Protocol

Immersion Freezing of Suspended Particles and Cells for Cryo-Electron Microscopy

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INTRODUCTION

Immersion freezing of thin aqueous specimens is an essential preparation technique for cryo-transmission electron microscopy (cryo-TEM), aiming to preserve fragile biological structures such as molecules and cells in their hydrated environment for a close-to-native visualization. For successful experiments, vitreous ice must be produced, surface contamination must be avoided, and, most important, the natural state of the structure must be preserved. This protocol describes immersion freezing of biological samples, such as purified protein complexes, viruses, liposomes, synthetic cytoskeletal filaments, isolated organelles, or small cells suspended in an aqueous solution, using the new Leica “EM GP” grid plunger. It includes a discussion of issues of general importance for cryo-EM, such as the properties of the sample and the pretreatment of the specimen carrier. It also provides details on how to make the most of the special features of this instrument to obtain good specimens and reproducible results. Troubleshooting issues concerning the operation of the GP in particular, as well as common problems in immersion freezing encountered on manual and semiautomatic instruments, are addressed.

RELATED INFORMATION

For a brief introduction to cryo-EM, immersion freezing, and the instrumentation available for this purpose, see Immersion Freezing of Biological Specimens: Rationale, Principles, and Instrumentation (Resch et al. 2011a). A second protocol is available for Immersion Freezing of Cell Monolayers for Cryo-Electron Tomography (Resch et al. 2011b). Parts common to both protocols were duplicated so that each protocol would be self-contained. For other lab protocols describing immersion freezing of suspended specimens on a homemade instrument or on the Vitrobot, see Grassucci et al. (2007), Iancu et al. (2007), and Frederik et al. (2009).

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Ethane gas, 99.995% (4.5; e.g., Air Liquide)
Liquid nitrogen (LN₂), 99.95% (3.5; e.g., Air Liquide)
Specimen at a concentration of ~1 mg/mL (this is a rough guideline that will vary from one specimen to another; between one and 10 times what is used for negative staining), suspended in an aqueous solution of low ionic strength with near-native buffer conditions
Freezing on perforated carbon substrates usually requires a significantly higher concentration of specimen as compared to continuous carbon. Compounds such as glycerol that increase the viscosity of the solution should be avoided or kept at a low concentration, as they make blotting more difficult and contribute to background noise (Grassucci et al. 2007). If the sample (e.g., nonadherent cells) will be visualized with cryo-electron tomography, supplement it with gold fiducials (see Immersion Freezing of Cell Monolayers for Cryo-Electron Tomography [Resch et al. 2011b]).

**Equipment**

Cryo-grid boxes with lid, either 15-×15-mm square (e.g., Ted Pella 160-44) or 14-mm diameter round (e.g., Leica Microsystems 16706039; Ted Pella 160-40)

*Each of these boxes can accommodate four grids.*

EM grids with mesh size and support film suitable for your experiment

*See Table 1 for examples and Dobro et al. (2010) for an in-depth discussion of the best choice of grids. Both continuous as well as perforated (holey) carbon films can be made with a high vacuum evaporator (Grassucci et al. 2007) or purchased from EM suppliers (e.g., Quantifoil Micro Tools GmbH, jena, Germany, www.quantifoil.com).*

Glow discharge unit, either:

- Bal-Tec SCD005 sputter coater (now Leica Microsystems, Vienna, Austria) with the Au target removed
  *This provides more gentle discharge; it is used for thin continuous carbon.*

- Instrument built according to Aebi and Pollard (1987)
  *This is stronger and renders the films more hydrophilic, but should only be used for very stable films such as Quantifoil.*

Hair dryer (e.g., Steinel HG 2000 from VWR International) or Leica EM CTD drying platform (available from mid-2011 on)

Leica EM GP main unit (Leica Microsystems, Vienna, Austria; see Immersion Freezing of Biological Specimens: Rationale, Principles, and Instrumentation [Resch et al. 2011a]) set up in a well-ventilated area with low environmental humidity and good lighting

*The instrument can be upgraded with a number of options:*

- The foot switch (16654925) makes the workflow more comfortable; it takes the user to the next step in the freezing cycle.

- A binocular (“viewing system;” 16706433) mounted in front of the environmental chamber can be used to closely monitor the filter paper alignment and the blotting process. In practice, however, it is not being used much.

