Cryo electron tomography studies of bacterial chemosensory arrays

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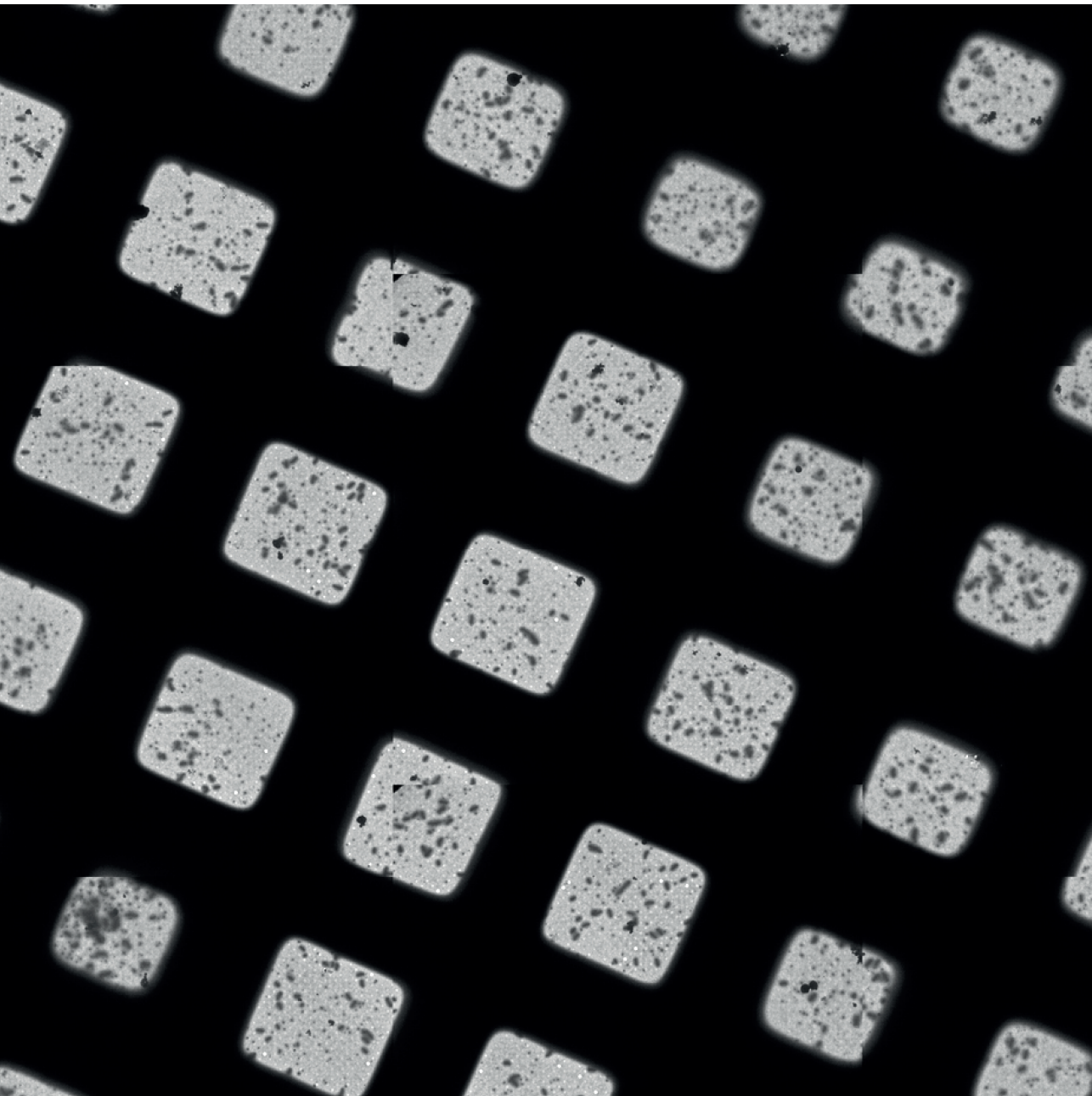
CHAPTER 6



