Appendix B

Electron cryotomography sample preparation using the Vitrobot.

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ABSTRACT

Electron cryotomography (ECT) is the highest-resolution structural technique currently available that can be applied to unique objects such as flexible large protein complexes, irregular viruses, organelles, and small cells. Specimens are preserved in a near-native, "frozen-hydrated" state by vitrification. The thickness of the vitreous ice must be optimized for each specimen, and gold fiducials are typically added to facilitate image alignment. Here, we describe in detail our protocols for ECT sample preparation including (1) introduction of fiducial markers into the sample and (2) sample vitrification. Because we almost exclusively use an automated, climate-controlled plunge freezing device (the FEI Vitrobot) to vitrify our samples, we discuss its operation and parameters in detail. A session in which 8 grids are prepared takes 1.5 to 2 hours.

INTRODUCTION

Electron cryotomography (ECT) is an increasingly important technique for determining the structure of unique objects such as flexible large protein complexes, irregular viruses, organelles or small cells (Lucic *et al.*, 2005; Nickell *et al.*, 2006). Specimens preserved in a near-native, "frozen-hydrated" state are tilted incrementally around one or two (orthogonal) axes while being imaged in a transmission electron microscope (TEM). The resulting "tilt-series" of projections are aligned with respect to each other usually using fiducial markers (electron-dense reference objects such as colloidal gold) embedded with the sample in the ice. The aligned images are then computationally merged into a three-dimensional (3-D) reconstruction.

As in all types of microscopy, sample preparation is vital. Three factors are key: the sample itself must be maintained in as near a native state as possible, the vitreous ice must be thin and uniform, and the fiducial markers should be abundant and evenly distributed. To minimize artifacts and perturbations, samples can be plunge-frozen in complete growth media or physiologic buffers. Because the surrounding media or buffer produces background scattering that degrades the image, however, it is generally advantageous to thin the ice as much as possible, as long as the structure of the sample is not distorted. In extremely thin ice, even individual strands of duplex DNA have been seen in electron cryomicrographs (Adrian *et al.*, 1990). Thicker ice seems to have improved mechanical stability, however, in other contexts (Li *et al.*, 2002), so ice thickness must be optimized for every situation. The fiducial markers need to be abundant and distributed uniformly to ensure accurate image alignment.

ECT sample preparation typically comprises two major steps: (1) introduction of gold fiducial markers and (2) sample vitrification. In order to increase the chances of having uniformly distributed fiducials in any particular area, we apply colloidal gold to both the grids and the sample solutions before they are united. Aqueous solutions are vitrified by cooling them so quickly (~ 10^7 K/s) that individual water molecules do not have opportunity to move around and form an extended, hydrogen bond-mediated crystal lattice with long-range order. Rather, they become "trapped" in an amorphous state resembling liquid water (Angell, 2004). Using a strategy pioneered by Jacques Dubochet and his colleagues (Adrian *et al.*, 1984; Dubochet and McDowall, 1981; Lepault *et al.*, 1983), this can be accomplished in four steps: (1) applying the sample onto an electron microscopy grid; (2) blotting excess fluid to produce a thin film; (3) plunge-freezing the grid into liquid ethane or propane; and (4) transferring the grid to a storage box immersed in liquid nitrogen.

Today many labs have custom-made, "in-house" plunge-freezing devices where the sample is blotted manually (by hand with a filter paper) and the plunger is accelerated by gravity or air pressure. We are not aware of any that give robustly reproducible results, however, even for expert practitioners, because they depend on user dexterity, timing, and the local climate conditions. More recently, sophisticated commercial devices have become available that allow precise control of nearly all the parameters including the temperature and humidity of the blotting chamber; the frequency, duration, and pressure used for blotting; and the plunge velocity. In our lab we use the FEI Company's automatic plunge-freezer, which they named the "Vitrobot" (Frederik and Hubert, 2005). In addition to the aforementioned parameters, which are self-explanatory, a "blot offset"

parameter controls the position of the grid between the blotting pads, which controls in part the pressure exerted by the filter paper on the grid. "Drain time" is a surprisingly useful parameter that specifies an optional delay between blotting and plunging when the sample simply equilibrates in the blotting chamber ("drain time" is actually a misnomer, since no fluid is actually draining). In general, the blotting chamber is humidified to 100% to minimize potential imbalances in evaporation and condensation that might develop during the drain time, and the temperature is generally set to the preferred temperature of the sample (can be the incubation temperature of a cell culture, for instance).

