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Three-Dimensional Analysis of Single Particles by Electron Microscopy

Sample Preparation and Data Acquisition

Teresa Ruiz and Michael Radermacher

Summary

Electron microscopy of single particles has recently become a very popular field in both biological and material sciences. It might be difficult for a novice researcher new to this field to know how to start tackling a new project. This chapter is designed to serve as a guideline for anyone starting a new project to determine a three-dimensional structure using single-particle techniques. The chapter describes the basic techniques necessary to prepare the samples and acquire the data to calculate a three-dimensional reconstruction in easy-to-understand, step-by-step instructions. It starts with the basic preparation of support films and the usage of a variety of staining techniques needed to assess the quality of the sample and the viability of the project. It ends with a detailed description of vitreous ice preparations designed to acquire high-resolution structural information. Guidelines and tips are given on how to record the best images with an electron microscope. Although this chapter is geared to researchers new to the field, experts might find it not only useful as a reference but also valuable because of the number of practical tips included.

Key Words: Three-dimensional analysis; single particles; cryo-electron microscopy; sample preparation; data acquisition; data collection; deep staining; methyl amine tungstate; ammonium molybdate; uranyl acetate; vitreous ice; random conical; electron microscope alignment; improved coherence; point mode.

1. Introduction

Three-dimensional cryo-electron microscopy of single particles has matured into a well-established biophysical technique *(1)*. Outstanding are the reconstructions of the ribosome in different functional states *(2–7)*. Recently, the reconstruction of phosphofructokinase from *Saccharomyces cerevisiae* at better than 11 Å resolution *(8)* in combination with molecular replacement has led to an initial model to phase native X-ray data, in a situation where heavy-atom derivative data was not available. During the past 15 yr, X-ray structures of subdomains have been

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fitted into cryo-electron microscopy structures to either localize them in the whole macromolecule or understand conformational changes in different states *(9–12)*. At the resolutions currently achieved by cryo-electron microscopy of single particles, we would not be surprised if using electron microscopy data to provide starting phases for X-ray data when heavy-atom derivatives cannot be obtained would become a standard technique and not a singular event as for phosphofructokinase.

There is no general technique for preparing and analyzing single particles that can be applied to all samples. Each sample has its own characteristics and requires tailoring of the preparation and image processing techniques to accommodate them. It is true that our final goal is to determine the structure in its most native environment from vitrified samples *(13–15)*; however, mastering a variety of staining techniques is a necessity. Any new molecule should be first analyzed in negative-stain preparations, carefully selecting the stain that least affects the structure. Stained specimens are highly visible and the effort in data collection and image analysis is much less than for cryo-specimens. The analysis of stained samples allows one to optimize the biochemical purification and is of invaluable help to reach a first understanding of the molecular architecture and variability of the sample *(16)*. Moreover, the correct image analysis strategy can be more easily established in these preparations. For example, in the study of phosphofructokinase (an elongated molecule), the superiority of using Radon transform algorithms became evident *(17)*. In particular, the possibility to perform simultaneous rotational/translational alignments presented a great advantage in this study and it should benefit all studies of nonglobular molecules. Only after a thorough analysis using negative-stain techniques has been carried out can the quality of frozen hydrated samples be properly judged. Imaging a new specimen in ice without prior analysis in negative stain can lead to many weeks of lost work and frustration.

A very important fact to remember is that all electron microscopy preparations are radiation sensitive *(18–20)*. This is most visible when examining vitrified or glucose-embedded samples where bubbling of the media can easily be observed *(21,22)*. However, negatively stained specimens also suffer radiation damage. Stain redistribution and microcrystal formation occur, which prevents the imaging of high-resolution details. This loss of high resolution might often pass unnoticed. By burning samples needlessly you might miss important features of your specimen that otherwise would have been obvious. Therefore, minimal or low-dose settings should always be used in the microscope when collecting data *(22,23)*.

This chapter was designed for researchers with some basic knowledge of electron microscopy techniques. Depending on your background, some topics might seem either too basic or too advanced; we hope that, on average, you find them useful. We will first describe the preparation of support films best suited for single-particle imaging and a variety of different staining techniques. This will be followed by detailed procedures for the preparation of frozen-hydrated samples

on holey grids coated with a thin carbon layer. The settings to obtain high-resolution results using a microscope equipped with a LaB_6 cathode and the data collection procedures optimum for imaging single particles are described.

2. Materials

Necessary materials and small tools for general electron microscopy and negative stain preparations are as follows:

1. Precision tweezers (either with an O-ring for closure or with reversible action).
2. Grids (the material depends on the sample; for our samples, copper is acceptable).
3. Carbon-coated grids.
4. Holey grids.
5. Carbon-coated holey grids.
6. Negative-staining solutions or the powder to make the solutions.
7. Filter paper for blotting: Whatman no. 40 or no. 1 (medium) or Whatman no. 41 or no. 4 (fast).
8. Parafilm.
9. Pasteur pipet (glass, quartz, or plastic).
10. Automatic micropipettor.
11. Pipet tips.
12. Water.

Many of the materials (staining solutions, carbon-coated grids, holey grids) needed for sample preparation can be purchased from electron microscopy supply companies:

Ladd Research (www.laddresearch.com); Ernest F. Fullam (www.fullam.com); Structure Probe (www.2spi.com); Ted Pella (www.tedpella.com); Electron Microscopy Sciences (www.emsdiasum.com); Nanoprobes (www.nanoprobes.com); Quantifoil (www.quantifoil.com).

However, in most cases, and to have a tight and better control on the quality, the materials are prepared in the laboratory. The same applies to certain small tools not available commercially. We will recommend to the real beginners to purchase the materials off the shelf from any of the electron microscopy supply companies. Those with certain experience in electron microscopy and with a minimally equipped laboratory for electron microscopy might benefit from the following instructions for the in-house preparation of the materials and small tools described here.