Use cryo electron microscopy to study the chemosensory arrays *in vivo*

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Abstract

Cryo-electron microscopy (cryo-EM) allows the imaging of intact macromolecular complexes in the context of whole cells. The biological samples for cryo-EM are kept in a near-native state by flash freezing, without the need for any additional sample preparation or fixation steps. Since transmission electron microcopy only generates 2-D projections of the samples, the specimen has to be tilted in order to recover its 3-D structural information. This is done by collecting images of the sample with various tilt angles in respect to the electron beam. The acquired tilt series can then be computationally back-projected. This technique is called electron cryotomography (ECT), and has been instrumental to unravel the architecture of chemoreceptor arrays. Here we describe the method of visualizing *in vivo* bacterial chemoreceptor arrays in three main steps: immobilization of bacterial cells on EM grids by plunge-freezing, 2D images acquisition in tilt–series, and 3D tomogram reconstruction.

Introduction

Electron cyrotomography (ECT) is a new technique that has recently been used to gain insight into structure and function of macromolecular complexes inside intact cells, such as bacteria, archaea, and even small eukaryotic cells (223, 224). Rapid progress in the development of both hard- and software involved in ECT in the past decade has provided the technological basis necessary for the understanding of structure and function of bacterial chemoreceptor arrays. The arrays were first identified as a cytosolic plate-like structure at cell poles parallel to the membrane, with the perpendicular pillar-like densities of the chemoreceptors spanning into the periplasmic space (42). The plate like density is called the base plate and is comprised of the histidine kinase CheA and the linking protein CheW. Subsequent resolution improvement revealed the hexagonal packing of chemoreceptors above the CheA/CheW base plate with a center-to-center spacing of 12 nm (225). Further improvement was achieved by implementing image correction procedures such as contrast transfer function (CTF) correction and subvolume averaging. This finally revealed the native architecture of the chemoreceptor arrays: they consist of a hexagonally packed lattice of the trimers of receptor dimers networked by rings formed by CheW and the P5 domain of CheA. Neighboring CheA/CHeW rings are structurally connected via the dimerization domain P3 of CheA (43, 44). In addition to the abundant structural information, ECT has also provided important insights in understanding the molecular mechanism of receptor signaling and CheA kinase functioning (54, 141).

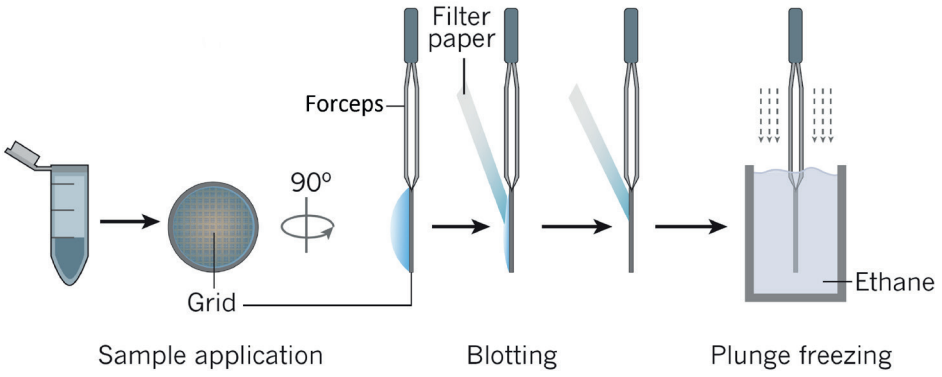

Figure 1. Sample preparation procedure for cyro-EM. A few microliter of bacterial cells suspension mixed with gold nanoparticles is applied to an EM-grid held by forceps. During blotting, filter paper wicks off the excessive liquid and leaves a thin aqueous layer. Plunge freezing fixes this aqueous layer into vitreous ice, while bacterial cells are preserved at their near-native state. Figure is adapted from (226).