Using the Vitrobot, we have successfully prepared a variety of samples for ECT including large protein complexes, viruses, organelles and narrow whole cells (Benjamin *et al.*, 2005; Briegel *et al.*, 2006; Henderson and Jensen, 2006; Iancu *et al.*, 2005; Iancu *et al.*, 2006; Komeili *et al.*, 2006; Murphy and Jensen, 2005; Murphy *et al.*, 2006; Wright *et al.*, 2006). The protocol and discussion presented here is the result of our laboratory members' collective experience with several different Vitrobots and in-house plunge freezing devices and will describe details of both the introduction of fiducial markers and the specific Vitrobot conditions used for different samples. (Additional information on the use and applications of the Vitrobot, including a video demonstration of its operation, can be found on the company website at www.vitrobot.com.)

MATERIALS

REAGENTS

- sample
- 10 nm (or 5 nm) colloidal gold (SPI Supplies, West Chester, PA, USA, www.2spi.com or Sigma, St Louis, MO, USA, www.sigma.com)
- liquid nitrogen
- ethane
- bovine serum albumin (BSA).

CAUTION: Liquid nitrogen and ethane are cryogens and should be handled with care and appropriate personal protective equipment (goggles, gloves, lab coat). Additionally, ethane gas is flammable and along with nitrogen poses an asphyxiation hazard and should only be used in a well-ventilated, spark-free environment.

CRITICAL: Small gold fiducials can be more precisely localized and obscure fewer specimen details, but they can be difficult to see at high tilt angles in thick samples. For objects larger than ~ 30 nm, use 10 nm colloidal gold, but for smaller specimens (for example, protein complexes under 1 MDa), 5 nm colloidal gold will likely be better. Some batches of colloidal gold are prone to clumping. In some cases, coating these particles with BSA helps prevent their aggregation (see the reagent setup section). In our experience this property neither improves nor degrades with shelf life, so if a good batch is found it can be used successfully for even years. Store colloidal gold solution at 4° C

in 1 ml aliquots, in 1.5 ml centrifuge tubes in order to minimize repeated contact with air and, implicitly, variations in temperature and possible contamination that may cause further aggregation.

EQUIPMENT

- lacey carbon film on 200 mesh copper grids (Ted Pella, Redding, CA, USA, www.tedpella.com) or Quantifoil holey carbon films (various sizes) on 200 mesh
- copper/rhodium grids (Quantifoil Micro Tools, Jena, Germany, www.quantifoil.com)
- plasma cleaner (Harrick, Model PDC-32G)
- filter paper (pre-punched from FEI Co or Whatman #1)
- grid boxes (usually custom-made)
- anticapillary tweezers (SPI Supplies, West Chester, PA, USA, www.2spi.com)
- vortexer
- FEI Vitrobot (FEI Company, Hillsboro, OR, USA, www.vitrobot.com)
- forceps or long tweezers
- cryo-transfer dewars

CAUTION: Any metallic tool such as a forceps or tweezer in contact with liquid nitrogen will be extremely cold and should be handled with care and appropriate personal protective equipment (gloves).