2.1. Support Films

Many substrates have been assayed for their viability to be used as supports for electron microscopy samples *(24,25)*. A good substrate should be highly transparent to electrons, have minimal intrinsic structure, and be stable upon electron irradiation. For cryo-electron microscopy preparations researchers started favoring grids coated with perforated carbon films (holey grids)—the

optimum holes having diameters between 1.5 and 10 µm. Images were taken of the sample in the vitrified water layer spanning the holes (suspensions) where the background noise coming from the support film was zero. A further advantage was, as a general accepted rule, that particles adopted many different orientations in suspension, unlike particles supported on a thin carbon film, which lie in preferred orientations in response to their interaction with the support film. However, the effect of the air–water interface was neglected and, as it has been observed in several cases, also gives rise to preferred orientations. In our laboratory, we favor the use of holey grids coated with a thin carbon film (5–10 nm); the holey film providing a strong support for the thin carbon. Images are collected over the holes with a minimal background arising from the thin carbon. This approach presents two general advantages. First, once the optimum sample concentration has been found using staining techniques, the same concentration is good for vitreous ice preparations (usually below 100 µg/mL). Second, the later correction of the transfer function of the microscope will be more accurate using the thin carbon on which the sample lies rather than the thick carbon that makes the holes. Moreover, it facilitates the preparation of membrane protein samples, avoiding the meniscus problems encountered in suspensions attributable to the presence of detergent in the buffers and also the need for using very high concentrations (approx 10 mg/mL) to have a significant amount of sample in the bare holes.

2.1.1. Holey Grids

Several techniques have been used to produce perforated films ***(26)***. Grids with perforated films are available from many manufacturers, in particular Quantifoil, which is a perforated film with holes very well defined in size, shape, and arrangement ***(27)***. Here, we present a method that produces reliable holes independent of the ambient conditions (e.g., humidity) and was passed on to us by the group of J. Frank in Albany, NY.

2.1.1.1. Cleaning of the Slides

1. Select approx 20 microscope glass slides without scratches.
2. Rinse the slides thoroughly with distilled water. Many suppliers coat the slides with a thin film of detergent that must be removed.
3. Dry the slides with lint-free Kimwipes.
4. Coat each slide very lightly with nose grease or, if you prefer, spread some light facial cream on the top of your hand and use this instead (Yves Rocher's cream for mixed skin types works well; however, the common Nivea cream does not).
5. Remove the grease from the slides using lint-free Kimwipes. Wipe always in the same direction along the long axis of the slide. This helps the production of holes that are aligned along the long axis of the slide.
6. Put the treated slides in a Petri dish and cover them to avoid dust deposition.

2.1.1.2. Preparation of the Formvar Solution

1. Prepare 1.2 mL of 90% solution of glycerol (anhydrous of high purity) in double-distilled water (ddH_2O).
2. Put 45 mL of chloroform (99.0–99.5% purity) in a 100 mL bottle.
3. Weigh 0.2 g of Formvar (polyvinyl formal). First add some of the Formvar to the surface of the chloroform and dissolve it gently (mimic a centrifuge motion with your wrist; do not shake).
4. Keep adding the Formvar slowly until all of it is dissolved.
5. Add 45 mL of acetone (99.5% purity); the bottle will become slightly warm.
6. Add 0.92 mL of the 90% glycerol solution to the bottle.
7. Close the bottle tightly, cover it with aluminum foil, and shake energetically for 10 min.
8. Remove the foil, open the bottle, and sonicate with a sonic probe for 4 min without cooling. Dip the sonicator tip approx 1 cm below the liquid surface level. After sonication, the bottle will be lukewarm and the solution clear.
9. Close the bottle, cover it with the aluminum foil, and wait 15 min before preparing the Formvar films.

2.1.1.3. Preparation of Formvar Holey Films

1. Fill a 50-mL beaker with the Formvar solution and a 100-mL beaker with acetone.
2. Dip the slide into the 50-mL beaker; let it sit for 10 s.
3. Slowly withdraw the slide at an angle from the solution. The surface that makes an acute angle with the surface of the solution (the bottom surface) is the one that will be used.
4. Let the slide dry. You can lean it against a rack in an almost vertical position to dry.
5. Dip the slide in the beaker with acetone and stir it gently for 5 s. Keep the slide vertical. Let it dry as in **step 4** before (*see* **Note 1**).
6. Observe the slide with a phase-contrast microscope to asses the amount of holes obtained and their size. If the holes are too small, wait a few minutes before dipping a new slide in the Formvar solution (*see* **Notes 2–4**).
7. Prepare as many slides as you can before the Formvar solution is exhausted or the holes become too large (usually 1 h after sonication).

2.1.1.4. Preparation of Formvar Holey Grids

1. Fill a crystallizing dish to the rim with ddH_2O.
2. Cut the edges of the plastic film with a diamond knife so that it will strip easily off the glass slide.
3. Breath gently on the edges of the slide to facilitate striping and dip the slide at an angle into the crystallizer. The plastic film should float off on the surface of the water.
4. Place 300 or 400 mesh grids on top of the film, try to always put the same side facing the plastic.
5. Pick the film with a piece of parafilm supported by a glass slide. Lower the parafilm perpendicular to the water surface touching the plastic film, keep lowering it into the water until all the grids are attached to it, and then lift the parafilm out of the water at a shallow angle.

6. Trim slightly the parafilm and let the grids dry on a Petri dish.
7. Once the grids are dry, remove them from the parafilm with a pair of tweezers and put them on a glass slide for carbon coating. At this point, you can proceed to a preselection of your grids by looking at them in the phase-contrast microscope.
8. Coat the grids with a very thick layer of carbon (80–100 nm).

2.1.2. Holey Grids With Thin Carbon

1. Select some holey grids. Grids with 2- to 3-µm holes and very few broken squares are good.
2. Put holey grids, carbon side up, on a Petri dish with filter paper (Whatman #4 is good) and gently add approx 2.5 mL of chloroform at the periphery of the paper to wet it slowly (use the same solvent to remove the plastic as was used to prepare the plastic films).
3. Keep the Petri dish closed for 30 min or overnight if you prefer.
4. Open the Petri dish for at least 10 min to let the rest of the chloroform evaporate. If the bottom of the Petri dish is cold, the chloroform has not finished evaporating.
5. Coat the holey grids with a thin layer of carbon (5–10 nm) either using a Smith carbon coating trough (from Ladd Research Inc. *[28]*) or a homemade device based on the same principle.
6. Let them dry for about 30 min before using them.