- The forceps-adjusting tool for the quick lock forceps (16706442) and the filter paper punch (16706443) come in very handy when using different types of forceps or filter paper (and can help to save money on consumables).

Leica EM GP accessories:

- GP quick-release forceps (set of two, well aligned to each other; one piece: Leica Microsystems 16706435)

- Special forceps with insulation coating (set of two; Leica Microsystems 16701955)

- Cryo-transfer container (Leica Microsystems 16706439)

- Cryo-tool with M4 thread (Leica Microsystems 16701958)

- Secondary cryogen liquefier (Leica Microsystems 16706438)

- Filter paper for blotting; either Whatman No. 1 filter paper with precut hole (Leica Microsystems 16706440) or filter paper of any type with an outer diameter of 55 mm and a central 15-mm hole, made with a filter paper punch (see above)

Light microscope slides (e.g., VWR International 631-9461)

LN₂ dewar vessels, 1-L (e.g., VWR International)

LN₂ specimen storage system

Pipette tip (200-µL) or hollow needle (if Leica liquefier is not used; see Step 20)

Pressure regulator for ethane gas, 50-mbar (GDK)

Screwdrivers (or Allen key) to open/close cryo-grid boxes

Syringe

Tubing for ethane gas

- Silicone tubing, 3-mm ID, 1-mm wall for the Leica liquefier

- Tygon R-3603 tubing, 5-mm ID, 1.5-mm wall from Saint-Gobain/Performance Plastics for a needle or pipette tip

Tweezers, long (300-mm; e.g., VWR International)
Table 1. Summary of freezing conditions for specimens

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<tbody>
<tr>
<td>Startup protocol</td>
<td>1.4 mg/mL</td>
<td>100 mM borate, pH 8.3</td>
<td>Cu 400 mesh, QF R1.2/1.3</td>
<td>Glow discharge</td>
<td>90%</td>
<td>4 µL on C side</td>
<td>30 sec</td>
<td>0.7–2.2 sec from film side</td>
<td>0 sec</td>
<td>Hasanovic et al. (2011)</td>
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<td>Rhinovirus, human</td>
<td>2.5 mg/mL</td>
<td>Unbuffered</td>
<td>Cu 400 mesh, QF R3.5/1</td>
<td>Glow discharge</td>
<td>50°C</td>
<td>90%</td>
<td>4 µL on C side</td>
<td>30 sec</td>
<td>0.5–1.0 sec from film side</td>
<td>0 sec</td>
<td>Wagner et al. (2010)</td>
</tr>
<tr>
<td>Type III secretion system</td>
<td>1.0 mg/mL</td>
<td>500 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.1% LDAO, pH 8.0</td>
<td>Cu 400 mesh, continuous carbon</td>
<td>Glow discharge</td>
<td>25°C</td>
<td>50%</td>
<td>5 µL on C side</td>
<td>30 – 120 sec</td>
<td>0.8–1.0 sec from back side</td>
<td>0 sec</td>
<td>Wagner et al. (2010)</td>
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<tr>
<td>Microtubules</td>
<td>10 µM</td>
<td>1 mM EGTA, 1 mM MgCl₂, 80 mM Pipes-KOH, pH 6.8</td>
<td>Cu 400 mesh, QF R1.2/1.3</td>
<td>Glow discharge</td>
<td>30°C</td>
<td>75%</td>
<td>4 µL on C side</td>
<td>30 sec</td>
<td>1.0–1.5 sec from film side</td>
<td>0 sec</td>
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All specimens were blotted once with Whatman No. 1 filter paper and plunged into liquid ethane cooled to just above the freezing point. For new specimens, perform initial experiments using blotting time and/or concentration series according to “Startup protocol” to identify optimal freezing conditions and the optimal specimen concentration. Once these parameters have been worked out, produce larger batches of frozen grids.

*Dipalmitoylphosphatidylcholine  
*bLauryldimethylamine-oxide
METHOD

Pretreatment of EM Grids

Before applying a suspension of macromolecules onto grids, glow discharge (plasma clean) grids in air to render the carbon support film more hydrophilic. This helps aqueous solutions to spread better and can avoid loss of carbon film upon applying liquid to the hydrophobic surface.

