A simple pressure-assisted method for cryo-EM specimen preparation

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Abstract

Cryo-electron microscopy (cryo-EM) has made great impacts on structural biology. However, specimen preparation remains a major bottleneck. Here, we report a simple method for preparing cryo-EM specimens, named Preassis, in which the excess liquid is removed by introducing a pressure gradient through the EM grid. We show the unique advantages of Preassis in handling samples with low concentrations of protein single particles and micro-crystals in a wide range of buffer conditions.

Main text

Single particle cryo-EM is a powerful tool for structure determination of biological macromolecules at near-atomic resolution, which has been revolutionising the field of structural biology. Cryo-EM has undergone enormous technological innovations in both hardware and software over the past decades. However, the most widely used specimen preparation method has still been the pipetting-blotting-plunging routine first reported in 1981[1]. A major drawback of the method is the large sample consumption because more than 99.9% of the sample is lost to filter papers [2,3]. Therefore, it is of great importance to develop new methods that handle proteins samples only available at low concentrations, which is often the case for membrane proteins and complexes extracted from biological sources. Recently, a number of blot-less methods have been reported, such as multiple glass capillaries[4], contact pin-printing[5], and ink-jet spotter/Spotiton[2,6]. However, their implementations require special instrumentations and often non-standard EM grids.

Recently 3D electron diffraction, known as MicroED[7,8], has shown great potential for the determination of biomolecule structures from crystals too small for X-ray diffraction[9,10]. Protein crystallisation is often conducted in viscous media by introducing e.g. polyethylene glycols (PEGs)[11,12], which is difficult to remove by the paper-blotting method. Currently, there is no report of efficient methods that can handle highly viscous samples. There is an urgent need to develop new cryo-EM specimen preparation methods for handling protein micro-crystals grown in a wide range of buffer conditions.

Here, we demonstrate a simple pressure-assisted method, named Preassis, for preparing cryo-EM specimens of both single particles and micro-crystals. By using Preassis, suitable cryo-EM specimens can be prepared from proteins with concentrations as low as 0.18 mg/ml. In addition, Preassis can handle protein crystal suspensions with both low and high viscosity.

More importantly, the method is simple and easy to implement, and no special EM grids are required. With minor modifications, the Preassis setup can be adapted to existing cryo-EM vitrification devices, making it widely accessible to various cryo-EM labs.

The basic concept of Preassis is to pull a portion of sample suspension through an EM grid by introducing a pressure gradient near the grid and simultaneously remove the extra liquid from the backside of the grid by suction. The thickness of the liquid on the EM grid is controlled by the pressure gradient and suction time. We propose two setups, setup A where the EM grid is held by a tweezer (**Fig. 1a**) and setup B where the EM grid is rested on a filter paper (**Fig. 2a**). Both setups can be applied for single particles and micro-crystals. In the setup A, a suction tube is placed near the grid to remove the liquid, which is blot-free. This setup can be easily implemented as an add-on to existing cryo-EM vitrification devices (such as FEI Vitrobot, **Supplementary Fig. 1**) that control the humidity, temperature, and plunge-freezing. In this setup, the EM grid can be pre-clipped (e.g. the sample clipping process used for auto-loading) to prevent it from bending caused by the suction force during liquid removal. Alternatively, the EM grid can be supported using a small strip of filter paper. The setup B (**Fig. 2a**) is simpler and more efficient to remove liquids, making it ideal for preparation of samples that are less sensitive to humidity and temperature, e.g. protein micro-crystals, especially those in viscous buffers. The setup A (**Fig. 1a**) is more flexible and can adapt different conditions, making it more suitable for protein single particle.

The setup A of Preassis was applied to three single particle samples: apoferritin, a yeast respiratory supercomplex[13], and a membrane protein. Normal Quantifoil grids were used for all experiments. First apoferritin was used as a test sample. As shown from the TEM images of apoferritin prepared by Vitrobot (3.5 mg/ml, **Fig. 1b**) and Preassis (0.35 mg/ml, **Fig. 1c**), 10 times lower protein concentration was needed by using Preassis to achieve the same particle density than that by using Vitrobot. For a good single particle data collection of

apoferritin, the concentration was further lowered to 0.18 mg/ml (**Fig. 1d**). Single particle 2D classification and 3D reconstruction demonstrate that the protein structure was intact throughout the specimen preparation process of Preassis (**Supplementary Fig. 2a**).

This method can also be applied to proteins with a strong affinity to carbon films (e.g. yeast respiratory supercomplexes), which are often prepared on EM grids coated with a carbon film, leading to reduced contrast of the images[13]. As shown in **Fig. 1e** and **f**, the supercomplexes (1.5 mg/ml) prepared using Preassis are nicely distributed within the holes of a normal Quantifoil grid. 2D classification shows that the structure of the molecules is preserved (**Supplementary Fig. 2b**). The third sample was a membrane protein (transporter) with a concentration of 0.4 mg/ml, 10 times lower than what was required by Vitrobot. Using Preassis, cryo-EM grids with a good distribution of the membrane proteins in the hole could be obtained, as shown in **Fig. 1g**. These results demonstrate that Preassis has great advantages for samples with very low concentrations and those with a strong affinity to carbon films.

Cryo-electron tomography (cryo-ET) was performed on the three protein specimens prepared by Preassis in order to understand the particle/ice behaviours. It was found that while a majority of apoferritin molecules are near the air-water interface (**Supplementary Video 1, Supplementary Fig. 3**), the supercomplex and the membrane protein particles are evenly distributed inside the amorphous ice layer (**Supplementary Video 2** and **Video 3**, **Supplementary Figs. 4** and **5**). This shows that particle and ice behaviours can vary widely among different samples, which was also observed by other specimen preparation methods[14]. The parameters used for specimen preparation need to be tuned based on sample conditions. For Preassis, the thickness of the vitrified ice can be adjusted by changing the pressure gradient, the time over which the suction is applied, and the hole size of EM grids. By

optimising these parameters, suitable ice thickness and particle distributions can be obtained over a large area on the EM grid (**Supplementary Fig. 6**).

The setup B of Preassis was applied for the preparation of three types of protein micro-crystals for MicroED specimens. First, micro-crystals of lysozyme and ribonucleotide reductase R2 subunit (R2), grown from low viscous media, were tested. The lysozyme crystals prepared by Preassis diffracted to 2 Å (**Supplementary Fig. 7a** and **b**), similar to those prepared by Vitrobot[15]. The R2 crystals diffracted to about 4 Å (**Supplementary Fig. 7c** and **d**). In addition, the number of crystals on the cryo-EM grid prepared by Preassis was significantly increased compared to that prepared by Vitrobot (**Supplementary Fig. 8**). It is of interest to note that the thickness of the vitrified ice layer can be adjusted by selecting grids with different hole sizes and by changing the strength of the suction (**Supplementary Fig. 9**). By reducing the ice thickness, the signal-to-noise ratio of the measured electron diffraction intensities and resolution of the diffraction data can be greatly improved. However, when too much water is removed, the crystallinity deteriorates due to dehydration.