2.2. Stains

Many articles and books have been written describing stains for electron microscopy *(24,25,29)*. In this chapter, we will focus on the most commonly used in our laboratory: uranyl acetate, methylamine tungstate, ammonium molybdate, and Nanovan (sold as a 2% solution by Nanoprobes *[30,31]*).

2.2.1. Uranyl Acetate

Uranyl acetate can be purchased from Ted Pella (ref. no.: 19481). This stain can be used at different concentrations (0.5–4%); however, one important thing to remember is that at lower concentrations, the grain is finer. A solution of approx 4% uranyl acetate is saturated, the exact value depends on the manufacturer. The pH of the staining solution should be around 4.0; attempts to raise the pH might result in the precipitation of uranyl acetate.

1. To prepare a 1% solution of uranyl acetate, put 200 mg of uranyl acetate in 20 mL of ddH_2O.
2. Vortex until all the stain is dissolved.
3. Filter the solution with a 0.22-µm filter.
4. Store the solution either covered with aluminum foil or in a dark container.

2.2.2. 2% Ammonium Molybdate

Ammonium molybdate can be purchased from Agar Scientific Ltd (ref. no.: R1156) or from Ted Pella (ref. no.: 19482).

1. To prepare 5 mL of stain, put 100 mg of ammonium molybdate in 5 mL of ddH_2O and vortex until it is dissolved.
2. Check the pH with pH paper; the starting value is acidic.
3. Adjust the pH to 7.0 with NH_4OH (250 µL or 300 µL of a 5% solution should be enough). Be careful because when pH 6.5 is reached, the pH seems to stabilize for a while, and adding a little more makes the pH jump to 7.5 or 8.0. Sometimes it is safer to stay at pH 6.5. Do not titrate back; if the pH gets to high, start again.
4. Filter the solution with a 0.22-µm filter, discarding the first few drops, and store the filtered solution in the refrigerator until use.
5. The staining solution is good for 1 wk if stored at 4ºC, but the best staining is achieved with stain prepared fresh.

2.2.3. 0.25% Methylamine Tungstate With 10 µg Bacitracin

Methylamine tungstate can be purchased from Agar Scientific Ltd. (ref. no.: R1219) or from Ted Pella (ref. no.: 18353). Bacitracin can be purchased from Sigma. A stock solution of bacitracin (1 mg/mL) in water is stable for 1–3 mo in the refrigerator.

1. To prepare 20 mL of stain, put 50 mg of methylamine tungstate in 20 mL of ddH_2O and vortex until it is dissolved (the solution might still be slightly cloudy).
2. Check the pH with pH paper; it should be around 5.5 or 6.0.
3. Adjust the pH to 7.0 with NH_4OH (5 µL or 6 µL of a 5% solution should be enough). Methylamine tungstate is not a good buffer; therefore, the pH will change very fast. Do not titrate back; if the pH gets too high, start again.
4. Filter the solution with a 0.45-µm filter, discarding the first few drops.
5. To the filtrate, add bacitracin from the stock solution to a final concentration of 10 µg/mL.
6. Cover the stain with aluminum foil and keep in the refrigerator until use. To achieve the best results, prepare the solution fresh the same day or the night before because the nice staining capabilities diminish the longer the solution is stored. Only in extreme cases can a 1-wk-old solution that has been stored at 4ºC in the dark should be used, with less than optimal results.

2.3. Vitreous Ice

Necessary materials and small equipment specific for vitreous ice preparations are as follows:

1. Safety glasses.
2. Liquid nitrogen.
3. Ethane gas.

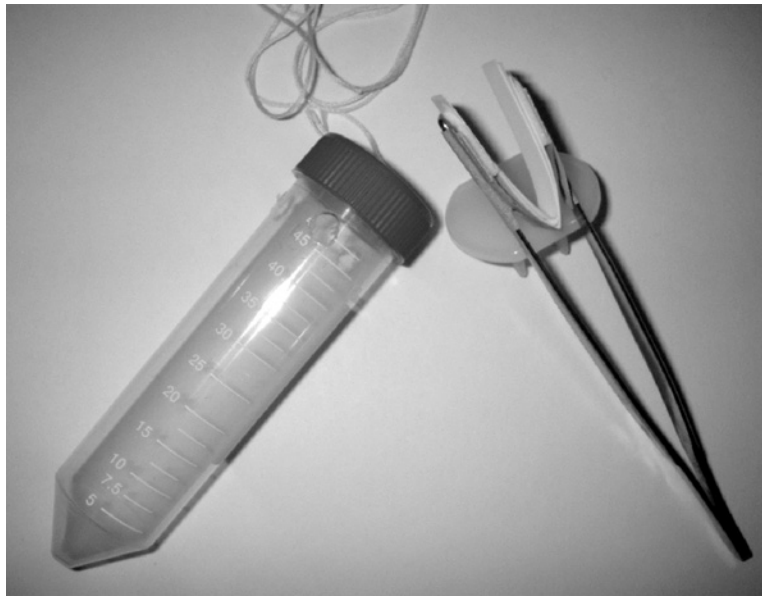

Fig. 1. **Left**: 50-mL Falcon tube modified to be used for long-term storage of cryo-samples; **right**: blotting tweezers for double-sided blotting.

4. Liquid nitrogen container with a small neck (4 L).
5. Small nitrogen container for storing the falcon tube with the frozen grids (1 L).
6. Plunger *(32)*.
7. Blotting tweezers to perform blotting from both sides of the grid (*see* **Fig. 1**); to make them, proceed as follows:
   a. Take a pair of tweezers with round tips (12/8).
   b. Take a thin piece of cardboard (1 cm wide and 6 cm long), bend it to a V-shape, and glue it with tape to the tweezers bridging the tips.
   c. Stick a piece of soft padding (1 cm wide and 1.5 cm long) to each side. Either a thin piece of styrofoam or a piece of mounting tape is good.
   d. Put a double-stick tape over the soft padding.
   e. Cut a piece of filter paper (1 cm wide and 5 cm long), bend it to a V-shape, and bridge the tips as in **step b**. This will prevent the blotting paper from slipping off the blotting tweezers.
   f. The blotting paper also will be bent to a V-shape and will be placed in the gap between the tips.
8. Two long tweezers with round tips (18/8 is a good size).
9. Short tweezers with round tips (10/8 is a good size).
10. 200-µL pipet tips with the nose slightly cut (to serve as the nozzle of the tubing used to pour the ethane).
11. Filter paper for blotting: Whatman no. 40 (medium) or Whatman no. 41 (fast).
12. Three to six precision tweezers for plunging (e.g., Dumont N5 biology).