1. Place the grids to be discharged with the carbon film side facing up onto a clean glass slide.
2. Place the glass slide into the vacuum chamber of the glow discharge device and evacuate according to the instructions.
3. Expose the grids for 30–60 sec to the homogeneous purple discharge.
   On the SCD005, use a current of 20 mA; on the homemade device (Aebi and Pollard, 1987), set the high frequency generator to full power.
4. Vent the vacuum chamber carefully so as not to blow away the grids.
5. Use the grids as soon as possible, but in any case within 1 h.

Preparation of the Instrument

6. Power on the main unit.
7. Transfer the black secondary cryogen container into its cup-shaped holder. Remove any grids that are left inside.
   It is mandatory to use this inner container in order to remove the secondary cryogen before baking out the instrument!
8. Position the transfer container holding a marked and empty cryo-grid box onto its platform.
9. Insert a new piece of filter paper, and mount it on the magnetic holder with the metal ring provided. Confirm the corresponding warning message on the touch screen.
10. Using a syringe, fill the water container for the humidiﬁer with 60 mL of water. Close the valve and attach the tubing to the back side of the humidiﬁer.
11. Press the environmental parameters display to show controls. Set the chamber temperature $T_C$, humidity $H_h$, and cryogen temperature to the desired values. Keep the following points in mind:
   i. The chamber temperature is generally set to the preferred temperature of the sample or +4°C for improved homogeneity of the specimen (Fischer et al. 2010).
   ii. To avoid evaporation of the solvent, the humidity is typically set to 90% or above.
   iii. The temperature of the secondary cryogen should be set to the lowest value that just prevents solidiﬁcation of the secondary cryogen (e.g., −185°C).
12. To set up the position of the ﬁlter paper relative to the grid, pick up a blank grid with one of the quick lock forceps that will be used for freezing. In the “Load forceps” position, attach the forceps to the interlock protruding from the bottom of the environmental chamber. Go to the “Setup” page of the software.
13. With the environmental chamber lowered, adjust the horizontal position of the ﬁlter paper (i.e., the pressure exerted onto the grid for blotting; “Blotter Setting”). We use a position where the grid is bent slightly by the ﬁlter paper pressing against it. If the blot sensor (Step 18) is to be used, this position has to be set up as well, as it is used as a reference point for the sensor measurements!
   Note: As the grid can be rotated around the vertical axis, there are two slightly different positions to set up: one with the forceps in “home” position, the other one rotated by 180°. Set up the position that will be used for blotting.
14. Adjust the vertical position of the grid relative to the ﬁlter paper (“Grid Blot Position”).
   We prefer a position in which the upper edge of the ﬁlter paper and the upper edge of the grid coincide.
15. If you intend to use the “Transfer” position for lifting the grid to the surface of the secondary cryogen before transferring it to the grid box, set up the desired vertical position of the grid relative to the container (e.g., just at the level of the secondary cryogen surface).
16. In “Setup,” “Settings,” set the TF heater (which produces a stream of gaseous nitrogen [GN₂] through the working area to prevent contamination) to full power, unless you are planning to use the GP for a very long time without refilling the LN₂. Increase the power of the window heater if you cannot see the grid/filter paper contact clearly due to a fogged front window.

17. Return to the main screen. Reset the filter paper counter to “10” to have the full number of blotting positions available.

18. Open the program list and set up/select a program suitable for freezing your specimen.

For most biological specimens, a single blot as described in this protocol will suffice. Viscous industrial samples might benefit from blotting multiple times or sequentially from both sides (“Multiblot”). For a single blot, up to 10 different programs, including the parameters explained below, can be saved. Due to the different concentration of solutes, different particle sizes, and different support films with varying surface properties used, each specimen will require its own, optimized program. If there is any previous experience from manual freezing of a specimen, start with similar conditions. For entirely new biological samples in physiological buffers, do a first run with the settings outlined in Table 1, varying the blotting time.

i. Enable/disable rotation of the grid around the vertical axis. The grid can be rotated by 180° at two stages in the preparation process: either when the environmental chamber is lowering after attaching the forceps (first check box), and/or between applying the specimen and blotting the liquid (second check box).

This is useful if you are right-handed and would like to apply the specimen onto the same side of the grid as it is blotted from (always the left side): Activate both rotations and use the port on the right side of the instrument to apply your specimen.