ECT allows the study of chemoreceptor arrays in the context of whole cells at near-native state. In order to withstand the high vacuum of the cryogenic electron microscope, the cells have to be embedded in a thin layer of vitreous ice. The most commonly used method to generate thin, vitirified samples is called plunge-freezing (223) (Fig.1). A few microliters of sample (cell culture) is spread out over an EM grid. Excess water is blotted away either manually or automatically with filter paper, and lastly plunged into liquid ethane or ethane/propane mixture that is cooled by liquid nitrogen. This allows freezing of the sample at an extremely high cooling rate (>10000 K/s) and prevents water from crystallizing and instead forms virtrous ice. Thus, cellular structures will be well preserved in a near-native state (227). Vitrified specimens on EM grids need to be transferred and stored below 120 K (-153°C) to maintain the "frozen-hydrated" state (228).

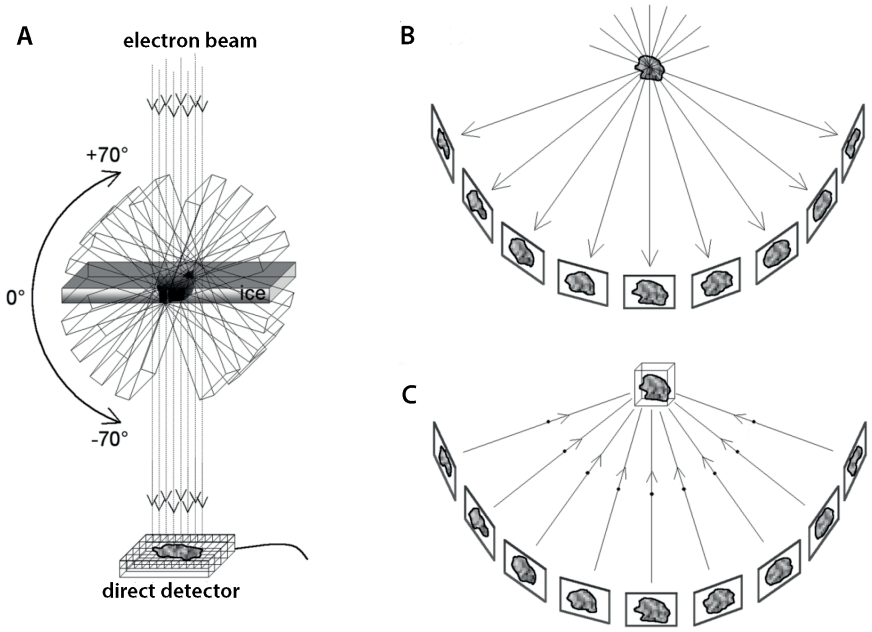

Figure 2. Basic principle of cryotomography. (A) The specimen holder tilts according to the preset tilting scheme around tilting axis perpendicular to the electron beam, and projection images of the same illumination area on the specimen are recorded on the detector. (B) The 2D images captured in a tilt-series correspond to a set of projections of the 3D sample with different tilt angles in respect to the electron beam. (C) After precise alignment, the successive projection images are merged computationally into one density map (referred to as tomogram) correspond to the specimen volume for example by weighted-backprojection (229) . Image is adapted from (230).

In order to gain 3D information of the biological specimen, a series of 2D projection images is collected while the sample is incrementally tilted along the axis perpendicular

to the electron beam (Fig. 2) (231). However, this typically requires the acquisition of ~100 or more individual 2D images of the sample. Since the vitrified samples are very electron-dose sensitive, the most critical factor is to limit the total electron dosage in order to allow complete tilt-series acquisition before structures are irreversibly destroyed by radiation damage (232). Based on the type of sample, imaging condition such as total electron dose, magnification, defocus value, tilting range and angular increment, need to be selected carefully for data collection (233, 234).