The most important feature of Preassis is its ability to handle protein crystals grown in highly viscous buffers. We applied Preassis to micro-crystals of *Sulfolobus acidocaldarius* R2-like ligand-binding oxidase (*Sa*R2lox) (**Fig. 2c** and **f**) grown with 44% polyethylene glycol (PEG) 400, which is highly viscous. It was difficult to remove the liquid by Vitrobot even using extreme blotting conditions (2 layers of filter paper on each side, strong blotting force 16, and long blotting time 10 s); the vitrified ice was still too thick and very few crystals remained on the grid (**Fig. 2b-d**). By using the setup B and EM grids with a large hole size, the viscous liquids could be efficiently removed and many *Sa*R2lox crystals were found over a large area on the EM grid (**Fig. 2e-g**). Importantly, by reducing the vitrified ice thickness, the resolution of the electron diffraction data was significantly improved from 9 Å to 3.0 Å. This allowed the collection of good MicroED data on *Sa*R2lox crystals (**Supplementary Video 4**), which

was crucial for the structure determination of *Sa*R2lox, the first novel protein structure solved by MicroED[9].

Preassis is a simple and promising method for preparing cryo-EM specimens of single particles and micro-crystals. Our results demonstrate that the method can greatly reduce the sample consumption compared to the conventional pipetting-blotting-plunging method, which is important for proteins that are difficult to purify or concentrate. In addition, this method is widely applicable for MicroED specimen preparation, especially for crystals grown in highly-viscous media. We believe that, unlike the blotting routine, Preassis can be applied to targets from the vast majority of the commercially available crystallisation screen conditions, including crystals grown in lipid cubic phase. We are currently prototyping an add-on to the Vitrobot to make Preassis more controllable in order to improve the reproducibility. New implementations of Preassis in order to reduce the preferred orientation of single particles[16,17] are also being investigated, such as manipulating the directions of the suction, speeding up the plunge-freezing time and applying magnetic fields during the plunge-freezing process. Preassis is simple and can be easily implemented in any cryo-EM labs. We believe that this method will provide new opportunities for cryo-EM studies of samples that cannot be studied today and make large impacts in structural biology and life science.

Acknowledgments

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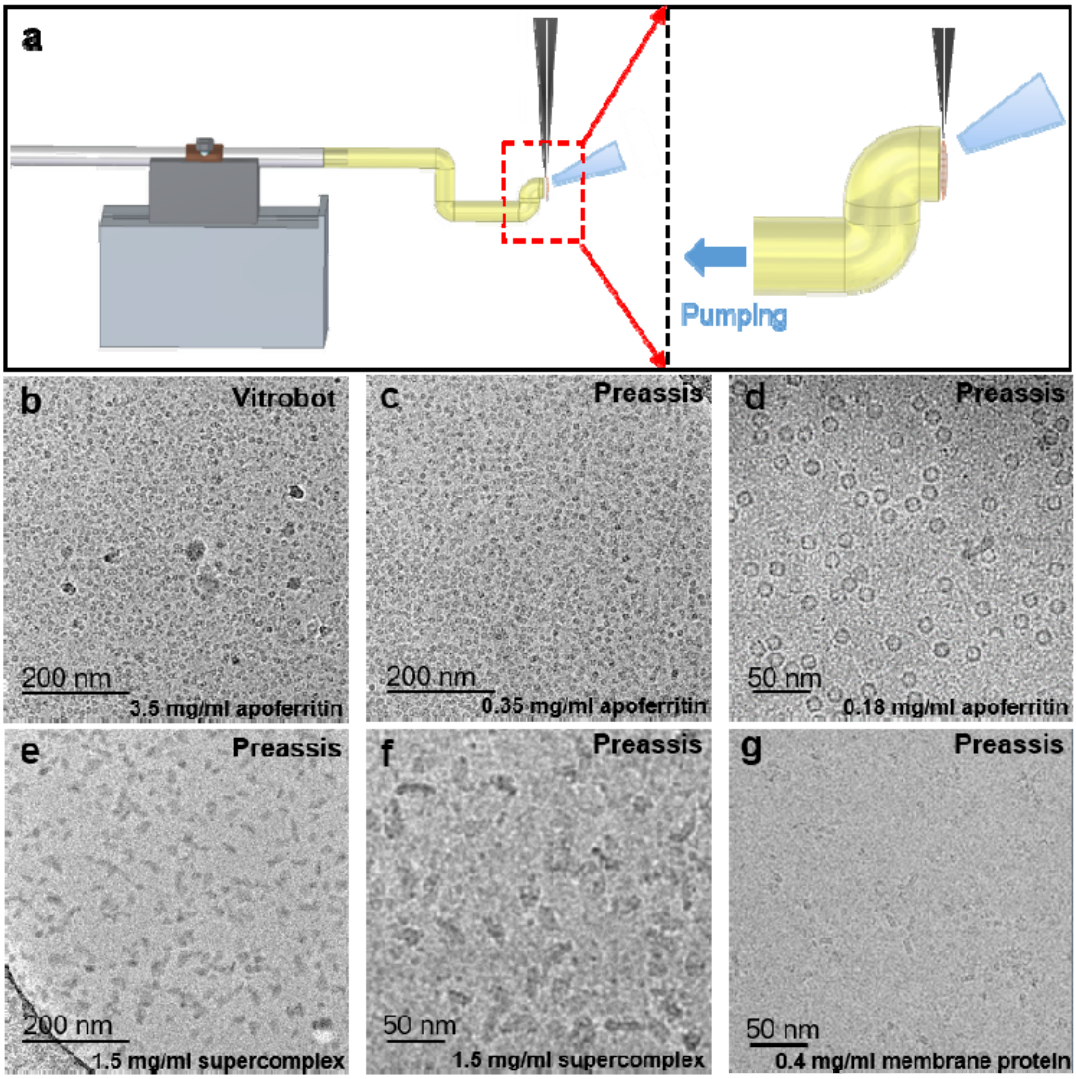

Fig. 1 | Schematic of the setup A of Preassis and comparison of results using Vitrobot and Preassis. a, Schematic of the setup A of Preassis, where the TEM grid is vertically held by a tweezer. By introducing a pressure, the extra liquid is removed through the EM grid to obtain suitable ice thickness. The yellow part is a glass tube, which is replaceable as it will be contaminated by the sample. **b-d**, TEM images of apoferritin particles taken from specimens prepared by Vitrobot (**b**, 3.5 mg/ml) and Preassis (**c**, 0.35 mg/ml, **d**, 0.18 mg/ml). **e-f**, TEM images of yeast respiratory supercomplex (1.5 mg/ml) from the specimen prepared by Preassis. **g**, TEM images of the membrane protein with a low concentration (0.4 mg/ml) showing good particle distribution. The gauge pressure used in these three experiments were -25 mbar, -32 mbar, and -40 mbar, respectively. The applied volume was 3 µl for all these experiments.