EQUIPMENT SETUP

Grids CRITICAL: Before starting, carefully choose which type of grid to use. In our experience, the carbon film of lacey grids is more sensitive to dose than that of Quantifoil grids. Therefore, if the intended total dose per tomogram exceeds ~ 60 electrons/Å^2, Quantifoil grids are a better choice. Furthermore, the selection of the type of Quantifoil film (hole size) depends on the type of sample and the targeted magnification (pixel size of the image). During data collection, successive tilts are correlated with each other to maintain tracking, and it is helpful to have contrast-rich features in the field of view. For small objects (10–100 nm) such as protein complexes or viruses that are imaged at 4–5 Å per pixel and have little contrast, Quantifoil holey film grids such as R 0.6/1 or R1.2/1.3 are most suitable because the holes are smaller and their edges guide the tracking. On the other hand, for cells that are around 2 μm in length, R 2/2 or R 2/1 grids are more appropriate because the entire cell can be housed within a hole. Although we have not tried this specifically, for much longer cells, we would predict that R 3.5/1 or S 7/2 grids would be even better.

In our experience, grids that are nominally identical but were manufactured on different days can behave quite differently with regards to dose (they may bubble earlier) or wetting (the ice distribution may be uneven). We think that grids bubble sooner if they are dirty and distribute ice more poorly as they age. Because of this we procure freshly manufactured grids. Our main grids supplier, Quantifoil, labels the batches of grids with the date on which the carbon film was applied. One way to clean the grids is to simply rinse them in methanol before glow-discharging. An easy way is to float them on a drop

of methanol on parafilm for 30 seconds, then pick them up with anti-capillary tweezers, remove most of the methanol by touching the grid edge to filter paper, and then let them finish drying by evaporation.

Because the cooled freezing apparatus becomes increasingly contaminated with ice during the procedure, we recommend that only two boxes of grids (8 grids total) should be frozen per session.

Vibrobot Turn on the tank of compressed air ensuring that the proper air pressure (at least 6 bar) is applied to the Vitrobot. Fill the humidifier with distilled water using the special 60 ml syringe. Mount the filter paper on the blotting pads, securing them with the clip rings (it is better to do this while the Vitrobot is off because the plunger is not in the way). Turn on the Vitrobot and set the desired parameters in the Console and Options screens: temperature, humidity (100%), type of application (manual or automatic), the blotting time, blotting offset, drain time, wait time, etc.

REAGENT SETUP

Sample Depending on the molecular weight of a purified specimen, the concentration of the sample should be somewhere between 0.3–8 mg/ml. In our hands, saturated bacterial cultures produce well-populated grids when the cells are concentrated about 20 times by very gentle centrifugation. Care must be taken, however, because centrifugation or turbulent ejection from a small-diameter syringe can shear off external structures such as pili or flagella and can distort cell shape. Likewise, extensive centrifugation may distort

the shapes of viruses or cause other structural damage. Whenever possible, use buffers low in salt and without glycerol or sucrose to increase contrast.

Bovine serum albumin (BSA)-treated colloidal gold Coating the gold fiducials with BSA can help prevent clumping and promote even distribution on the grid. To do this, make a stock solution of 5% (w/v) BSA solution in distilled water. Using a tabletop centrifuge, pellet a volume of 10 nm colloidal gold for ~ 10 minutes at 18,000X gravity (25 minutes for 5 nm colloidal gold). Carefully remove the supernatant with a micropipette. Resusupend the pellet of colloidal gold in the same volume of 1 % (w/v) BSA solution and incubate at 4 °C or room temperature for 30 minutes. Centrifuge the colloidal gold-BSA solution again for 10 minutes at 18,000X gravity to pellet the gold. Remove the supernatant and resuspend the gold pellet in a large (~ 500 μl) volume of distilled water (rinse step). Centrifuge one more time as before to pellet the gold and finally resuspend in deionized water at the desired concentration (typically 5X) for the treatment of grids or resuspend in some volume of the sample solution. Note that excess BSA will, of course, contaminate the sample. If it dries on the grid in abundance it can interfere with vitrification. Note also that even when BSA-treated, gold will precipitate in sample solutions if the solute concentration is too high. If the gold precipitates, this is immediately visible because the normally red color of the gold solution turns purple.

PROCEDURE

First phase: Preparation of gold, grids, and the sample. Timing: 30–60 min

1 Briefly vortex (~ 10 s) a tube of 10 nm colloidal gold solution. (Two concentrations of colloidal gold solution will be prepared: a lower concentration to be applied to the grids (2X-concentrated) and a higher concentration to be mixed with the sample (4 to 7X-concentrated). Expect to use 5 μl of each concentration of gold per grid.)