Once a tilt series of a biological sample is collected, the tomogram (the 3D density map) can be computationally calculated by back-projection of the 2D images into the sample volume. A successful 3D reconstruction relies on precise alignment of the 2D images of the tilt-series. Alignment of the 2D images prior to 3D reconstruction is therefore essential. Instead of cross correlation methods, an alignment based on gold fiducial particles is commonly used, especially in the case of thicker samples such as intact bacterial cells. Here, gold colloidal particles of typically 5-15 nm in diameter are mixed together with the biological sample. These highly electron dense particles are easily visible in the individual 2D images of a tilt series and can be used to align the individual 2D projections (235). Several software packages are available for tomogram reconstruction, either fully automatic or interactive through a graphic user interface. Several different reconstruction schemes are available, such as for example weighted back-projection (229), SIRT (236)or SART(237). In-depth information on these computational methods can be found in the respective publications. Additional data processing steps such as contrast transfer function (CTF) correction (157, 238) and subvolume averaging (239, 240), can also be applied accordingly to obtain higher signal to noise ratio cryo-EM maps to study the structure of chemoreceptor arrays.

While ECT is an important tool for structural studies of molecular machines, the high cost of both, the required specialized equipment as well as the in-depth expertise of the operators, is preventing many laboratories from utilizing this technique. Therefore, this chapter provides methods that focus primarily on cryo-EM sample preparation and evaluation that is essential to generate adequate samples for ECT. The method description below is based on the particular instrument equipped in our lab; adjustment of parameters and condition may be required for alternative instrument setup. Unless your laboratory is a dedicated cryo-EM facility, access to high-end equipment is available at one of several open-access EM facilities worldwide (see note 3). While this chapter provides suggestions for data collection parameters for imaging chemoreceptor arrays, the on-site expert staff of the cryo-EM facility should provide the necessary in-depth advice on proper data acquisition and processing.

Materials

Materials

Quantifoil grids (Cu 200 mesh R2/2; Quantifoil Micro Tools GmbH, Germany)
Colloidal gold solution (10 nm) (Sigma, St. Louis, MO, USA)
Bovine Serum Albumin solution (5%) (Sigma, St. Louis, MO, USA)
Bacterial culture
Ethane
Liquid nitrogen

Equipment

Sputter Coater (e.g. Quorum Technologies, UK)
Leica EM GP immersion freezer (Leica Microsystems, Vienna, Austria) or similar (see Note 1)
Tecnai Talos G2 (FEI Company, Hillsboro, OR, USA) or similar (see Note 2)
Cryo-transfer holders (Gatan Inc., Pleasanton, CA, USA)
Titan Krios (FEI Company, Hillsboro, OR, USA) (see Note 3)

Softwares

FEI Tomography (FEI Company, Hillsboro, OR, USA) (see Note 4)
IMOD (179, 216) (see Note 5)

Methods

Plunge freezing bacteria cells on EM grids

1. Grow bacteria culture to mid-exponential phase at an OD600 above 0.5 (see Note 6).
2. Place EM grids Quantifoil side up, glow discharge under 10^{-2} Mbar pressure, at 25 mA current for 45 seconds (see Note 7).
3. For the blotting chamber on Leica freezer, set the temperature to room temperature; set the humidity to 95%; set the temperature of cryogen container to -183 °C.
4. Fill up the liquid nitrogen tank, let the cryogen container cool down to the target temperature; and then fill up cryogen container carefully with ethane (see Note 8).
5. Wait until all parameters set up for the blotting chamber are reached, secure one EM grid with the forceps provided with the plunge freezer and load it into the blotting chamber.
6. Mix 100 µl colloid gold solution with 25 µl 5% BSA solution and vortex briefly; spin down the BSA treated gold nanoparticles at 14,000 rpm for 10 min; discard the supernatant and keep the pellets of colloidal gold for next step.