13. Millipore tweezers for closing and transferring cryo-boxes (or tweezers and screwdriver).
14. Cryo-boxes to store the frozen grids.
15. A 30- or 50-mL Falcon tube for long-term storage of the grid-boxes in nitrogen with appropriate holes for airing and a string to retrieve them from the liquid nitrogen refrigerator (*see* **Fig. 1**).
16. Automatic micro-pipettor.
17. 10 µl pipette tips.
18. Holey carbon grids coated with a thin carbon film.

3. Methods

3.1. Staining Techniques

3.1.1. Uranyl Acetate Staining

This is the first step that one uses when analyzing a new sample. There are many different ways of staining with uranyl acetate, and many readers probably know one or two of them; therefore, we will not describe them in this chapter. However, we will take the time to describe here a method not often used in our field, which we have called the drop method. This method is especially useful when samples are in buffers containing either detergents or phosphate. Moreover, it is very successful for removing unwanted precipitates that form on the grid and that otherwise would not permit the correct visualization of the sample.

1. Coat the outer surface of a large Petri dish with parafilm (approx 15 cm in diameter).
2. Put 3 drops of stain (approx 150 µL each) on the parafilm.
3. Add 5 µL of sample to the grid and wait 30–60 s.
4. Place the grid on top of the first drop and move it gently over the surface. Be careful not to allow it to go inside of the stain drop.
5. Move the grid to the next drop and do the same as before.
6. Move the grid to the last drop and let it float on the surface for 30 s.
7. Pick up the grid, remove the excess liquid by touching the edge of the grid with a filter paper (wick), and dry it fast.
8. Remove stain drops with a pipet.
9. Set up new stain drops for the next grid in a clean area of the Parafilm.

3.1.2. Deep Staining

This procedure, first described by Stoops et al. for methylamine tungstate ***(33)***, is used in our laboratory with either methylamine tungstate, ammonium molybdate, or Nanovan. We have found that most soluble proteins stain well with methylamine tungstate, whereas membrane proteins stain better with ammonium molybdate. Nanovan is our favorite stain when looking at large complexes or vesicles coated with membrane proteins (*see* **Notes 5–10**).

1. Add 6 µL of sample to the grid.
2. Wait 30–60 s.

3. Wick the sample with filter paper (Whatman no. 4 or no. 41, fast papers). A small layer of liquid should be left on the surface of the grid. The grid should never be allowed to dry. Before wicking, you should have ready the pipet with the next drop of stain.
4. Add 6 µL of the staining solution.
5. Wick the stain.
6. Add 6 µL of the staining solution.
7. Wick the stain.
8. Add 6 µL of the staining solution.
9. Let it stand for a few seconds, wick the stain, and air-dry fast.

3.2. Vitreous Ice Preparations

We carry out all our vitreous ice preparations in the cold room to minimize evaporation. The evaporation rate is proportional to the surface/volume ratio of the drop; therefore, the most critical point in the preparation is the time between blotting the grid and vitrification of the sample in liquid ethane *(13,32,34)*.

1. Clean cryo-boxes with ethanol and dry them well with a lint-free Kimwipe. Make sure that the holes are not wet. Outline with a permanent marker the outside of the lid and also the slit of the screw (this will allow you to see them easily under liquid nitrogen). Check that all the boxes open/close easily because things get tighter at liquid nitrogen temperatures.
2. Cut Whatman no. 40 paper to size (1 cm wide and 5 cm long), fold into a V-shape, and store it in Petri dishes. For each grid that you plan to make, you need two pieces of paper (*see* **Note 11**).
3. Clean the tips of the plunging tweezers with ethanol. Make sure that all the tweezers hold the grids properly and check that they do not show capillarity. Normally, we add 5 µL of sample; if your sample buffer contains detergent, test with the buffer to determine the maximum amount that you should use to avoid capillary effects. This amount depends on the buffer; 3–4 µL might be fine.
4. Fill the 4-L container with liquid nitrogen.
5. To prepare ethane nozzles, cut off 1 mm of the 200-µL pipet tips and store them in a Petri dish. As the tips are sold, the hole is very small and it might take a long time to fill the ethane pot or, worse, it might clog, preventing the ethane from coming out.
6. Put the 30- to 50-mL Falcon tube inside of the small liquid nitrogen container and fill it with nitrogen.
7. Transport the ethane bottle to the cold room (*see* **Note 12**).
8. Transfer all of the necessary equipment to the cold room.
9. Fill the styrofoam plunger box with liquid nitrogen. Make sure that the level of nitrogen is high enough to liquefy the ethane gas. A few refills will be necessary to allow all of the large surfaces to cool down (*see* **Notes 13** and **14**).
10. Open the main valve of the ethane bottle.
11. Use a new, and therefore clean of water contamination, ethane tip. Every time that you need to pour ethane, take a new tip. In the cold room, the cold tips get a lot of condensation, and if you reuse them, this condensation will end up in your ethane pot and later on your grids.