2 Pipette into one tube (tube "A," for grid treatment) 10 μl of unconcentrated gold per grid.

3 Pipette into a second tube (tube "B," for sample treatment) 30 μl of unconcentrated gold per grid.

4 Centrifuge the colloidal gold solutions using a tabletop microfuge at 18,000X gravity for 10 minutes to pellet the gold. If 5 nm colloidal gold solution is used, the centrifugation duration should be extended to 25 minutes.

5 Remove the supernatants, being careful to avoid losing gold.

6 Resuspend the pellet from tube A in 5 μl filtered deionized water per grid with a pipet.

7 Glow-discharge the grids in a plasma cleaner for between 30 seconds and 2 minutes, ensuring that the carbon film of the grids is exposed to the plasma.

8 Pick up the grids with anticapillary tweezers so that the carbon face is upward.

9 Vortex the tube of 2X-concentrated colloidal gold solution (tube A) at maximum speed for ~ 3 minutes and apply 5 μl of this solution onto the carbon side of the first 2–3 grids (part of the vortexing can be done while the grids are being glow-

discharged). If more than 3 grids are to be prepared, one may briefly vortex the 2X-concentrated gold solution after treating every 2–3 grids to decrease clumping.

10 Bake the grids in a 50 °C oven (drying will take less than 10 minutes) or let them air dry (drying may take 30 minutes or more).

11 Add an appropriate amount of sample to the gold pellet in tube B (see comments on ideal sample concentrations above). As soon as the grids have dried, be ready to proceed with the vitrification.

TROUBLESHOOTING

Second Phase: Cooling the Vitrobot cup. Timing: ~ 10 min

CAUTION: This step should be done in a fume hood or a well-ventilated area, as a large amount of nitrogen and some ethane is released. Liquid nitrogen, liquid ethane. and cryogen-cooled metallic tools (such as forceps or tweezers) are extremely cold: be sure to wear appropriate personal protective equipment (gloves, goggles, lab coat, and closed-toe shoes) and be careful when handling these reagents and tools.

12 Start cooling the Vitrobot cup while the grids are drying. Fill both the central ethane cup and outer nitrogen ring initially with liquid nitrogen to cool them down quickly. (The Vitrobot cup is cylindrical and consists of a central cup for liquid ethane into which the grid is plunged and a larger peripheral ring for liquid nitrogen. Between these chambers is a gap spanned by carefully designed thermal connectors that

conduct just enough heat that the ethane will first liquefy and then very slowly freeze.) Grid boxes may be placed in their holders in the outer ring at this time and cooled along with the Vitrobot cup. Keep replenishing the liquid nitrogen in the outer ring so that it is at least 75% full at all times.

13 When vigorous liquid nitrogen bubbling has ceased, begin liquefying ethane in the central cup. To do this, open the ethane tank slightly to produce just a modest flow rate directed towards the bottom or side of the cold cup wall. (If the flow rate is too low, the ethane will solidify. If the flow rate is too fast, it will not liquefy and will blow out of the chamber. Holding the tip of the ethane supply tube against the inner wall of the ethane cup facilitates condensation.)

14 Continue filling the ethane cup until it is full.

15 Wait until a thin layer of solid ethane coats the inner walls of the ethane cup to ensure that the liquid ethane is cool enough. If too much of the ethane becomes solid, melt it by blowing in more warm ethane gas.

16 When necessary, refill the ethane cup to the brim. To avoid ice contamination, make sure that the tip of the ethane supply tube is dried completely each time you introduce it into the ethane cup.

CRITICAL: The rate of heat transfer between the ethane cup and the outer nitrogen ring is important. If the heat transfer rate is slow, it takes a longer time to liquefy the ethane. If the heat transfer rate is too fast, the ethane may solidify too quickly. For the newest Vitrobot model (Mark III) there is a metal frame that should be placed over the ethane compartment during the liquefaction process. It helps to transfer heat and liquefy the ethane more quickly. After filling the ethane compartment with

liquid ethane, the metal frame should be removed to prevent the ethane from rapidly solidifying.