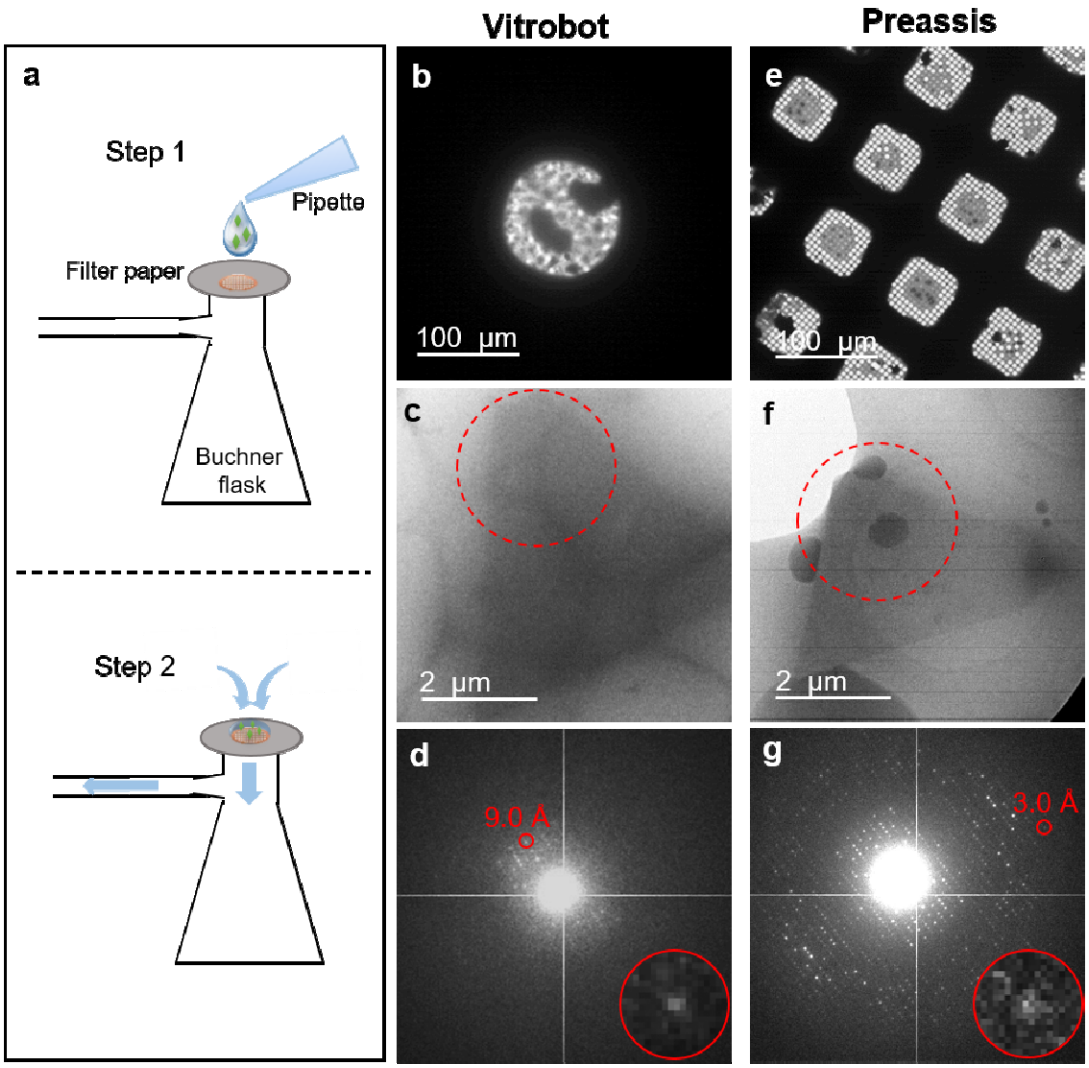

Fig. 2 | Schematic of the setup B of Preassis and specimen preparation results on *Sa*R2lox crystals. a, Schematic of the setup B of Preassis, where the TEM grid is rested on the filter paper horizontally. This model is especially useful for crystals grown in a viscous mother liquid. **b-d**, TEM images and electron diffraction pattern taken from a specimen of *Sa*R2lox crystals prepared by Vitrobot with optimised conditions. The *Sa*R2lox crystals were grown in a highly viscous mother liquid with 44% PEG 400. **e-g**, TEM images (**e**, **f**) and electron diffraction pattern (**g**) taken from a specimen of *Sa*R2lox crystals prepared by the setup B of Preassis. An exposure time of 2 s was used for taking the diffraction patterns in **d** and **g**, where the insets are enlarged views of the spots marked by the red circle. Using Preassis, much thinner vitrified ice was obtained, and the resolution of electron diffraction data was improved significantly from 9 Å to 3.0 Å. A normal lacy carbon grid was used in Vitrobot, and Quantifoil grid R 3.5/1 was used in Preassis.

Methods

Single particle samples

Apoferritin. Horse spleen apoferritin was bought from Sigma (A3641, 481.2 kDa). The original concentration was 35 mg/ml. A buffer solution (20 mM Tris-HCl, pH 7.5; 150 mM NaCl) was prepared and filtered using a 0.2 μm filter. The apoferritin concentration was further diluted using the buffer to 3.5 mg/ml, 0.35 mg/ml and 0.18 mg/ml, respectively.

Yeast respiratory supercomplex. The yeast strain BY447 from Saccharomyces cerevisiae with a FLAG tag on cytochrome *c* oxidase subunit Cox6 (DiVA, id: diva2:1296858) was grown in YPG (2% peptone, 1% yeast extract, 2% glycerol) at 30 °C in a shaker. The cells were harvested by centrifugation and disrupted with a Constant Systems cell disrupter. The isolated membrane fragments were solubilised in 0.5% GDN. Purification was done in 0.01% GDN using an Anti-FLAG M2 resin. The supercomplexes were further purified using size exclusion chromatography with a Yarra SEC-4000, 300 x 7.8 column. The original concentration was 3 mg/ml, which was further diluted to 1.5 mg/ml using a buffer (20 mM KH_2PO_4-KOH pH 7.4, 150 mM KCl, 0.0084% GDN (Anatrace)). A detailed purification protocol will be published elsewhere.

Membrane protein (transporter). For a large scale production, 12 L of S. cerevisiae FGY217 cells were grown at 30 °C in –URA medium containing 0.1 % (v/v) glucose. The target protein containing membranes were solubilised for 1 h at 4 °C in a buffer consisting of 1×PBS and 1% (w/v) DDM. The supernatant containing the target protein was incubated with Ni^{2+}-agarose affinity resin (Ni-NTA; Qiagen) at 4 °C for 2 h. For the elution, a 500 mM imidazole containing buffer (50 mM Tris-HCl pH 8.0 and 300 mM NaCl) was used. The eluate was incubated at 4 ºC with equimolar TEV-MPB-His6 protease overnight to cleave the GFP-His8 tag. After the cleave, the solution was collected and concentrated. The concentrated solution was applied onto Superdex200 (GE) with running a buffer containing

(50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% n-Nonyl-β-D-Maltopyranoside). Finally, the protein was concentrated at 4 mg/ml. For our study, the protein suspension was further diluted to 0.4 mg/ml by a buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% n-Nonyl-β-D-Maltopyranoside (Anatrace)).

Protein micro-crystal samples

Lysozyme. Hen egg-white lysozyme micro-crystal sample was produced as described by Xu et al.[15]. In brief, the protein was crystallised using the hanging drop vapour diffusion method where 2 µl of an 8 mg/ml protein solution in 10 mM Tris-HCl pH 8.0 was mixed with 2 µl of reservoir solution consisting of 1 M potassium nitrate, 0.1 M sodium acetate trihydrate pH 3.4, to produce thin fibrous needle-shaped crystal clusters within 48 h at 21°C.