12. Put on the safety glasses.
13. Filling the ethane pot for the first time. First, make sure that the level of nitrogen is high enough; if the level is low, the ethane gas will not liquify. Hold the tubing approx 10 cm from the tip to prevent getting your hands too cold or your fingers in contact with the liquid ethane. Position the tip inside of the ethane pot approximately at the center and about 0.5 cm from the top. Open the fine valve of the bottle to allow the ethane gas to flow. Increase the flow until steam forms at the tip. At this moment, lower the tip all the way to the bottom of the ethane pot, making sure that it is touching one of the walls. You can increase the flow of ethane a little more. Now you should see that the steaming has stopped and you will start hearing a sizzling noise. This means that the ethane is starting to liquify. If you look carefully inside of the pot, you should see how it is starting to fill up with liquid. As the pot fills, raise the tip along the pot wall with the liquid level, always keeping the tip about 1–2 mm below the liquid surface while filling the pot. Once the pot is filled to the rim, slightly decrease the gas flow, take the tip away from the pot, and close the fine valve completely. The main valve will remain open for the duration of the plunging (*see* **Notes 15–23**).
14. Take one of the cryo-boxes. Open it slightly so that the lid moves freely. Pick it up with the small tweezers and put it inside of the liquid nitrogen in the plunger box. With a pair of tweezers, move the lid of the box so that one of the holes is ready to accept a grid (*see* **Fig. 2**).
15. Set a pair of tweezers with a grid in the guillotine; tighten them well but not excessively.
16. Put a drop of sample (size depends on your test for capillarity [*see* **step 3**]) and wait for 20–30 s.
17. Place a fresh set of blotting paper in the blotting tweezers. Turn the guillotine to the position that best fits your blotting technique (*see* **Fig. 3**). Hold the blotting tweezers with one hand and the manual guillotine release with the other. Blot the grid for 4–5 s, quickly remove the blotting tweezers out of the way, and release the guillotine to plunge the grid into the ethane.
18. Loosen the tweezers from the guillotine, always keeping the grid inside of the ethane.
19. Transfer the grid from the ethane pot into the cryo-box. Once the grid is inside of the hole, move the lid over the grid and make another hole available for the next grid (*see* **Notes 24–28**).
20. Repeat **steps 15–19** for the other grids in this box.
21. Take the Millipore tweezers, the large tweezers, and the nitrogen container with the Falcon tube. Cool down the Millipore tweezers. Close the lid of the cryo-box using just one of the legs of the Millipore tweezers as a screwdriver. Keep the Millipore tweezers inside of the nitrogen. Take the Falcon tube out of the nitrogen container, holding it with the large tweezers along the wall and place it as close to the plunger box as possible. Take the cryo-box with the Millipore tweezers, holding it from the screw, and drop it very fast into the Falcon tube. Place the Falcon tube back into the nitrogen container and close the lid.
22. Refilling the ethane pot. If the ethane level has dropped low, you have to refill it. If the ethane is frozen, you have to reliquify it (*see* **Notes 15–23**). Put a new tip on

Fig. 2. Bottom part of the plunger device showing the disposition of the ethane pot and the cryo-box.

the tubing. Hold the tubing carefully, as before. Position the tip just above the ethane. Open the fine valve on the bottle to start the flow of ethane gas. Increase the flow until you see steam coming out from the tip. At this moment, lower the tip 1–2 mm below the liquid level, keeping it close to the cold wall. Again, once the pot is filled to the rim, slightly decrease the gas flow, take the tip away from the pot, and close the fine valve completely.

23. Use the same procedure (repeat **steps 14–21**) to plunge a new set of grids.
24. When you have finished all of the plunging, close the Falcon tube that contains all of the cryo-boxes and transfer it to a liquid nitrogen refrigerator (approx 35 L).
25. Close the main valve of the ethane bottle and take the bottle close to a fume hood. With the main valve closed, open the fine valve to release the pressure in the line. When the meter shows zero pressure, close the fine valve again and take the bottle to its storage place.
26. Clean all the plunging tools (dry the moisture with a lint-free Kimwipe).

3.3. Electron Microscope Alignment

3.3.1. Standard Alignment

Align the microscope following the manufacturer's instruction manual. The procedure described here should be taken as a guideline for the order in which the different optical elements should be aligned, as it is based on the alignment of the FEI CM microscope series with which we are most familiar.

Fig. 3. Double-blotting technique.

1. Insert a known specimen into the microscope.
2. If the voltage was changed to a new value, wait 30–60 min to allow the lenses to equilibrate.
3. Adjust the eucentricity of the grid at low and high magnification (×3000 to ×60,000).
4. In the mode where the image will be acquired, start fine-tuning the alignment for the specific conditions where the data will be collected (e.g., magnification, C_1 lens excitation, bias, C_2 aperture, and objective aperture). Remove the objective and selected area or diffraction apertures.
5. Correct first gun tilt and gun shift (*see* **Note 29**).

6. Center the condenser aperture. Focus the beam to a small spot. Center the spot using the beam deflection coils. Spread the beam and center it using the condenser aperture adjustments. Repeat a few times until the beam is well centered.
7. Correct condenser astigmatism.
8. Check again the eucentricity of the grid and focus the specimen.
9. Align the beam deflection coils, the objective lens, and the image deflection coils according to the manufacturer's instructions. For the CM microscope series, align the beamcoils pivot points first, then the rotation center alignment. Loop around these two alignments until they are both correct. Finally, align the image shift (*see* **Notes 30** and **31**).
10. Center the objective aperture. Insert the objective aperture, focus the specimen, and focus the beam to a small spot. Go to diffraction mode and center the aperture. Focus the aperture (make it sharp) with the imaging lens system. Make sure that under these conditions that the aperture is larger than the diffraction spot. If the aperture is smaller than the diffraction spot, you will get distortions in the negatives. To solve this problem, either select a larger objective aperture or change the C_1 lens excitation to get a smaller spot. Go back to the imaging mode.
11. Go to the center of the grid square and correct the objective astigmatism.
12. Align properly all of the modes of your minimal-dose or low-dose kit (search, focus, and exposure).
13. Select an area of the grid without carbon to set up the illumination. Go to the exposure mode. At a magnification of approx ×60,000, (good for data collection of single particles), spread the beam to cover at least the large viewing screen. Adjust the illumination to obtain a meter reading of 1.5–1.7 s. When the sample is in the beam path, the meter reading will show increased exposure times. Set the manual plate exposure mode to 1 s.

3.3.2. Improved Illumination Conditions for High Coherence

The beam coherence can be improved by adjusting the C_2 lens settings and the gun settings and by finding the optimal combination of C_1 lens excitation and C_2 aperture ***(8)***.

3.3.2.1. C_2 Lens Settings

The C_2 lens should be over focused and the illumination should cover at least the whole area of the large viewing screen. A beam focused to a small spot on the screen worsens the effects of spherical aberration because of the large aperture angle. A focused beam is almost incoherent because of the large difference in the electron path.