Third phase: Sample vitrification. Timing: 3–5 min

17 Pick up the edge of a grid with the Vitrobot tweezers (tweezers especially constructed for the Vitrobot so that the fixed end can be attached onto the Vitrobot plunger and the pincers can be closed or opened by changing the position of a black clamp ring) and secure their grip on the grid by sliding the black clamp ring into the first notch.

18 Gently tap the tweezers on a finger to ensure that the grid does not fall off.

19 Bring the plunger in the appropriate position and mount the tweezers onto the tip of the plunger. (The Vitrobot provides two ways to apply samples to the grid. In the "manual application" mode, the sample is applied to the grid by hand with a pipetman through a side port in the blotting chamber. In the "automatic application" mode, the sample is placed in a 1.5 mL tube and secured in a holder in the blotting chamber, and the Vitrobot dips the grid into the tube before blotting. The choice of manual or automatic sample application depends on the amount of sample available. The depth to which the grid is dipped in the sample tube can be set. To use the automatic application mode, at least 50 μl of the sample must be available, but with samples smaller than even 100 μl, the grid and tweezers are sometimes damaged by hitting the tube walls near the bottom.) If the manual application mode is to be used, be sure the carbon side of the grid faces the sideport.

20 Double-check the parameters in the Console and Option screens. Different blotting parameters are needed for different samples. Our most successful parameters are summarized in Table 1.

21 Enable the humidifier to reach 100 % humidity.

22 Wait until the blotting chamber reaches the desired temperature and humidity.

23 Refill the nitrogen and ethane chambers of the Vitrobot cup as necessary.

24 Activate the plunger to bring the grid in the blotting chamber and enable the holder housing the Vitrobot cup to move it right under the blotting chamber.

25 If the 'automatic application' mode has been selected, the grid will be automatically dipped in the sample tube, blotted, and plunged in the ethane. Skip to Phase Four, Placing the grid in the storage box. Otherwise proceed with the manual application as follows.

TROUBLESHOOTING

26 Mix the sample again with a pipet or briefly vortex it.

27 Draw 3–5 μl of the combined sample and colloidal gold into the pipet, insert it through the sideport of the blotting chamber and discharge the sample onto the grid.

28 Close the entry port and wait until 100% humidity is restored in the climate chamber, if opening the port reduced the humidity.

29 Press the button to initiate grid blotting and plunging.

TROUBLESHOOTING

Notes: When using the automatic application option, we found that protein orientations in the ice were more randomly distributed and, for the same concentration of protein, the particles were more numerous in a given area than in the case of manual application. The increased range of views is important for single particle analysis. If the sample is fairly dilute and for some reason cannot be further concentrated, one way we have been able to increase the number of objects per field of view is to use the sequential application option, which was not described earlier for simplicity. This allows for the sample to be applied and blotted several times before plunge freezing. We have used at most two sequential applications.

If no sample is seen on the grids, it may be preferentially adhering to the blot paper (see Troubleshooting table). In this case, the grid can be manually blotted from its edge (rather than "face on") with a small piece of blotting paper inserted through the side port with tweezers. This minimizes the contact area while maintaining the controlled environmental conditions.

The drain time may be adjusted to 1 s to increase the likelihood of thin ice and/or to get more uniform ice (instead of the typical gradient), in cases where the increment in the blotting time (0.5 s) is insufficient to define optimal sample preparation conditions. For example, in the case of *Caulobacter crescentus* cells, 2 seconds of blotting is too long, and 1.5 seconds without drain time is too short, so we use 1.5

seconds with a 1 second drain time. Additionally, using a drain time of 1 s or more may also avoid formation of non-vitreous ice with samples where this may be a problem.

There is an optional wait time between the application of the sample to the grid and blotting, during which time the sample solution may reach equilibrium with its environment. For some of our protein complexes, a 15–30 second wait time resulted in grids with more randomly distributed and numerous particles in a given area than 0 seconds waiting. In the case of some of our viral samples, a 15 second wait time increased the number of viral particles present. Presumably this is because some samples adhere to the grid in time and remain throughout the blotting process.