Ribonucleotide reductase R2 subunit (R2) Crystal. Crystal sample of the R2 protein from *Saccharopolyspora erythraea* was produced as described by Fuller et al.[18] In brief, the protein was crystallised using the batch method where a crystallisation buffer consisting of 10% (w/v) polyethylene glycol (PEG) 3350 and 1% (v/v) tacsimate pH 4.0 was added to the protein solution at 20 mg/ml in a 1:1 protein solution to crystallisation buffer ratio. Square-bipyramidal-shaped crystals appeared overnight at 21 °C. The original size of R2 crystals was too large for MicroED data collection. Vortexing was used to fragment these crystals as described by Cruz et al.[19].

Sulfolobus acidocaldarius *R2-like ligand-binding oxidase (*Sa*R2lox)*. Crystal sample of the *Sa*R2lox protein was produced as described by Xu et al.[9] In brief, the protein was crystallised using the hanging drop vapour diffusion method where a volume of 2 µl of an 8 mg/ml protein solution is mixed with 2 µl of reservoir solution consisting of 44% (v/v) PEG 400, 0.2 M lithium sulphate and 0.1 M sodium acetate pH 3.4. Plate-like crystals grew within 48 h at 21 °C.

Specimen preparations by Preassis

Single particles. Single particle cryo-EM specimens were prepared using the setup A of Preassis (**Fig. 1a)** together with a Vitrobot Mark IV (Thermo Fisher Scientific), which was used to control the humidity and temperature. The humidity and temperature were set to 70 % and 4 °C for apoferritin and 100 % and 4 °C for the yeast respiratory supercomplex and the membrane protein. The blotting process was disabled. Quantifoil (R1.2/1.3) grids were used in all experiments. Glow discharging was performed using a PELCO easiGLOW with a current of 20 mA, and a duration of 40 s for apoferritin and the membrane protein and 120 s for the respiratory supercomplex. The pressure (suction) used for the specimen preparation was -25 mbar for apoferritin, -32 mbar for the respiratory supercomplex, and -40 mbar for the membrane protein. The duration of suction was approximately 10 s in all experiments. The cryo-EM images are shown in **Figs. 1c-g**, and results of 2D classifications and particle distributions are given in **Supplementary Figs. 2-6**.

Lysozyme and R2 crystals. Cryo-EM specimens of lysozyme and R2 crystals for MicroED experiments were prepared using the setup B of Preassis (**Fig. 2a**). The results are shown in **Supplementary Figs. 5-9**. The preparation conditions were the same for both samples. Two types of Quantifoil grids with different hole sizes were tested, Quantifoil R 3.5/1 (**Supplementary Figs. 7-9**) and Quantifoil R 1.2/1.3 (**Supplementary Fig. 9**). The grids were glow discharged with 20 mA current for 60 s (10 s hold). The pressure used to produce the suction was -17.3 mbar. The time from applying the sample to plunge-freezing was ca 5 s.

SaR2lox crystals. The setup B of Preassis was also used for the preparation of the *Sa*R2lox crystals. Quantifoil grids (R 3.5/1) were used. The grids were glow discharged with 20 mA current for 60 s (10 s hold). Due to the large viscosity of this sample suspension, higher

pressure (-30.7 mbar) was used to produce the suction. The time from applying the sample to plunge-freezing was ca 5 s. The results are shown in **Fig. 2b-g.**

Cryo-EM specimen preparations by the conventional pipetting-blotting-plunging method

For comparison, cryo-EM specimens of the apoferritin particles, lysozyme crystals, and *Sa*R2lox crystals were also prepared by the conventional pipetting-blotting-plunging method using Vitrobot Mark IV.

Apoferritin. A droplet of 3 μl of apoferritin suspension (3.5 mg/ml) was applied to a glow-discharged (40 s, 20 mA, PELCO easiGlow) Quantifoil grid (R 1.2/1.3). The operation parameters of the Vitrobot Mark IV (Thermo Fisher Scientific) were 4 °C, 100% humidity, 3 s blotting time, 0 blotting force, and one blotting paper on each pad.

Lysozyme crystal. A droplet of 3 μl of lysozyme crystal suspension was applied to a glow-discharged (60 s, 20 mA, PELCO easiGlow) Quantifoil grid (R 3.5/1). The operation parameters of the Vitrobot Mark IV (Thermo Fisher Scientific) were 4 °C, 100% humidity, 5 s blotting time, 1 blotting paper on each pad, and -6 blotting force. The experimental results are shown in **Supplementary Fig. 8 a**.

Sa*R2lox crystal.* A droplet of 3 μl of *Sa*R2lox crystal suspension was applied to a glow-discharged (60 s, 20 mA, PELCO easiGlow) copper lacey-carbon grid. It was found that lacey carbon grids allow more efficient blotting of viscous liquid compared to Quantifoil grids. The operation parameters of the Vitrobot Mark IV (Thermo Fisher Scientific) were 4 °C, 100% humidity, 10 s blotting time, two layers of blotting papers on each pad, and 16 blotting force.

Single-particle cryo-EM data collection

Apoferritin. Single-particle data of apoferritin were collected in counting mode on a Titan Krios (Thermo Fisher Scientific) equipped with energy filter and a K2 camera (Gatan, Inc). The microscope was operated at 300 kV with a nominal magnification of 130,000 ×, giving a calibrated pixel size of 1.06 Å/pixel. The dose on the camera was 4.8 e^-/pixel and the total dose was 39.6 $e^-/Å^2$ distributed over 44 frames.

Yeast respiratory supercomplex. Single particle data of yeast respiratory supercomplexes were collected on a Talos Arctica equipped with a Falcon 3 camera (Thermo Fisher Scientific) in linear mode. The microscope was operated at 200 kV at a nominal magnification of 92,000 × giving a calibrated pixel size of 1.54 Å/pixel.

Single-particle cryo-EM data processing

Movie frames alignment with dose weighting[20], CTF estimation with Gctf[21], automatic particle picking[22], particles extraction and 2D classification (Relion 3.0[23]) was performed on-the-fly using the Scipion-box suite[24]. Processing was continued using Relion 3.0 within the Scipion interface. After a series of 3D classifications and auto-refinements with octahedral symmetry, a final map at 3.2 Å resolution of apoferritin was obtained from 20,243 particles. For the respiratory supercomplex, the processing was performed up to 2D classification.

Cryo-ET data collection

Tomography data were collected on a Titan Krios (Thermo Fisher Scientific) equipped with an energy filter and a K2 counting camera (Gatan, Inc.). The tilt step was 2° and most tilt series were collected in the range of -50° to 50°. The image dose was 1.4 $e^-/Å^2$, and the pixel size was 3.45 Å/pixel.

Cryo-ET data processing and subtomogram averaging

The IMOD software package was used for the alignment of the image tilt series, using paths as fiducial markers before tomograms were reconstructed by weighted back-projection[25]. Tilt series were not CTF-corrected. Particles in the reconstructed tomograms were picked manually using 3dmod in IMOD. Subtomogram averaging was done using the PEET software[26] to get 3D models of the apoferritin, yeast respiratory supercomplex, and membrane protein. The UCSD Chimera software package was used for 3D visualisation of the distribution of apoferritin particles[27]. The sizes of the supercomplex and membrane protein particles were artificially reduced in the reconstructed 3D tomography model for a clearer representation of their distributions.

