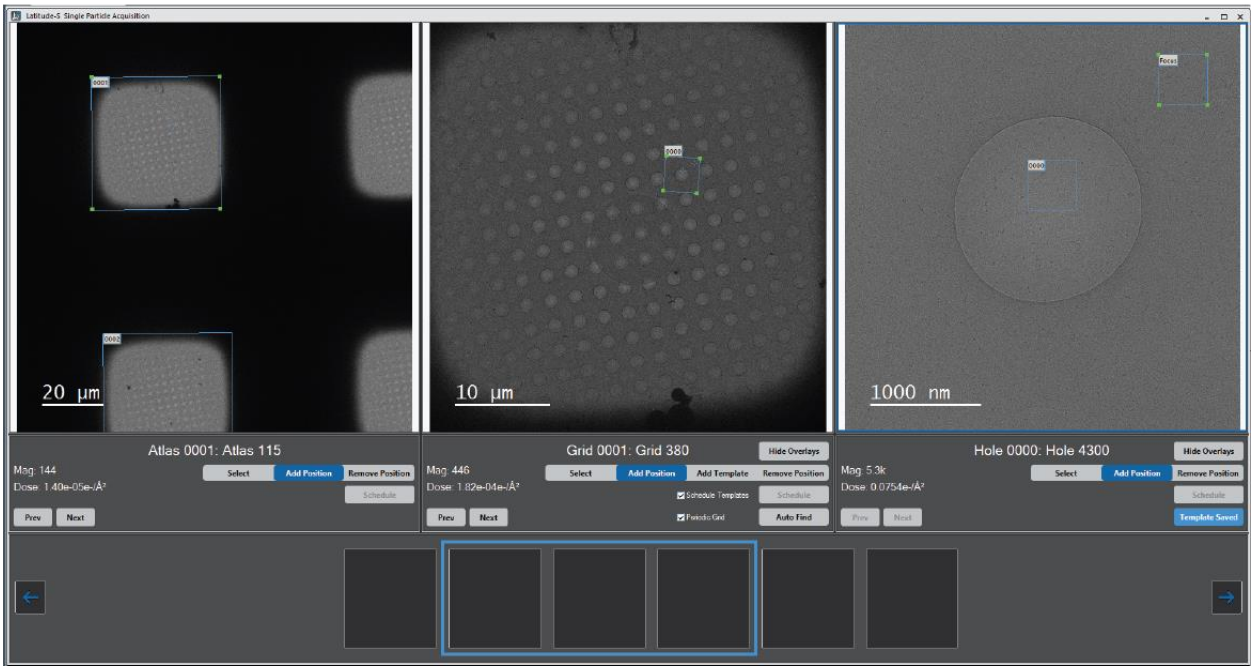


B. Latitude-S main navigation window showing three consecutive magnification

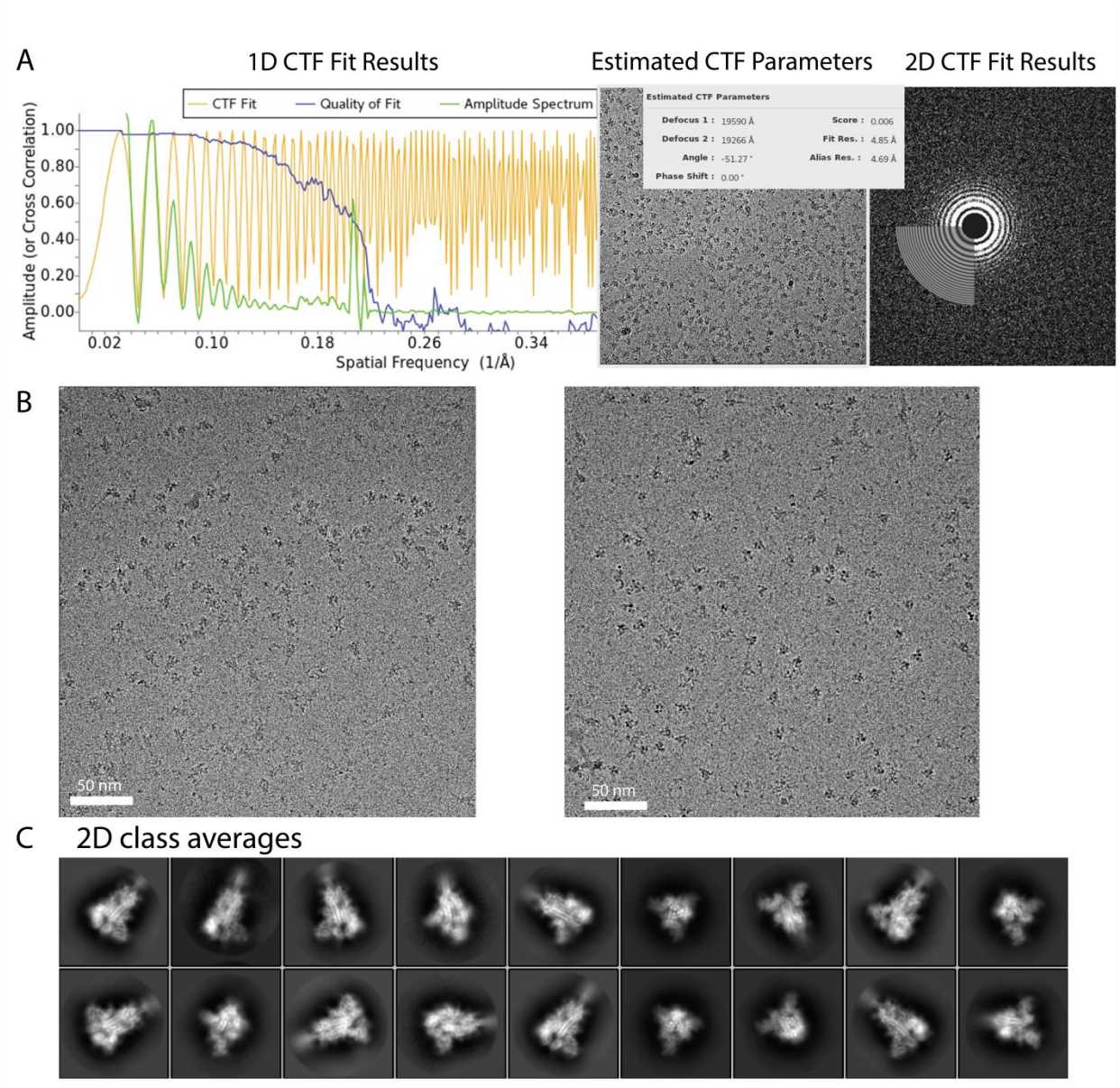