Fourth phase: Placing the grid in the storage box. Timing: 1–2 min

30 Slide the Vitrobot tweezers off the tip of the plunger while keeping the grid submerged within the liquid ethane.

31 Support the tweezers on the edge of the ethane cup, again keeping the grid completely immersed in liquid ethane, and stabilize your hand against the side of the Vitrobot cup.

32 Using both hands, move the cryogen cup from the Vitrobot to the bench so that there is more room to maneuver the grid and tweezers.

33 In one swift motion transfer the grid from the ethane to the liquid nitrogen ring. Alternatively, with the latest model of the Vitrobot (Mark III), the transfer of the tweezers from the ethane cup to the liquid nitrogen ring can be done automatically.

Before doing this, fill the nitrogen reservoir again after its descent and before activating the movement.

34 Slide the black clamp ring off the tweezers while keeping them pinched closed with your fingers.

35 Gently move the tweezers close to the grid box and place the grid in the desired slot.

TROUBLESHOOTING

Fifth phase: Iterations. Timing: 4–6 min per grid

36 Before proceeding with the next grid, if there is any condensation on the outside bottom part of the climate chamber resulting from the contact of the chamber with the cold Vitrobot cup, wipe it off with a paper towel and/or warm it with a heat gun.

37 Dry the Vitrobot tweezers with a Kim wipe.

38 Repeat steps 17–37 for each new grid.

Sixth phase: Sample storage. Timing: 2–3 min

39 When all the grids have been frozen and placed in the grid boxes, cool the grid box lids in liquid nitrogen and secure them on top of the grid boxes. (If the lids are fastened by screws, pre-cool the screwdriver before touching it to the grid boxes.)

40 Cool a pair of forceps or long tweezers in liquid nitrogen and use them to move the grid box into a liquid-nitrogen-filled transfer dewar.

41 Store the grid box under liquid nitrogen until ready for observation.

PAUSE POINT: If the grids will not be used immediately, the grid box can be stored essentially indefinitely in a 50 ml screw-cap conical tube. We typically punch two holes on opposite sides of the tube, approximately 1 cm below the bottom thread, and loop an ~ 4 ft long labeled nylon or polyester string through the holes. We then fill the 50 ml tube with liquid nitrogen, drop the grid storage boxes into it, and place the tube in a cane which is finally lowered into a large nitrogen cryostorage dewar with the labeled string hanging out the top.

TIMING

Steps 1–11 (Preparation of gold, grids, and the sample) take 30–60 minutes depending on which method is used to dry the gold solution onto the grids. Steps 12–16 (Cooling the Vitrobot cup) take about 10 minutes. Steps 17–29 (Sample vitrification) take 3–5 minutes. Steps 30–35 (Placing the grid in the storage box) take 1–2 minutes. Steps 36–38 (Iterations) take 4–6 minutes per grid. Finally, steps 39–41 (Sample storage) take 2–3 minutes.

ANTICIPATED RESULTS

This protocol typically produces plunge-frozen electron cryomicroscopy grids with suitably thick ice covering most of the grid (~ 70% or more) and a uniform gold distribution (see Fig. B-1 for an example). The preparation of 8 grids takes less than two hours and most of these grids are suitable for single- or dual-tilt tomography data collection.