TEM image collection

TEM images were collected at 200 kV under cryogenic conditions using a Gatan 914 cryo-transfer holder. Images in **Fig. 1b**, **c**, and **e**, and **Supplementary Fig. 6** were collected on a JEOL JEM-2100FEG transmission electron microscope (TEM) using a Gatan Ultrascan 1000 CCD camera. Images in **Supplementary Figs. 7- 9** were collected on a JEOL JEM-2100LaB_6 TEM using an Orius detector.

Electron diffraction data collection

MicroED data and selected-area electron diffraction (SAED) patterns were collected under cryogenic conditions using a Gatan 914 cryo-transfer holder on a JEOL JEM-2100LaB_6 TEM operated at 200 kV. The MicroED data of *Sa*R2lox and R2 crystals were collected by continuously rotating the crystal under the electron beam whilst simultaneously collecting the diffraction patterns on a fast Timepix hybrid pixel detector (Amsterdam Scientific Instruments). The conditions used to collect MicroED data were: spot size 3, cameral length 100 cm, and exposure time 2 s per frame. The rotation speed of the goniometer was 0.45 °/s. The SAED patterns of lysozyme crystal in **Supplementary Fig. 7b** and **Supplementary Fig.**

9 were collected on the same microscope using the same detector. The conditions used to collect SAED patterns were: spot size 3, camera length 60 cm, and exposure time 2 s.

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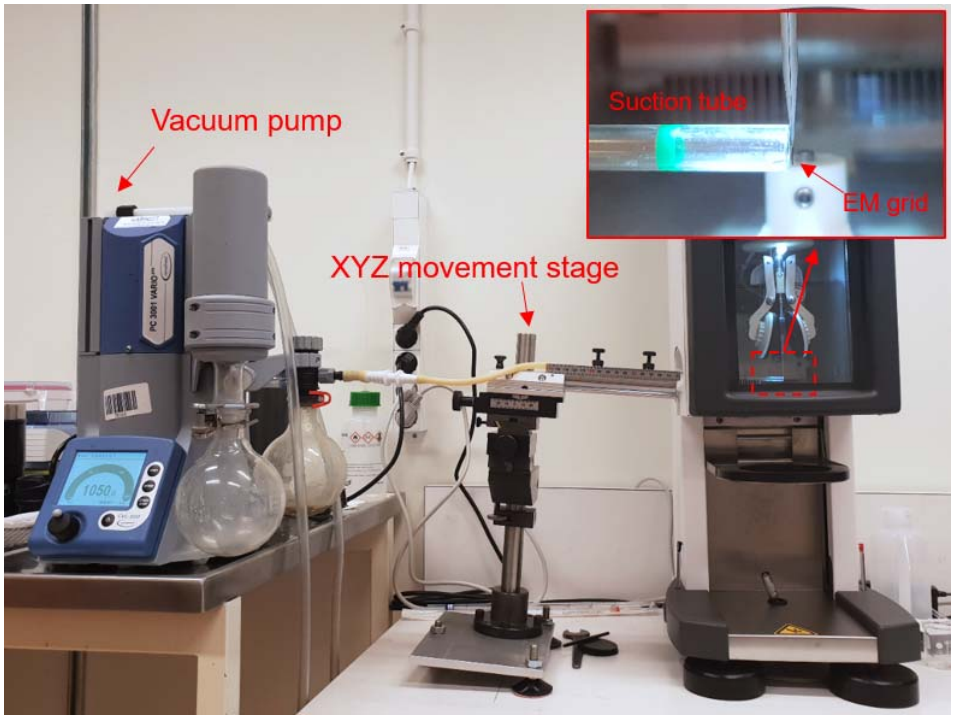
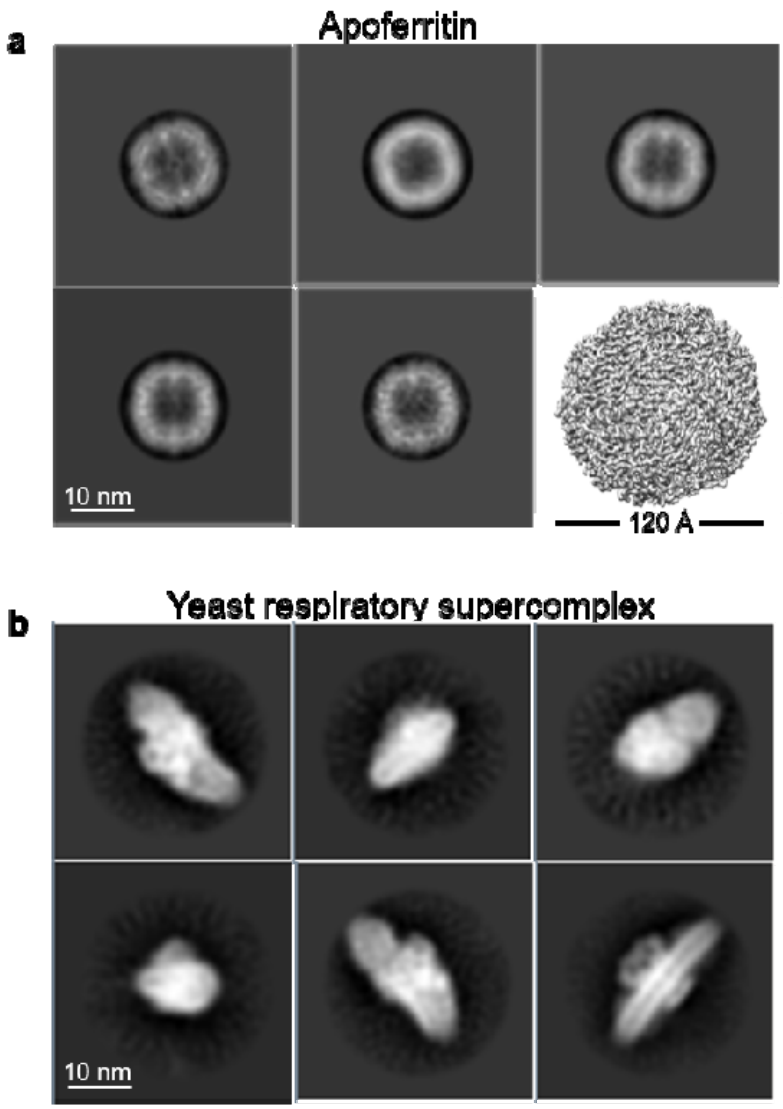
Supplementary Figures



Supplementary Figure 1

Setup A implemented in a FEI Vitrobot®.