3.3.2.2. Gun Settings

1. Mechanical settings of the gun.
   a. Use a LaB_6 cathode.
   b. Use the smallest Wehnelt aperture available (in our case, for the CM120 FEI microscope, it was 0.3 mm).

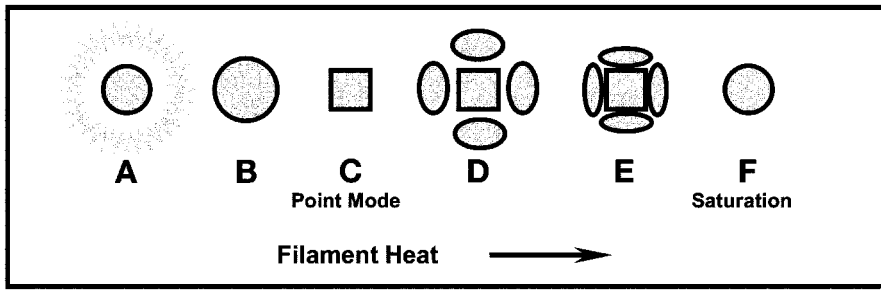

Fig. 4. Drawing of the different stages of heating of a LaB_6 cathode.

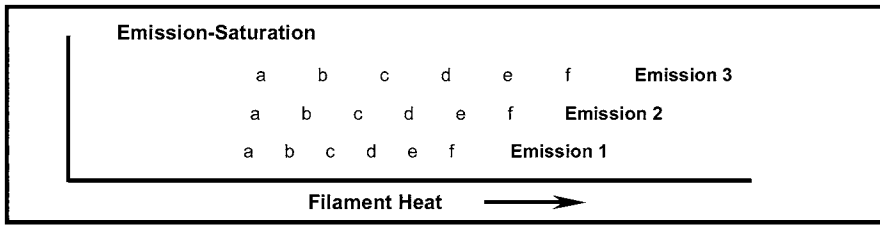

Fig. 5. Relationship between the different stages of heating of a LaB_6 cathode and the filament emission.

c. Use a small distance between the tip and the Wehnelt aperture (in our case, for the CM120 FEI microscope, it was 0.12–0.13 mm).

2. Electronic settings of the gun.
   a. In **Fig. 4** are represented the different stages through which a LaB_6 cathode passes while it is heated. Most laboratories collect their images with the filament saturated (stage f). We use the filament slightly unsaturated in the stage c or point mode. This provides the highest spatial coherence.
   b. The Wehnelt is set at a voltage slightly more negative than the cathode, therefore preventing the free flow of electrons toward the anode. The voltage difference between the cathode and the Wehnelt can be set at different values via the bias or emission controls. As the voltage difference is decreased, more electrons pass through the Wehnelt aperture and the emission increases. Another effect of reducing the bias or increasing the emission is that, as is shown in **Fig. 5**, the filament saturation is achieved at higher currents or higher filament heat values.

3.3.2.3. Optimal Combination of C_1 Lens Excitation and C_2 Aperture

1. The beam coherence is adjusted by the spot size (C_1) and condenser aperture (C_2).
2. Increasing the spot size increases the coherence.
3. Decreasing the size of the C_2 aperture increases the coherence.
4. As a guide, in **Table 1** we present some of the combinations tested for coherence in a CM120 FEI microscope equipped with a LaB_6 cathode (after spreading the

Table 1
Different Combinations of C_1 Lens Excitation and C_2 Aperture Tested for Coherence

	Spot size			
	3	4	5	6
	(500 nm)	(300 nm)	(200 nm)	(150 nm)
C_2 aperture	100 µm	100 µm	200 µm	200 µm

beam as described earlier, and still being able to collect low-dose images in 1 s). The best conditions were found for spot size 4 (300 nm) and 100-µm C_2 aperture.

3.3.2.4. Simple Protocol to Obtain Point Mode at 100 kV

1. Preparatory settings at 120 kV where the filament is saturated. We have not determined the optimum values for point mode at this voltage because we collect our data at 100 kV.
   a. Settings: Emission 1; C_2 aperture 100 µm; spot size 4.
   b. Saturate the filament (standard procedure).
   c. At ×60,000, expand the illumination to cover the large screen; check that the meter reading is 1 s or less.
   d. If not adjust, it to 1 s by increasing the emission (decreasing the bias).
   e. If necessary, increase the filament limit by one click.
   f. This determines the final filament limit, which should not be exceeded.
2. Final settings at 100 kV for point mode operation.
   a. Wait at least 30 min for the lenses to equilibrate at 100 kV.
   b. Start with the final conditions obtained previously for 120 kV.
   c. Decrease filament heat to obtain the point mode.
   d. At ×60,000, expand the illumination to cover the large screen; check that the exposure time is 1 s or less.
   e. If not, increase the emission setting by 1.
   f. To obtain the point mode again, it might be necessary to increase the heat (never exceed the limit!).

3.4. Data Collection

This section has been divided into two parts. The first one describes the basic collection procedure for untilted images and the second one describes the collection of Random Conical datasets (*see* **Notes 32–38**).

3.4.1. Untilted Images or 0° Images

1. Align the microscope using the normal holder and a negative-stain grid. When finished, reset the stage to the zero position and remove the holder.