With the second check box activated, the grid will be taken back to the “home” position before blotting. If it was not rotated before—either manually or automatically—this setting will have no effect!

ii. Activate the sensor unless it does not work with the setup of your specific experiment.

See Troubleshooting.

iii. Leave the option “A-Plunge” active to automatically plunge the specimen after blotting, unless your thin liquid film after blotting requires some special treatment.

iv. Preblot time (a wait time after application of the specimen but before blotting, for adsorption of the sample or temperature equilibration): This value can be set from 0 to 600 sec with 0.1-sec increments. We use 30 sec as a starting value for suspensions of macromolecules.

v. Blot time (the time the filter paper contacts the grid for blotting liquid): Allowed values are from 0 to 60 sec in 0.1-sec steps. Good starting values for most suspensions of macromolecules are 0.8 sec to 2.5 sec (see Table 1).

vi. Postblot time (an optional time after blotting but before plunging the specimen, used for equilibration of the liquid layer [Iancu et al. 2007]).

We obtain very even ice layers (Fig. 1A) without this additional wait time.

19. Cool down and fill the 1-L dewar at the base of the main unit with 2 L of LN₂. Bring the LN₂ level up to the grid at the bottom of the working area. Wait for the vigorous bubbling of the LN₂ to stop. Fill the transfer container holding the grid box with LN₂ and close its lid.

20. Condense the secondary cryogen (ethane) using one of the two following methods:

Using the Leica liquefier

i. Connect the liquefier to the ethane bottle and place it over the secondary cryogen cup. (Contrary to the manual, we recommend not to cool the instrument with the liquefier in place, as this might trap humidity in the secondary cryogen container!) The temperature reading of the secondary cryogen container will rise. Pour LN₂ over the liquefier to speed up cooling and wait for the temperature to equilibrate at the preset value.

ii. Fully open the main valve of the ethane bottle. Open the needle valve on the ethane bottle slowly.

After some time, white gas and subsequently liquid ethane will fill the secondary cryogen container.
iii. Close all valves when the container is full. Do not overfill; excess ethane might freeze the liquefier onto the secondary cryogen container.

iv. Carefully remove the liquefier and place it into the small Styrofoam container provided by Leica for safe transport.

**Using a pipette tip (or a needle)**

i. Cut off the fine end of a 200-μL pipette tip and connect it to the tubing on the ethane bottle.

ii. Wait for the temperature of the cryogen container to equilibrate to the preset value (e.g., −185°C).

iii. Hold the pipette tip to the bottom of the secondary cryogen container and slowly open the main and the needle valve of the ethane bottle. At the beginning, whitish gas fills the
chamber; use the sound of bubbling ethane to control tip position and to adjust the flow rate. Within 1 min, the rising level of liquid ethane is visible. Pay attention to the temperature of the secondary cryogen container as it increases during ethane condensation: If the temperature rises above −160°C, close the needle valve and wait for the cryogen container to return to the preset temperature. Resume ethane condensation.

iv. When the level of liquid ethane reaches the rim of the secondary cryogen container, move the pipette tip above the ethane surface. Ethane gas flows out of the tip replacing residual liquid ethane that could cause burns.

v. Close the needle valve and the main valve of the ethane bottle.

21. Wait until the environmental chamber has reached the preset temperature and humidity. Keep the working area covered, but not as tightly as to stop the gas flow.

   See Troubleshooting.

22. Prepare another (dewar) vessel with LN₂ for refilling the transfer container.

Freezing Grids

Repeat the following procedure for each grid.

23. Bring the instrument to the “Load Forceps” position.

24. Pick up a flat, glow-discharged grid with an intact carbon film with a quick-release forceps in the right orientation (depending on the setup of your experiment). When the instrument is used at high humidity and temperature, use prewarmed forceps to avoid condensation of humidity. Likewise, precool your forceps when used at 4°C. Close the black slider only to the first notch.

25. Attach the quick-release forceps to the interlock and lower the environmental chamber.

26. Wait for the chamber temperature and humidity to reach the preset values.

27. Open a port at the side of the environmental chamber and pipette 3–5 µL of your specimen onto the grid. Close the port immediately, to maintain efficient temperature control and humidification of the environmental chamber.