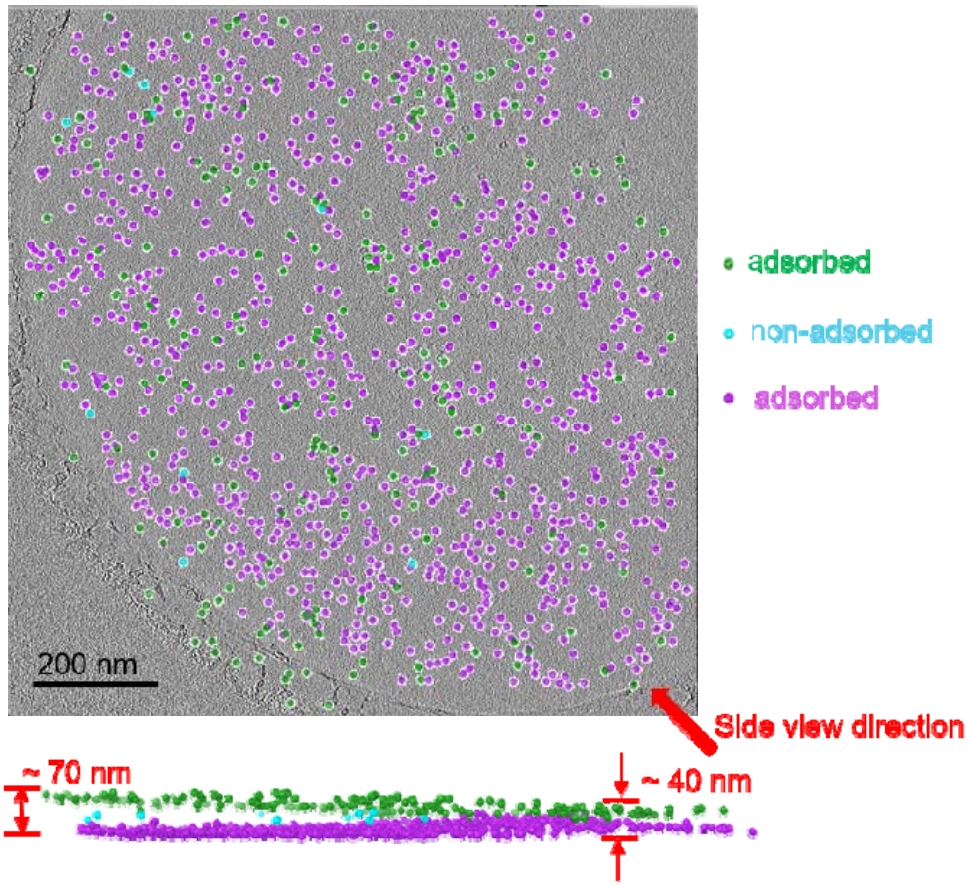
The setup A can be adapted to most cryo-EM vitrification devices, such as FEI Vitrobot, Gatan CP3, Leica EM GP, etc. The design of an automated setup is in progress.



Supplementary Figure 2

2D classifications of apoferritin and yeast respiratory supercomplex showing the capability of Preassis for single particle cryoEM applications**.**

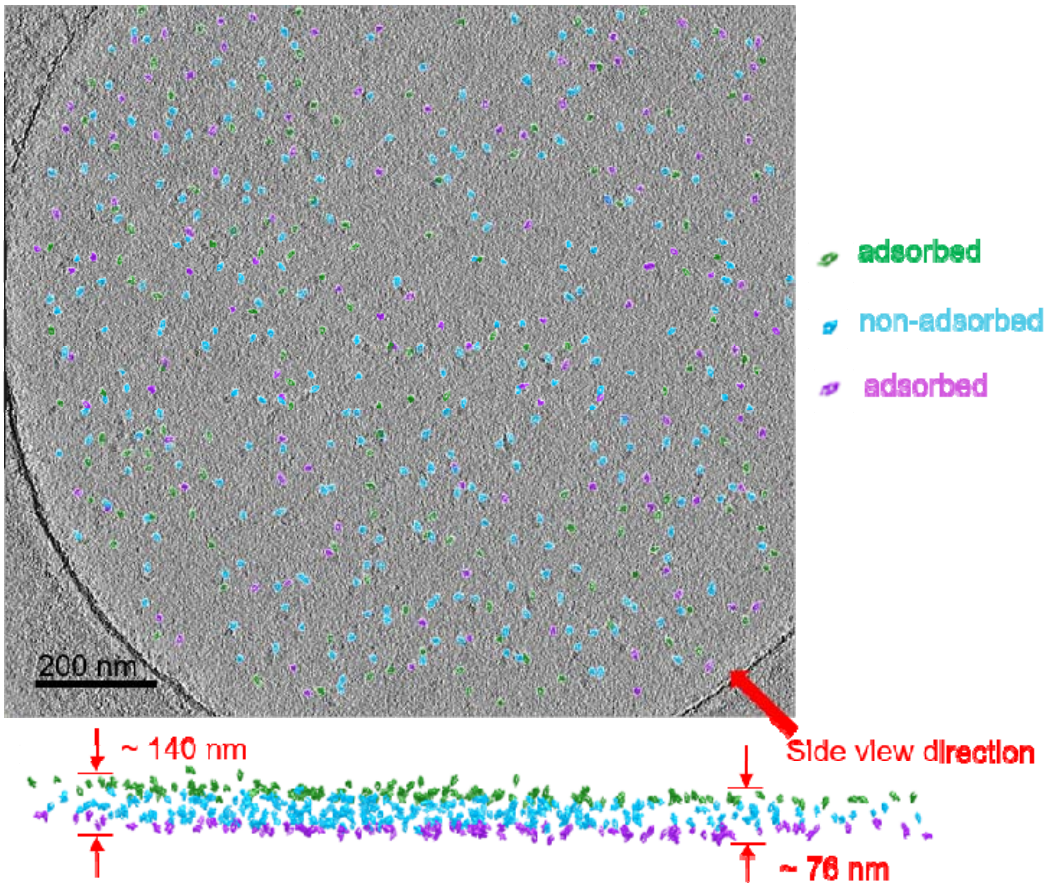
a, Representative 2D classification images of apoferritin and a 3D reconstruction map. The resolution of the map is about 3.2 Å. **b**, Representative 2D classification images of the yeast respiratory supercomplex.



Supplementary Figure 3

Particle distribution of apoferritin from the grid prepared by Preassis.