2. Switch the filament down all the way. Cover the lead glass window if you are doing cryo to prevent liquid nitrogen from falling on the glass window.
3. Transfer either the negative-stained grid or the frozen grid to the holder and insert the holder in the microscope.
4. Wait for the microscope to recover a good vacuum Ion getter pump ([IGP]=6, on FEI microscopes). If you are using a room-temperature holder, wait a few minutes for it to stabilize. If you are using a cryo-holder, wait 30–45 min for the temperature of the holder to stabilize. Make sure that the shutter in the cryo-holder is closed and that the beam is blanked. During this time, you can monitor the temperature of the holder with the Gatan temperature controller unit. When the cryo-holder has reached its minimum temperature, remove the plug of the temperature controller unit.
5. Switch on the filament. Remove the cover from the lead glass window. Release the beam blank, and for the cryo-holder open, also its shutter.
6. Go to search mode and find a visible area on the grid. Adjust the eucentric height at ×3000. Go back to the exposure mode and make a finer adjustment of the height at higher magnifications. This step is very important if you want to collect a Random Conical dataset or a tomography dataset.
7. Go back to the search mode, remove the objective aperture, and select a low magnification (×100 to ×200). Move around the grid to select areas with either the right thickness of ice or a good stain distribution.
8. Select all usable areas. Blank the beam. Insert the objective aperture. Go back to your normal search magnification.
9. Move to one of the selected grid squares. Because grids are not completely flat, go to a neighbor grid square that is not good (if you have many good squares in an area, just give one up), fine adjust the eucentric height, and make sure that the minimal-dose kit is still well aligned.
10. Go back to the good square and select a good area.
11. Focus the specimen and reset the defocus to zero. At a magnification of ×100,000 and dialing a small defocus, you can check if there is drift. Stare at the grain and make sure that the holder is stable.
12. Blank the beam and lift the screens.
13. Dial the desired defocus value. Because there is less contrast, frozen hydrated specimens will require larger defoci than negatively stained ones. The necessary defocus value depends on the voltage and the size of the specimen. At higher voltages, larger defoci will be necessary. The smaller the particle, the larger the necessary defocus. The first time that you look at your sample in a frozen hydrated state, it is recommended that you take pictures at different defoci to know what would be a good range later.
14. Acquire an image.
15. After recording the image, go to the exposure mode, put down both screens, depress the beam blank, and look at the meter to get a more quantitative idea of the thickness of the sample. If you have adjusted the intensity of the light to be 1.72 s in an area without carbon; good areas (for 0° images) should show meter readings of about 2.16–2.52 s.

16. Keep taking as many images as you can.
17. When you have finished your data collection, remove the holder (before removing the holder, empty the prevacuum buffer tank to avoid having the vacuum collapse while you remove the holder).
18. Place the holder in the transfer station and check if your grid was up or down in the holder.
19. If this was the last frozen hydrated grid, empty the nitrogen of the cryo-holder and warm it up by gently blowing nitrogen gas in the Dewar and also on the tip. If you remove the clip-ring to take your grid out, put it back in its place. It is cleaner than having it in the clip-ring tool.

3.4.2. Tilt-pairs for Random Conical Reconstructions

1. Do **steps 1–9** of **Subheading 3.4.1.** When selecting areas for tilt-pairs at low magnification, remember that close to the center of the grid you will be able to achieve higher tilts.
2. Go to the maximum possible tilt; 55°–60° would be a good value. Be careful when tilting.
   a. When you see the shadow of either the holder or the grid bar coming into the field of view, stop tilting and reduce the tilt angle by approx 5° from this value, otherwise you will get a pseudoastigmatism in your image.
   b. Tilt slowly, at least for the last 15° to keep the specimen drift to a minimum.
   c. Always approach the final tilt angle from the same direction. If you overshoot, go back several degrees and try again.
3. On the tilted specimen, select an area for imaging using the search mode.
4. Focus in the focus mode. If two positions are available for focusing, use them both.
   a. Make sure that all the focus positions are on the tilt axis. If not, there will be a difference between the focus and the exposure mode, even if the grid is 100% flat.
   b. On the FEI CM series microscopes, the focusing positions are defined by a radius and an angle. The angle of the two positions is rarely at 0° and 180°. Determine the correct angle for your microscope beforehand (see the microscope manual). Typical values for the CM series of microscopes are 145° and 325°.
5. Observe that there is no drift, dial the desired defocus, and record an image.
6. Go to 0° tilt.
7. In the search mode, select the same area as before. This has to be done precisely; the best is to center a small feature on a mark on the small viewing screen. If it is not possible to achieve a good eucentricity of the stage, you can observe the specimen in focus mode during tilting; tilt slowly and correct the position as you tilt. Be very careful to keep the position so that you do not accidentally expose the imaging area.
8. Focus specimen as in **step 4**.
9. Observe that there is no drift; dial the desired defocus and record an image.
10. If you have a slow-scan charge-coupled device (CCD) camera, record a second image with the camera. Observe that the area you photographed was good. On a TVIPS CCD camera, you should be able to observe not only the ice/stain

thickness but also the preservation of fine details in your particles. If you do not have a CCD camera, go to exposure mode and check the area you photographed. Also check that all imaging conditions were good. (illumination, defocus, etc.)

11. Repeat **steps 2–10** to collect more data within the same grid square. Make sure to avoid exposed areas.
12. Proceed to the next preselected grid square. You should check the eucentricity when you move far away. Repeat **steps 2–10** for each image. Continue until either all good areas are exhausted or the film in the camera is finished.

4. Notes

1. Do not keep slides immersed in acetone for long times because the plastic film will dissolve.
2. The quantity and size of the holes depends on the amount of glycerol added to the solution. It is a parameter that you can vary; at a higher concentration of glycerol, the holes are larger and more numerous.
3. The Formvar solution should be used after sonication; if the solution is left standing for a long time, it is better to make a new one.
4. The films cast on the glass slides should be transferred to the grids as soon as possible. The longer they sit around, the more difficult it is to remove them from the slide.
5. If the surface of the grid is allowed to dry when using methylamine tungstate, the chances of having deep stain are minimal. With ammonium molybdate, it is not as critical.
6. Deep-stained areas seem to have a preferential direction. This might be avoided by using a filter paper with a cutout of the shape of the grid. Cut triangles of paper and then using a hole-punch, cut the tip of the triangle. You cannot get the whole perimeter of the grid but at least one-half or two-thirds of it.
7. Avoid getting the solutions up the tips of the tweezers as a result of capillary action. This will produce undesirable staining artifacts.
8. Grids prepared with methylamine tungstate are only good for about 1 wk and grids prepared with ammonium molybdate are good for about 2 wk. Images taken after these periods will be of lesser quality.
9. The purpose of the technique is to obtain areas on the grid that show deep stain. These areas can be described as rivers or lakes of stain that extend many grid squares. Areas surrounding them are usually dry. Another useful tip for recognizing these good areas is by looking at the change that occurs upon irradiation. Before electron irradiation, these regions look very smooth; after irradiation (the dose to collect and image is enough), the particles are visible as white dots at the low magnifications used in search mode of the minimal-dose kit.
10. All of the images should be taken under low-dose conditions. These stains are very radiation sensitive.
11. Do not make the papers too wide or blotting will be a problem; look at the blotting tweezers to get the right size. Make some spares just in case.
12. Always transport the gas bottles using a bottle trolley. Tighten the bottle properly with ropes or belts to the trolley. When you tip the trolley to move the bottle, put your hand over the bottle for safety.