28. Press “(Rotate Home)/Blot (S)/A-Plunge” to trigger the freezing cycle. Subsequently, the preblot delay will run and the blotting mechanism will be triggered.

29. Follow the blotting process closely to observe any irregularities. Check if large patches of carbon film are left on the filter paper.

   After the postblot delay, the grid will be plunged into the secondary cryogen.

   See Troubleshooting.

30. Once the specimen is frozen, the chamber will lift up. From now on, approach the specimen with precooled tools only!

31. (Optional) Advance to the “transfer position” of the grid. This is slightly higher than the freezing position and allows one to blot off liquid ethane with a piece of filter paper.

32. Open the lid of the transfer container with precooled forceps.

33. Disconnect the forceps from the grid plunger. To avoid damaging the grid at the wall of the secondary cryogen container, hold the forceps at the level of the black slider and tilt it out of the beveled interlock.

34. Transfer the grid in one quick movement into the transfer container filled with LN₂. Do not expose the grid to the humidity of the air; do not lift it any higher than the gray ring surrounding the working area.

35. Place the grid into the right slot of the grid box and release it from the quick-lock forceps.

36. Refill LN₂ in the transfer container, if required—the grid box should always be covered with LN₂. Keep the secondary cryogen cup covered with the plastic lid provided by the manufacturer, so as not to mix liquid ethane and LN₂! Close the transfer container.
37. Watch out for any warning messages issued by the GP. Keep the following points in mind:
   i. If necessary, refill the humidifier with 20 (!) mL water.
   ii. Refilling LN\textsubscript{2} in the main dewar is only required when a warning is displayed (<25%).
   iii. If you need to replace the filter paper, allow new filter paper to equilibrate with the humidity in the environmental chamber.  
   *This can improve the reproducibility of freezing and the evenness of the resulting ice layer.*

38. Warm up all cold and frosted tools with a hair dryer to avoid contamination of the specimen or the cryogens with ice crystals.

**Shutting Down the Instrument**

39. With a precooled screwdriver or Allen key, close the grid box.

40. Refill the transfer container with LN\textsubscript{2} and close it.

41. Precool the handle with the M4 thread. Attach it to the specimen transfer container.

42. Lift the transfer container out of the working area and into a dewar filled with LN\textsubscript{2}. Then, transfer the frozen grids either directly to the cryo-TEM or to a LN\textsubscript{2} storage container for later use.

43. Remove the container holding the secondary cryogen with insulated forceps and place it into the small Styrofoam box. Allow evaporation to occur in a safe place.

44. Discard the filter paper. Leave the door of the environmental chamber open.

45. Drain the residual water from the water container.

46. Switch on a 1 h bake-out cycle.  
   *The timer will start once the LN\textsubscript{2} level in the dewar has dropped to 0%.*
   *The bake-out is not intended to sanitize the environmental chamber after freezing pathogenic specimens. Alternative means of decontamination such as ultraviolet (UV) irradiation of the reflective interior of the chamber, gas sterilization, or wiping with ethanol have to be tested, taking into account that most components inside the environmental chamber are not removable.*

47. Once the bake-out cycle is complete (at most 1.5 h), switch off the instrument.

**TROUBLESHOOTING**

**Problem:** The environmental chamber does not cool down to +4°C.  
*[Step 21]*

**Solution:** Consider the following:

1. Make sure the room temperature is lower than 25°C.
2. Keep the front door and the side ports closed.
3. Reduce the power of the window heater (Step 16) to the minimum required.

**Problem:** The environmental chamber does not reach the specified humidity at low temperature.  
*[Step 21]*

**Solution:** This is due to condensation and hence depletion of humidity at the coldest parts of the instrument, i.e., the cooling elements. The evaporation rate, however, drops significantly with temperature (Frederik and Hubert 2005); hence, loss of solvent is less of an issue even at just 75% relative humidity.

**Problem:** The two quick-lock forceps of the GP used in alternating fashion are not well aligned, causing the blotting positions for both forceps to differ significantly.  
*[Step 29]*

**Solution:** Use the centering tool to align the forceps properly or talk to your local representative to resolve the problem if these misaligned forceps are new from Leica.
Problem: The blotting sensor does not work (i.e., the instrument aborts with the error message “Blotting fault” before plunging).