7. Mix 20 µl of bacteria culture with the colloidal gold prepared in step 6 briefly on a vortex mixer.
8. Apply 3 µl cell culture mixture onto the carbon film coated side of the grid.
9. Set blotting time to 1 s and activate auto-plunge; blotting is done on the carbon film side followed by automatic plunging (see Note 9).
10. Transfer the frozen grid from liquid ethane swiftly to a labeled grid box in liquid nitrogen (see Note 10).
11. Screw tight the lid on the grid box and transfer the grid box swiftly to a 50 ml conical tube in nitrogen dewar.
12. Checking the quality of the ice on the grid with the cryogenic microscope operating at 120 kV to evaluate the plunge freezing method (see Note 11) (Fig 3).

Single tilt-series 2D images acquisition

In spite of the fact that different data acquisition softwares have various tilt series set-up schemes, certain parameters are common for visualizing the chemoreceptor arrays.

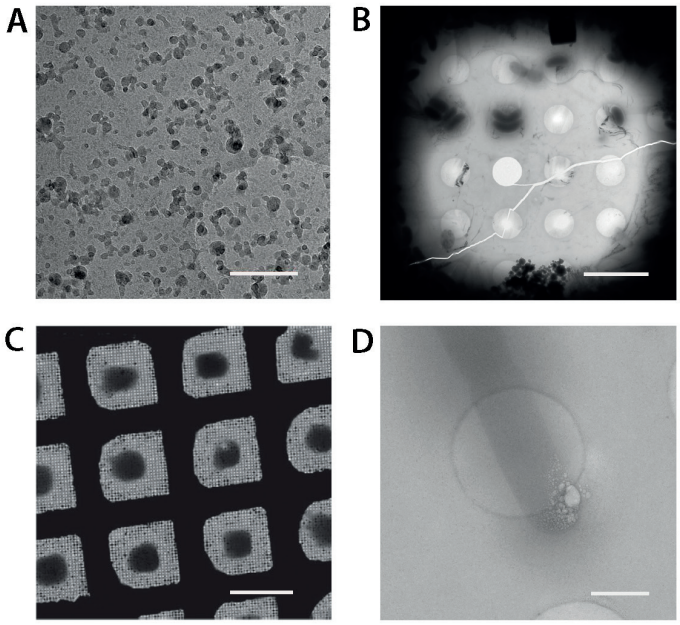

Figure 3. Common problems found on cryo-EM grids due to either contamination, mishandling, glow discharge failure or radiation damage. (A) The dark blobs are usually resulted from contaminants in the liquid ethane. Images is adapted from (241). Scale bar is 250 nm. (B) A mesh square shows a big crack of the carbon film, extensive crystalline ice as indicated by the irregular dark marks and chucks of ice contamination at the bottom of the image. Scale bar is 5 µM. (C) Failure in proper glow discharging results insufficient hydrophilicity on the grid, which leads to thick ice core in each grid square center. Image is adapted from (242). Scale bar is 100 µM. (D) The "bubbling" of cellular structures, here for example at an *E. coli* cell pole, is the typical result of radiation damage. Scale bar is 1 µm.

The general process of acquiring a tilt-series projection is outlined as follow:

1. Examine the loaded grid at low magnification with a low electron dose to find an area with optimal ice thinness; then localize the region of interest. For imaging bacterial chemoreceptors, it is preferred to target cell poles fixed in a hole of the carbon membrane where the sample thickness is minimized (See Note 12) (Fig 4).
2. Set the total electron dosage for single tilt-series as 120 electrons/Å^2 (See Note 13).
3. Set up the increment, range and imaging scheme for tilting. For chemoreceptor arrays in the context of a native cell, we usually record over an angular range of ±60° with 1° or 2° increment. We typically use a tiling scheme where imaging starts at 0° and continues tilting to one extreme angle, then returns to zero tilt before recording tilting through the other side.
4. Set the defocus to -10 µm.
5. The magnification suitable for imaging chemoreceptor arrays is roughly corresponding to 3.5Å pixel size (see Note14).
6. Most software for ECT support fully automated tracking and focusing for each tilt angle. For some software, it is important to select an area for tracking and autofocusing along the tilt axis close to the field of view but without overlapping to avoid damaging the data collection area. Depending on the software, camera hardware and the data collection parameters used, it typically takes around 20-60 minutes to collect the data recording for one single-tile series (see Note 15).