13. Do not pour nitrogen in the empty ethane pot or it will take a long time to evaporate. Do not pour nitrogen over ethane.
14. Always check that the plunger is well adjusted. Put a pair of tweezers with a grid in the guillotine. Plunge it gently by hand and make sure that the tip of the tweezers is 3–5 mm below the rim of the ethane pot and that it falls in the center.
15. Ethane is a flammable gas. Do not use close to open flames or sparks. Do not use close to electrical equipment during on/off operation unless they are explosion-proof.
16. Ethane is used for vitrifying specimens because it freezes them faster that liquid nitrogen. If for some reason you get in contact with liquid ethane, you will have a cold burn. Stop what you are doing safely and as fast as possible and pour cold water over the burn for 5–10 min. Afterward, treat it as a high-degree burn. If aloe is available, put some over the burned skin.
17. If there is no steam coming out of the tip when you start filling up the ethane pot, either the tip is clogged, the main valve of the bottle is closed, or the bottle is empty.
18. Frozen ethane: If the level of nitrogen in the plunger box is too high, the ethane will freeze first at the walls and at the bottom of the pot. Frozen ethane occupies a larger volume than liquid ethane and, as a result, you will observe a bulging of the ethane surface. To melt the frozen ethane, you can add some fresh ethane gas. Put a new tip on the tubing. Place the tip just above the ethane surface, open the fine valve, and when the steam starts coming out, dip the tip inside of the ethane pot. Open the fine valve a little more and direct the tip toward the frozen areas. As you do this, the ethane will melt. Stir gently the ethane with the tip, making sure that no frozen ethane remains at the bottom of the pot. When all of the ethane is liquid again, raise gently the tip toward the edge of the pot, lower the gas flow a little, remove the tip from the pot, and close the fine valve completely.
19. If you remove the tip from the ethane pot still having a large flow of gas, you will get a lot of steam on the plunger box.
20. If you close the fine valve before removing the tip from the ethane pot, some ethane will flow back into the tip and into the tubing. Try to avoid it.
21. If the ethane gas flow is too strong when you are either refilling the pot or melting the frozen ethane, some of the liquid ethane will splash out of the pot. Try to avoid having a strong flow of gas.
22. If the level of liquid ethane stopped rising and there is some steam coming out, the pot is not cold enough. Stop the flow of gas and add more liquid nitrogen to the plunger box. Be careful not to put the nitrogen inside of the ethane pot.
23. If the level of liquid ethane stops rising and there is no steam coming out, either the tip is clogged or the bottle is empty.
24. If you transfer the grid very fast, you will observe some white scum on it; do not worry because this is just frozen ethane and it will disappear after a few hours of exposure to liquid nitrogen. However, if you transfer the grid very slowly, then you risk getting it too warm and you might have initiated the transformation of amorphous ice into cubic ice. As always, a speed in between is the best.
25. If putting the grid inside of the cryo-box is taking too long, be careful because cold metal surfaces will burn your fingers. The tweezers will be getting colder, so

search for a new pair of clean tweezers, cool the tips down in the deepest part of the cryo-box pot, and try again.

26. Always cool down the tools in liquid nitrogen before you touch the frozen grids.
27. If a cold tool has been taken out of the liquid nitrogen, do not reuse it until it is warmed up and you have dried it with a Kimwipe.
28. Air currents are the main source of contamination on your grids. Try to avoid them. Your movements close to the liquid nitrogen should not be too rough, to minimize air currents.
29. If the gun is not well aligned, you will have a reduced illumination and also a reduced coherence in your beam.
30. If the beam deflection coils or the objective lens are not well aligned, either the beam, the image, or both will show displacements when focusing.
31. In the FEI CM microscope series, if the beam moves while focusing, you have to realign the beamcoils pivot points. However, if the image moves while focusing, then it is the rotation center alignment that requires adjustments.
32. Lots of drift: Check the nitrogen level in both the anticontaminator and the cryo-holder. The nitrogen inside the cryo-holder should not boil. Refill if necessary. If the nitrogen level was acceptable, move the holder slowly to another area and check the drift again. If it is still the same, it is best to remove the grid and put in a new one. Either the grid does not make a good contact with the holder, it is not sitting properly in its place, or it is too bent. In the end, you will save time if you put in a new grid.
33. For both 0° and tilted images, if there is a large difference in focus between the two focal positions of the minimal-dose kit, the grid might be warped. Another possibility in the case of tilted images is that the focal positions are not positioned on the tilt axis.
34. If there is contamination during transfer of the grid to the holder and the transfer was smooth, check whether there are air currents where you are doing your transfers.
35. Be careful when you collect tilt-pairs. Grid bars have a given thickness. If you look at your grid in search, you will observe that as you tilt from 0° to a high tilt, certain areas of the grid square start becoming visible while on the opposite side some areas become obscured by the grid bar. Select areas that are visible on both.
36. When specimens are tilted, you will observe that the value shown on the exposure meter reading increases. This is caused by the larger specimen thickness that the electrons have to penetrate (easy to calculate with simple trigonometric formulas). Do not increase the beam intensity to compensate for this effect or you will be irradiating your sample with a higher electron dose.
37. Images of tilted specimens can have defocus gradients in the micrometer range (1–3 μm). Select the defocus that ensures that the whole image is underfocused and where the least defocus area is defocused enough so that your particles are still visible.
38. Always record in which orientation the grid was placed on the holder to know which side of the grid was facing the electron beam. Please note that some standard or room-temperature holders turn the grid upside down when inserted in the microscope. This is important to be certain of the handedness of the structure and to make sure that enantiomorphic structures are not mixed in the reconstruction (*see* Chapter 20).

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