Supplemental Figure S6

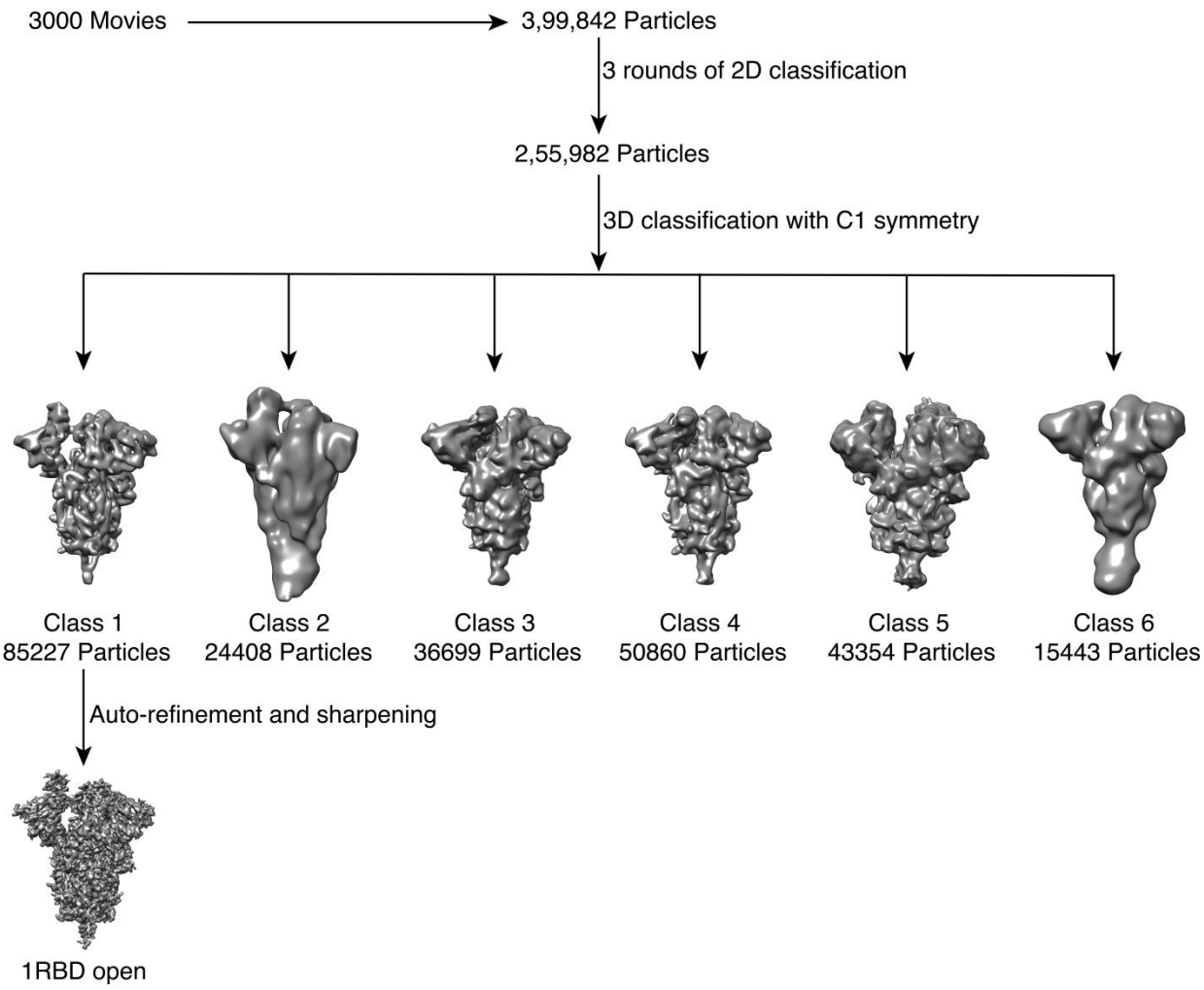
Template generation



Supplemental Figure S7

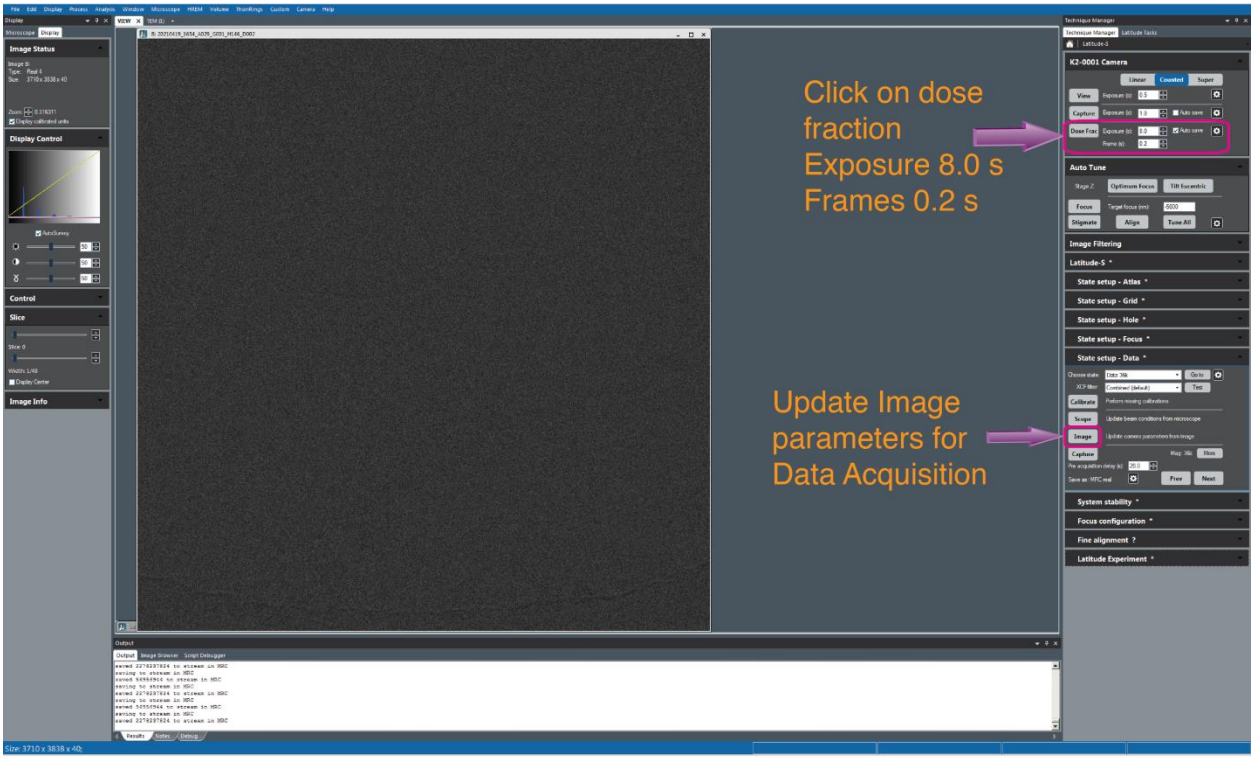


Supplemental Figure S8



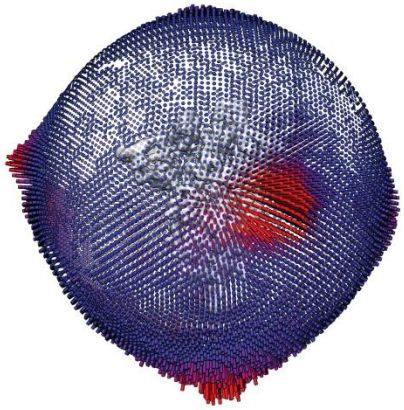
Supplemental Figure S9

Data Image update by dose fractionation (40 frames)



Supplemental Figure S10

A Angular distribution



B FSC curve