Building tomogram and visualizing chemoreceptor arrays

IMOD is the data processing software package used in our lab. As a widely distributed, well maintained and user-friendly software package, it offers informative description of each program on its website (http://bio3d.colorado.edu/imod/). A detailed description on cryo-tilt series data processing is also provided (http://bio3d.colorado.edu/imod/betaDoc/cryoExample.html). This open-source software package also allows for CTF correction and provides the option for either SIRT or weighted back-projection to generate the tomographic reconstruction. For subvolume averaging, the PEET software package has been developed by the same research group as IMOD and provides the means to aligning and averaging subvolumes extracted from tomograms (http://bio3d.colorado.edu/PEET/). Please note that there are multiple options for tomogram reconstruction and further data processing available (see further examples in Note 5).

The tomographic image directly shows the native location and assembly forms of chemoreceptor arrays, and higher resolution cryo EM maps (20-35 Å) can also be obtained with additional data processing (Fig 5).

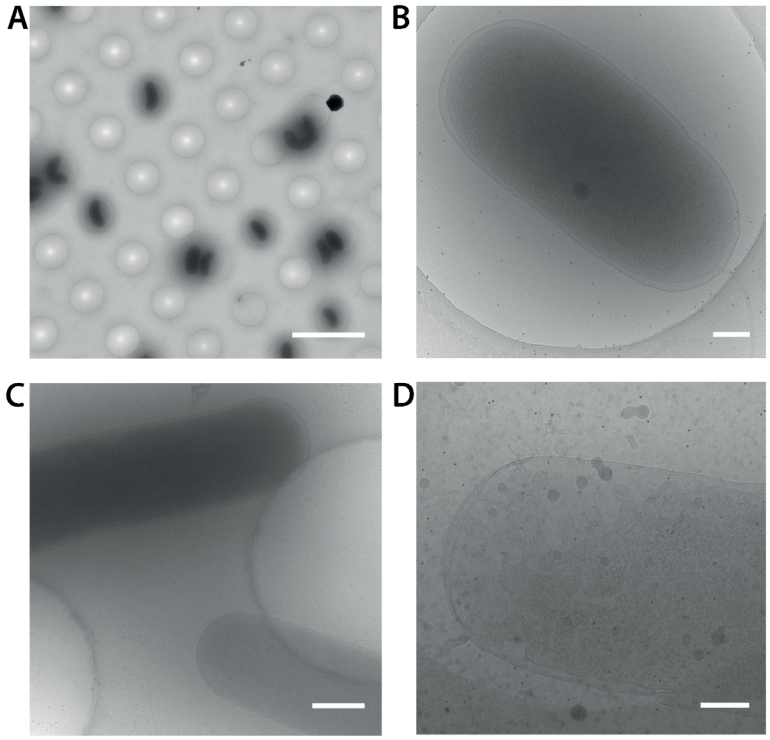

Figure 4. Good cryo-EM specimens for bacteria chemoreceptor arrays study. (A) An ideal ice thickness with abundant *Vibrio cholerae* cells embedded. Scale bar is 5 μm. (B) For small cells (< 2 μm in length) it is easy to find whole cells located in the holes of carbon film. The vitreous ice appears as transparent, and the well-distributed black dots are gold nanoparticles serving as fiducials for images alignment. Scale bar is 200 nm. (C) For large cells, gentle lysis can effectively help to reduce the sample thickness without structurally disturbing the chemoreceptor array. Intact *E. coli* cell on the left appears much darker compared to the lysed cell on the right. Scale bar represents 500 nm. (D) A gently lysed E.coli cell in the hole is considerably thinner compared to intact cells, and the ordered pattern of the chemoreceptors can be identified at high magnification. Scale bar is 200 nm.