[Steps 18.ii, 29]

Solution: Make sure you have set the reference point correctly in Step 13. Because the sensor comes with a factory side calibration of 3 µL water, different solvents, or smaller volumes might not trigger it.

Problem: The filter paper does not contact the grid strongly enough or contacts it too strongly when using the blotting sensor.

[Step 29]

Solution: After the filter paper makes contact with the water droplet on the grid, it keeps moving forward by a defined distance to touch the grid. If this distance is not properly set up, the contact the filter paper makes will not be appropriate in strength. Consider the following:

1. First, check if the correct volume (3–5 µL) is set in the “Blotter settings” dialog.
2. If that does not resolve the problem, go to “More>>” and change the corresponding value for “additional move.” A step on this scale corresponds to ~0.2 mm; positive values increase the pressure.

Problem: There are issues with freezing specimens on continuous carbon films.

[Step 29]

Solution: If you use continuous carbon for freezing, it is important throughout the whole procedure not to damage the carbon film and to achieve even and reproducible blotting. Therefore, the sample is primarily applied on the carbon side of the grid and blotted from the other (noncarbon) side. The blotting distance should be chosen so that the liquid is blotted by the filter paper without strongly bending the grid. To facilitate blotting, it is advisable to add a very small amount of sample (0.5 μL) to the noncarbon (“back”) side of the grid once the sample has been applied for settling.

Problem: The microscope vacuum crashes during the transfer of freshly frozen specimens into the microscope.

Solution: This is due to the sublimation of frozen ethane attached to the specimen, visible as a white crust (Benoît Zuber, personal communication). To reduce the risk of compromising the microscope’s vacuum, blot away liquid ethane in the transfer position (Step 31) or wait until the white crust has fallen off.

Problem: There is uneven ice thickness or patchy ice.

Solution: Consider the following:

1. This problem might be caused by an insufficiently hydrophilic carbon film.
   i. Glow discharging the grids for longer time or at higher current might help.
   ii. Introducing a postblot delay for equilibration of the liquid layer (Iancu et al. 2007), use of humidity equilibrated filter paper, or an increased pressure of the filter paper might also improve the results.
2. A bent grid can cause an uneven distribution of water; flat grids can help to avoid this issue and simplify data acquisition.
3. A large mass of ice on one side of the grid can be caused by gripping the grid too far inside with the forceps.
4. For some samples, for example those containing surface active components such as suspensions of liposomes, it is very difficult to obtain very even ice layers covering holes of a perforated film.

Problem: The ice on the grid is not vitreous but thin enough to be electron transparent (Fig. 2A).

Solution: Consider the following:

1. Check the temperature of the secondary cryogen, which should be as low as possible without having the ethane freeze.
2. Make sure that the secondary cryogen is filled up to the brim and that the forceps used are as fine as possible to minimize heat intake.

3. The more likely culprit is a secondary warm-up above devitrification temperature: Use exclusively precooled tools, grid boxes, and vials, and transfer the sample quickly between secondary cryogen and the transfer container or from the grid box to the cryo-holder.

**Problem:** Large portions of the carbon film are damaged.

**Solution:** Consider the following:

1. Check the film under a light microscope before and after glow discharging to see at what stage it is damaged (e.g., by the discharge).
2. Check the film after applying the specimen; damage at this stage can indicate that the film is not hydrophilic enough.
3. Blot from the back side of the grid, use grids with a smaller mesh size, test another batch of carbon film, and consider supporting your continuous carbon film with a perforated film with wide mesh.

**Problem:** No sample can be found on the grid or in the holes.

**Solution:** Consider the following:

1. Increase the concentration of the specimen.
2. It might help to increase the glow discharge time.
3. If no sample can be found in the holes of a perforated carbon film, you might be dealing with a particle (such as *Salmonella* Type III secretion system needle complex) or a buffer (e.g., detergent

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**FIGURE 2.** Examples of improperly immersion frozen cryo-specimens. Remedies for these issues are discussed in the Troubleshooting section. (A) Regions of crystalline ice, exhibiting pronounced diffraction contrast. (B) Ice contamination on top of a well-frozen ice layer, arising from excessive humidity around the frozen specimen. (C) *Salmonella* type III secretion system bases on a continuous carbon film, embedded in an overly thin layer of ice. Increased electron density surrounding each particle can be seen, representing a local increase in thickness (see also Fig. 1D). (D) Subviral particles of human rhinovirus frozen on this perforated carbon film are only found at the periphery of the hole, up to a certain point where many of them accumulate. This suggests that the ice layer at the center of the hole is too thin to allow them to enter. In the left bottom corner, contamination, possibly from ethane, is visible. Bar (D) = 250 nm (also applies to A,B,C).
containing), that strongly prefers to attach to carbon surfaces over staying in holes. In this case, try a continuous carbon film or a shorter glow discharge time.