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Figure and Tables

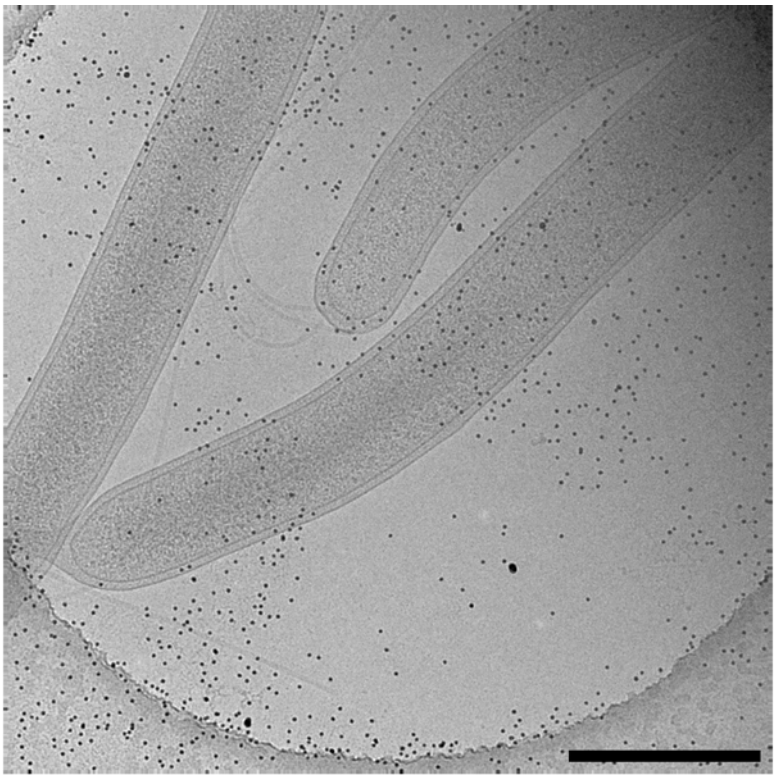

Figure B-1. Example image showing well-preserved bacterial cells, a good distribution of gold fiducials, and thin ice.

Energy filtered (20 eV slit) image collected on a 300 kV FEG "G2 Polara" TEM (FEI Company), using a 2048 x 2048 GIF CCD (Gatan, UK) and a pixel size of 0.98 nm. (Scale bar is 500 nm.)

Sample	Blot time (s)	Blot offset (mm)
Large protein complexes	3-4	2-3
Viruses	2-3 + drain time 0.5-1 s	2
Organelles	1-2	1-2
Cells	1-2 + drain time 1 s	1-2

Table B-1. Vitrobot blotting parameters for different samples.

"Blot time" is the period that the blot pads are pressed against the sample. "Blot offset" describes the position of the grid between the blot pads and partially controls the pressure exerted by the blotting paper on the grid. Larger absolute values of the blot offset produce a steeper ice thickness gradient on the grid. Blot offset values in the table are negative numbers. "Drain time" is the delay between blotting and plunging.)

Table 2. Troubleshooting.

Problem	*Possible reason*	*Solution*
Steps 1–11. Non-uniform dispersion of colloidal gold (associated with massive aggregation of gold particles)	The buffer is potassium phosphate or has high concentrations of salt or other additives promoting the aggregation of gold. If the gold solution changes color (like from pink or red to purple), this indicates it has precipitated.	Try to minimize salts and additives. Otherwise, apply gold only on the grids, avoid combining gold with the sample, or treat the gold with BSA.
Steps 12–35. Formation of non-vitreous ice on the grids	Humidity in the climate chamber may not have reached 100% in the blotting chamber.	Double-check that the humidity sensor is working properly.
	The transfer of the grid from the ethane cup to the liquid nitrogen ring was too slow.	Accelerate the transfer. If the Vitrobot Mark III model is available, use the automatic transfer option.
	The ethane may not have been cold enough.	Check that there is a thin film of solid ethane on the sides of the cup at all times and that the liquid nitrogen level is maintained throughout the freezing session.
Steps 25 or 29. Damaged grids (some of the carbon film gets stuck on the blotting paper)	The blotting pressure is too high.	Reconsider the blotting offset or ensure that the blotting pads function properly. The springs of the blotting pads may need to be adjusted or replaced.
	If, after the blotting, the wet spots on the blotting paper are significantly different in size for the two pads, the blotting mechanism is probably not centered correctly with respect to the plunger.	Recenter the blotting mechanism relative to the plunger.
Steps 25 or 29. No sample is visible on the grid	The sample is sticking to the filter paper.	Blot manually from the edge of the grid.