Notes

1. Other popular choices for commercial automatic plunge freezer include Cryoplunge (Gatan, Inc., Pleasanton, CA, USA) and Vitrobot (FEI Company, Hillsboro, OR, USA). Detailed protocol on how to use the Vitrobot has been described previously (243, 244).
2. A cryo-EM operating at 120 keV and a cryo holder are the basic requirement for assessing the sample quality and visualizing chemoreceptors *in vivo*.
3. Unless your laboratory is a dedicated cryo-EM facility, access to high-end equipment such as TITAN Krios, is available at one of several multi-user EM

facilities worldwide (245).

4. Automated tilt-series data acquisition schemes for cryo-EM samples have been under fast development, providing the user a wide variety og software packages to choose from. SerialEM (246), TOM (247), UCSF tomography (215), or commercial packages like FEI Tomography (FEI Company, Hillsboro, OR, USA) are a few of the most widely used choices.
5. There are numerous software packages available for tomogram reconstruction besides of IMOD, such as SPIDER (248), TOM(247), Bsoft (249), Protomo (250), UCSF tomography (215) and Xplore3D (FEI Company, Hillsboro, OR, USA). A complete list of software packages applicable for ECT data collecting and data reconstructing can be found on the website (https://en.wikibooks.org/wiki/Software_Tools_For_Molecular_Microscopy).
6. One fundamental limitation of this technique is the sample thickness. The vitrified sample has to be thin enough to allow the electrons to pass through the sample. Therefore, samples optimally are less than 500 nm when imaged with 300 keV instrument. Some of the model organisms that are used to study bacterial chemotaxis exceed an ideal cell diameter. Therefore, multiple methods have been developed to effectively reduce the bacterial cells thickness and facilitate the visualization of chemoreceptor arrays. Both antibiotic and lysozyme treatment can lead to cytoplasmic content release with the native structure of arrays preserved (43, 54, 251). Similarly, a phage gene has been engineered into *E. coli*, allowing for controlled inducible lysis to flatten the cells (142). In addition, small and DNA free "minicells" of model organisms are the result of strains with disrupted localization of the cell division machinery. Such cells are ideal candidates for ECT, and were previously used for describing the chemoreceptor array structure (43, 44).
7. The carbon side of EM grids normally remains hydrophilic within 2 hours after being glow discharged; if grids are not used in time, repeat the glow discharge process.
8. Wear safety glasses or face shield when using ethane.
9. 3 µl cells mixture with 1s blotting time usually yield vitreous ice layer thin enough for imaging; if the volume of the cell mixture is increased, consider extending blotting time accordingly. In addiction, sample-specific features like viscosity should also be taken into account for choosing the optimal blotting time in order to achieve ideal ice thickness.
10. Mishandling of the sample by warming it above 120 K (-153°C) will cause the crystallization of the ice film, which irreversibly damages the specimen; also, exposing the grid to atmospheric moisture results in ice contamination on the surface of the grids.