**Problem:** The specimens are contaminated (Figs. 2B,D).

**Solution:** Contamination can arise from different sources.

1. Rounded, high contrast blobs seen in a wide range of sizes are interpreted as water ice crystals (Grassucci et al. 2007; Crucifix et al. 2009) that arise from atmospheric humidity condensing on the specimen or from ice in the cryogens. The GP is designed to minimize ice contamination. In addition, we recommend a number of precautions against this type of problem:
   - Set the TF heater to 100% (Step 16).
   - Store the specimens in LN2 at all times or keep them in the GN2 atmosphere of the working area.
   - Work as quickly as possible with frost-free tools.
   - Work in a room with low humidity.
   - Avoid breathing on the specimen.
   - Use clean, ice-free LN2.

2. Filamentous and branched aggregates on the specimen are attributed to impurities of the ethane (Grassucci et al. 2007; Crucifix et al. 2009).
   - Use high-quality ethane and do not use up the bottle completely (Grassucci et al. 2007).
   - Crucifix et al. (2009) also recommend removing excess ethane after freezing (transfer position; Step 31) or before transfer into the cryo-EM.

3. Finally, contamination can also build up over time if the vacuum in the microscope is poor.

**Problem:** The ice layer is generally too thick or too thin (Fig. 2C,D).

**Solution:** Consider the following:

1. Increase or decrease the blotting time.
2. Preincubate the filter paper in the humid chamber, allowing it to saturate, which yields thicker ice.
3. Make sure the viscosity of the specimen solution is low enough if the film is too thick.
4. It is not advisable to produce thinner ice by decreasing the humidity in the environmental chamber, for reasons outlined in *Immersion Freezing of Biological Specimens: Rationale, Principles, and Instrumentation* (Resch et al. 2011a).

**DISCUSSION**

To assess the ice thickness all over the grid and to fully exploit the potential of well-frozen grids, we always prepare low magnification (~140×), minimal dose montages of our cryo-grids using Leginon (Suloway et al. 2005) or SerialEM (Mastronarde 2005). Optimal results in terms of evenness for a suspended specimen are shown in Fig. 1A. Doing pretests using blot time series (Table 1), we observe a very robust gradient in ice thickness from the shortest to the longest blot time. Once parameters for a specimen have been worked out, we can obtain >80% of almost identically frozen grids on the EM GP, allowing us to greatly improve our throughput.

Grid squares that have been selected at low magnification based on their electron transparency are then checked at higher magnification (e.g., 1350×) at appropriate underfocus. Good squares have thick ice close to the grid bars, making the corners of the square appear rounded, while the center of the square is evenly covered with thin ice. The holes of perforated carbon film are covered with a thin ice film, the support film is not ruptured, and they do not contain any coarse contamination. Only at high magnification (>20,000×) can the specimen finally be checked for successful vitrification, the presence of nanometer-size contamination (Fig. 2B), and particle density. The density, e.g., for single particle
analysis, is ideal if the particles are close to each other but do not touch or overlap. For examples of good results for various specimens, see Figure 1C–F.

The thickness of the ice layer is another key factor to be considered. In the optimal case, it is slightly bigger than the dimensions of the specimen to avoid both unnecessary deterioration of the signal and deformation of the specimen. Clear indicators that the ice film is too thin are an increased electron density around particles on continuous carbon (Fig. 2C) as the aqueous film is “bulging out” around each of them or a concentration of particles toward the periphery of holes, where the covering ice layer is the thickest (Fig. 2D; see also Troubleshooting). For data acquisition, homogeneous clusters of good grid squares are preferred due to smaller differences in eucentric height and stage movement.

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