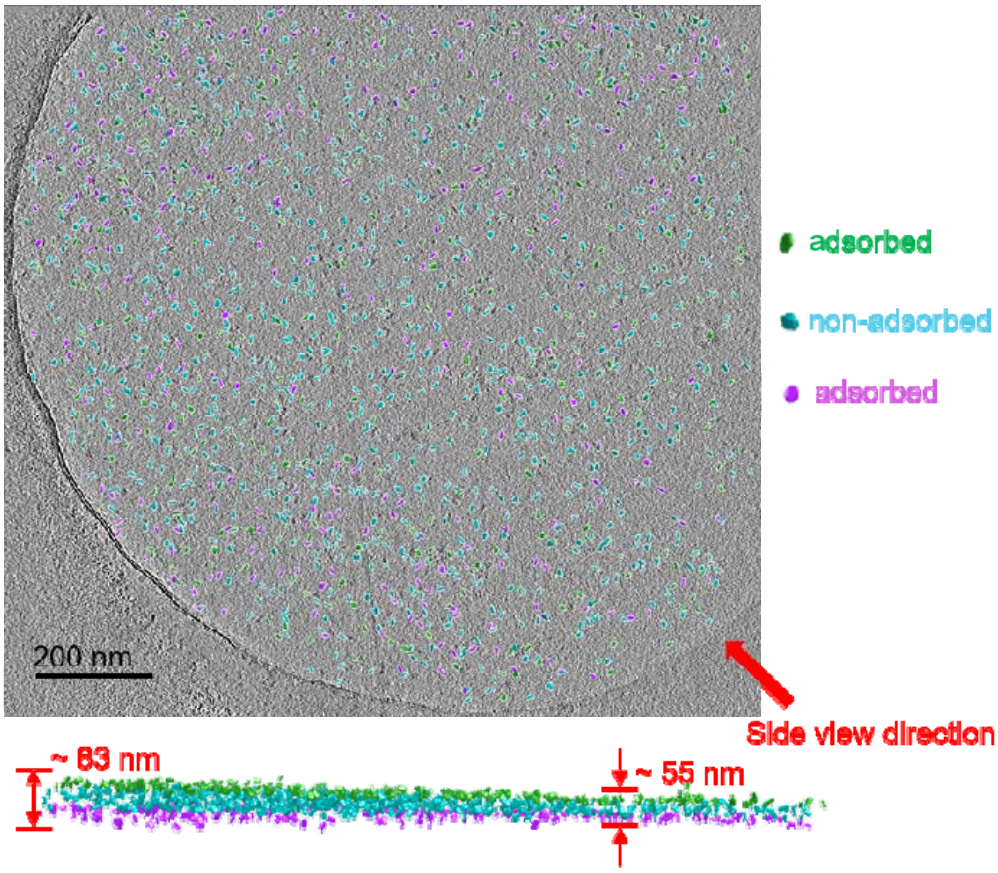
The particle distribution figure was obtained from the tomogram of **Supplementary Video 1**. Particles were picked manually in IMOD. The distribution information was obtained by re-projecting a subtomogram averaged 3D model obtained by PEET onto the original tomogram. Top depiction is a top-down view with a tomographic slice included. Bottom depiction is a side-view roughly perpendicular to the air-water interfaces. The thickest area is close to the edge of a hole, about 70 nm thick. In the middle of the hole, it is about 40 nm. Most particles were adsorbed to the air-water interface close to the suction side, which probably results from the suction direction. The sample concentration was 0.18 mg/ml.



Supplementary Figure 4

Particle distribution of yeast respiratory supercomplex from the grid prepared by Preassis.

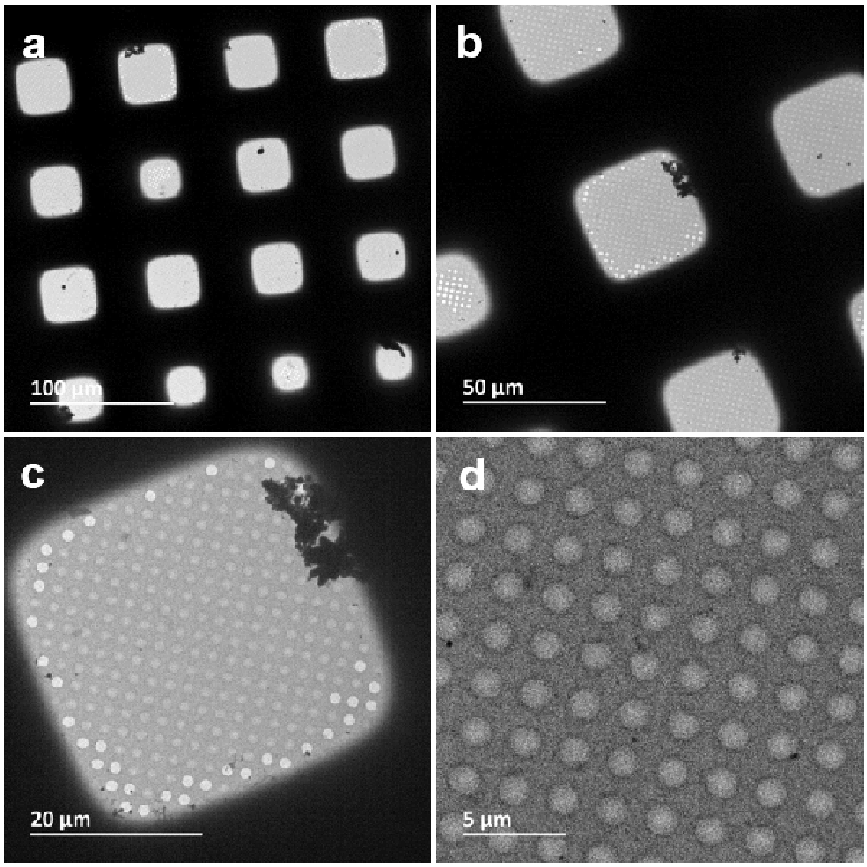
This particle distribution figure was obtained from the tomogram of **Supplementary Video 2**. Particles were picked manually in IMOD. The distribution information was obtained by re-projecting a subtomogram averaged 3D model obtained by PEET onto the original tomogram. Top depiction is a top-down view with a tomographic slice included. Bottom depiction is a side-view roughly perpendicular to the air-water interfaces. The thickest area is close to the edge of a hole, about 140 nm thick. In the middle of the hole, it is about 76 nm. Particles are evenly distributed in the ice. These particles prefer thicker ice, and have a strong affinity to carbon. The size of particles showed in the picture are smaller than the real structure. The sample concentration was 1.5 mg/ml.



Supplementary Figure 5

Particle distribution of a membrane protein from the grid prepared by Preassis.

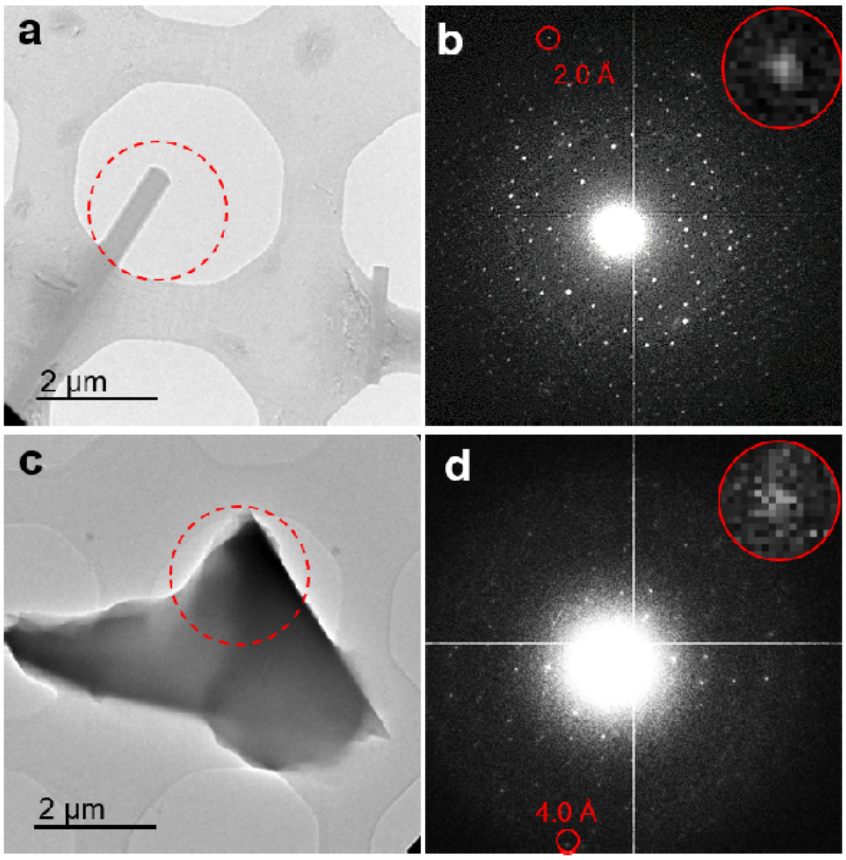
This particle distribution figure was obtained from the tomogram of **Supplementary Video 3**. Particles were picked manually in IMOD. The distribution information was obtained by re-projecting a subtomogram averaged 3D model obtained by PEET onto the original tomogram. Top depiction is a top-down view with a tomographic slice included. Bottom depiction is a side-view roughly perpendicular to the air-water interfaces. The thickest area is about 83 nm wich is close to the edge of a hole. In the middle of the hole, it is about 55 nm. Particles are evenly distributed in the ice. The size of particles showed in the picture are smaller to the real structure. The sample concentration was 0.4 mg/ml.