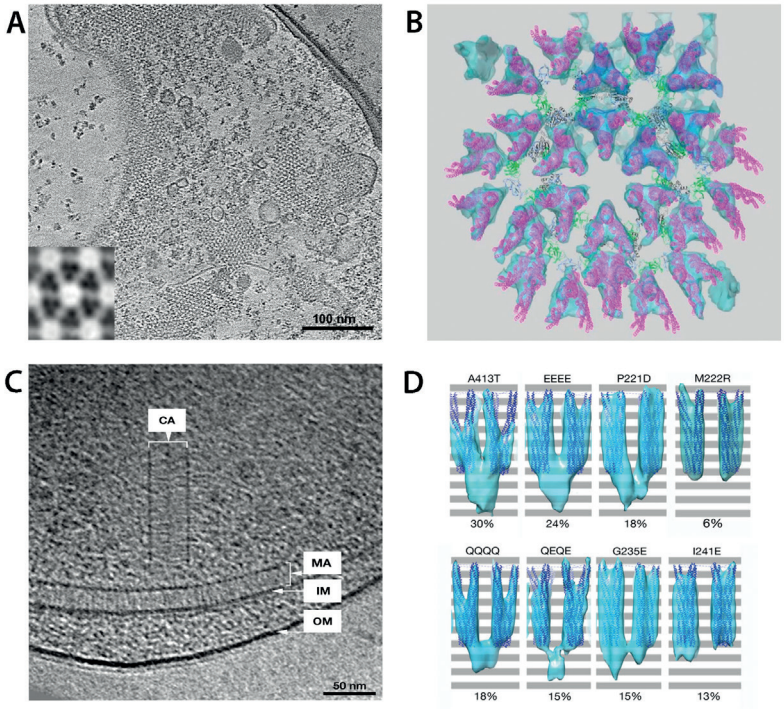

Figure 5. Images generated from cryo-tomography data for chemoreceptor arrays studies. (A) On the left panel, the top view of chemoreceptor arrays is shown in a slice through a tomographic reconstruction of an *E. coli* cell overproducing chemotaxis proteins. Inset shows the hexagonal packing of receptor trimmers. Image is adapted from(58). (B) The cryo-EM map after subvolume averaging allows the docking of the crystal structures of receptors (magenta). The receptors are networked by rings of CheW and CheA (cheW (green), CheA domains P3 (gray), P4 (black) and P5(blue)). Image is adapted from(43). (C) The side view of chemoreceptor arrays can be easily identified in tomograms due to the high electron density "baseplate" structure formed by cheA and cheW. A membrane-bound chemoreceptor array (MA) is parallel to the inner membrane (IM) while a cytoplasmic chemoreceptor array (CA) appears as a "sandwich" structure in *Vibrio Cholerae* with two baseplates on either side. OM: outer membrane. Image is adapted from (105). (D) Subvolume averaging of the tomographic reconstruction of chemoreceptor arrays locked in specific activation states by single point mutations of the receptor reveals that the electron density of the ternary signaling complexes varies in different functioning states. Crystal structures (purple) have been fitted into the EM density map (blue) by Molecular dynamics flexible fitting. Image is adapted from (54).

11. Examination is essential for new specimen; this step is optional for samples where the blotting conditions have been determined previously.
12. Small bacteria, such as *V. cholerae*, can be found lying entirely inside the holes of the carbon film, which is ideal for data collection. Other bacteria can span several holes as well as the carbon film in between due to their extended cell length of several microns or more. Since the chemoreceptor arrays are localized mostly at

the cell pole, pick the cell poles that are trapped in one of the holes.

13. The total dosage depends both on the specimen as well as the desired resolution. Some intact bacterial cell specimens have been shown to tolerate accumulative dosage up to 200 electrons/Å^2. A more moderate dose of 160 electrons/Å^2 has been widely used and is a good starting point for initial dose testing. However, these dose values are very high and will impact the finer structural details in your sample. Therefore, when aiming for higher resolution in lysed cell samples, it is suggested to keep the dosage well below 100 electrons/Å^2.
14. The choice of magnification depends also on the specific detector that is used for data collection.
15. Most software packages support automatic batch tomography for collecting datasets from multiple positions on one grid. In this case, the process of individual tilt-series acquisition remains the same as described previously. In addition, an "atlas" of the whole gird can be recorded at a relatively low magnification for picking target positions for data collection. Batch tomography also requires an additional calibration steps for accurate localization of the targets.