Supplementary Figure 6

Large areas suitable for single particle data collection of the apoferritin specimen prepared by Preassis**.**

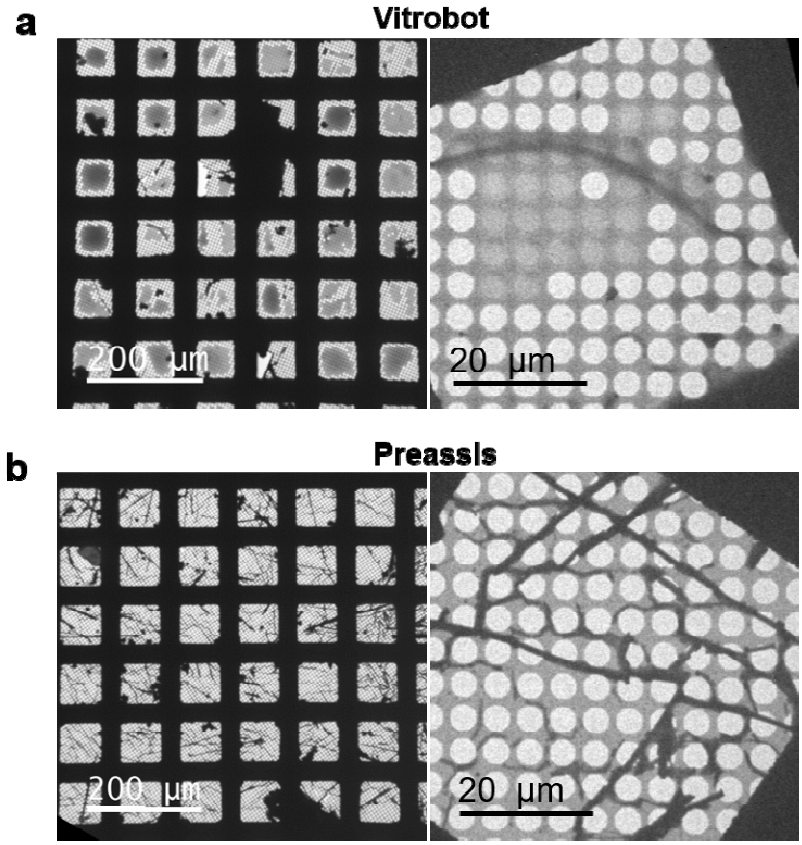
a, Low magnification image showing good control of vitrified ice thickness in a large area by Preassis. **b-d**, Sequentially higher magnification images of this specimen. Quantifoil R 1.2/1.3, and gauge pressure at -25 mbar were used in this experiment.



Supplementary Figure 7

MicroED specimen preparation of lysozyme and R2 protein crystals by the setup B of Preassis.

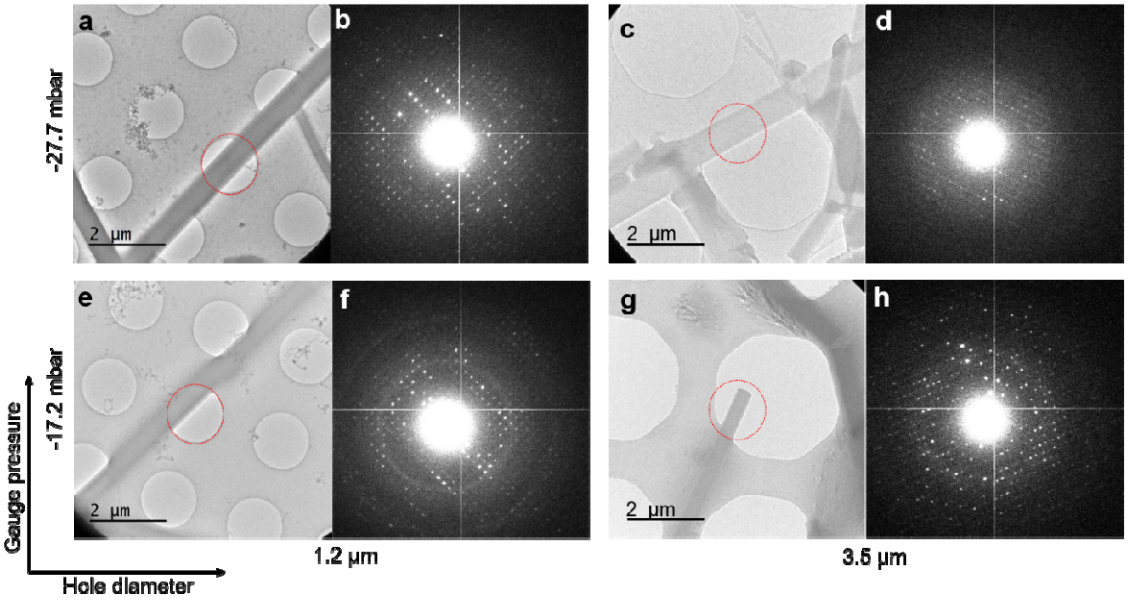
a-b, TEM images of a lysozyme crystal and its corresponding electron diffraction pattern, showing that the resolution reaches 2 Å. **c-d**, TEM image of a R2 crystal and its related electron diffraction pattern. The resolution is about 4 Å. Quantifoil grids R 3.5/1, and gauge pressure at -17.3 mbar were used for these two experiments. In the electron diffraction patterns **b** and **d**, the insets show a close-up of the spots indicated by the red circle.



Supplementary Figure 8

Sample consumption comparison between Vitrobot and Preassis for MicroED specimen preparation of lysozyme crystals.

a, Low magnification TEM images taken from the specimens prepared by Vitrobot. **b**, Low magnification TEM images taken from the specimens prepared by the setup B of Preassis. The same concentration of protein crystals was used for both techniques. This comparison illustrates that 10 times more crystals are kept on the grid prepared by Preassis.



Supplementary Figure 9

Adjust vitrified ice thickness of lysozyme MicroED specimens by changing the hole size of Quantifoil grids and the gauge pressure used to produce a suction.

Using gauge pressure at -27.7 mbar **a-d**, crystals on the grid with a hole size of 1.2 µm in diameter resulted in higher resolution electron diffraction. However, for -17.2 mbar pressure **e-h**, a hole size of 3.5 µm produced thinner ice, which improved the resolution and signal-to-noise ratio of the diffraction pattern in **h**. The comparison suggests that the thickness of vitrified ice can be adjusted by these two parameters.