Handbook of Cryo-Preparation Methods for Electron Microscopy

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Plunge-Freezing (Holey Carbon Method)

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Chapter 3

PLUNGE-FREEZING
(HOLEY CARBON METHOD)

Sacha DE CARLO
1. PRINCIPLES OF PLUNGE-FREEZING

Cryo-electron microscopy of frozen-hydrated macromolecules embedded in a thin layer of vitreous water is nowadays a well-established method. It was developed more than 20 years ago at the European Molecular Biology Laboratory (EMBL) in Heidelberg, by the pioneering group led by Jacques Dubochet. It is currently used in hundreds of labs worldwide in order to study biological complexes in their near-native state (see Chapter 1).

The main advantage of cryo-EM versus air-drying negative staining is that the biological object is fully embedded in its native environment, namely the vitrified buffer surrounding it, thus its three-dimensional structure is fully preserved down to atomic scale.

Typically, the frozen-hydrated specimen is observed suspended across the holes of a carbon support. The first part of this chapter describes how to prepare the holey carbon support grids. The second part describes how to apply the sample to the grid and the vitrification process in order to obtain a thin layer of suspension, to be observed in low-dose mode in the cryo-electron microscope.

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# 3. MATERIALS/SOLUTIONS

## 3.1. Materials

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<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone chamber</td>
<td>With grate</td>
</tr>
<tr>
<td>COLD aluminum block</td>
<td></td>
</tr>
<tr>
<td>Copper grids</td>
<td>200 or 400 mesh</td>
</tr>
<tr>
<td>EM-grade tweezers</td>
<td>Dumont biology N5; Ted Pella Inc., Redding, California</td>
</tr>
<tr>
<td>Filter paper, wiping paper</td>
<td>E.g., Whatman grade 1–5</td>
</tr>
<tr>
<td>Forceps</td>
<td>Clean</td>
</tr>
<tr>
<td>Glass microscope slides</td>
<td>For storage (Ted Pella Inc., Redding, California)</td>
</tr>
<tr>
<td>Glass Petri dish</td>
<td>With humidity between 45 and 50%</td>
</tr>
<tr>
<td>Glass pipette with rubber bulb</td>
<td>Ted Pella Inc., Redding, California</td>
</tr>
<tr>
<td>Glow-discharge apparatus</td>
<td>Homemade or commercially available</td>
</tr>
<tr>
<td>Grid-boxes</td>
<td>Or other commercially available plunging devices</td>
</tr>
<tr>
<td>Humidity chamber</td>
<td>Bigger dimensions than filter paper</td>
</tr>
<tr>
<td>Humidity reader</td>
<td></td>
</tr>
<tr>
<td>Paraﬁlm</td>
<td></td>
</tr>
<tr>
<td>Plastic container/liquid nitrogen Dewar</td>
<td></td>
</tr>
<tr>
<td>Razor blades</td>
<td></td>
</tr>
<tr>
<td>Screwdriver</td>
<td></td>
</tr>
<tr>
<td>Small plastic grid boxes</td>
<td></td>
</tr>
<tr>
<td>Stop watch</td>
<td></td>
</tr>
<tr>
<td>Vitrification device</td>
<td></td>
</tr>
<tr>
<td>Vitrobot</td>
<td></td>
</tr>
<tr>
<td>Water container</td>
<td></td>
</tr>
</tbody>
</table>

## 3.2. Solutions

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>90% acetone</td>
<td>With a pressure gauge and two security valves</td>
</tr>
<tr>
<td>Cellulose acetate solution (plastic)</td>
<td></td>
</tr>
<tr>
<td>Ethane-35 gas bottle</td>
<td>For grid storage</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>4 L bottle</td>
</tr>
<tr>
<td>99% ethyl acetate</td>
<td></td>
</tr>
<tr>
<td>25 L or 50 L liquid nitrogen Dewar</td>
<td></td>
</tr>
<tr>
<td>Liquid nitrogen</td>
<td></td>
</tr>
</tbody>
</table>
4. PROTOCOLS

4.1. Preparing Holey Carbon Grids

4.1.1. Making the plastic solution
To make the cellulose acetate solution, mix the dry cellulose acetate-butyrate with ethyl acetate to a final concentration of 0.15% (w/v).

As an example, if you use 0.026 g of the dry cellulose acetate, you would need to add 17.3 mL of ethyl-acetate so that the solution would be 0.15% (w/v).

4.1.2. Making “holey” plastic film
To be performed in the humidity chamber if lab’s humidity is not within the 45% to 50% range.

1. Remove aluminum block from refrigerator.
2. Put it on ice to keep it cold and wipe dry.
3. Wait for about 2–3 minutes before placing glass slide onto block.
   2 to 3 minutes waiting time is to form fine droplets (water condensation).
4. While waiting, clean a few (3 or 4) glass slides with ethanol.
   Use pure or 90% ethanol to clean glass slides.
5. Mark on which side of each slide the plastic film is to be deposited.
   Mark slides with a permanent marker (a fine dot is sufficient).
6. Place the glass slide onto the aluminum block, designated side up, for 10 seconds.
7. Remove slide from block.
8. While tilting the slide at a 60° angle, pipette a layer of plastic onto it.
9. Set the slide aside beveled and allow to dry.
10. REPEAT steps 5–9 with no more than 3 slides before wiping the block dry and starting over again with step 3.
4.1.3. Choosing the plastic film

1. Hold the slides up to the light to make sure that the plastic has dried evenly throughout.

Discard slides that do not meet this criterion.

2. Observe the slides in a phase contrast microscope with an EM grid on top of it for reference. The bubbles should be of homogenous shape and size. About 20 bubbles should fit across each side of each square (or more reasonably, most of the bubbles should be of that size, regardless of orientation).

Check to make sure that the entire length of plastic has bubbles of the appropriate size. If not, discard the section with poor bubble formation.

4.1.4. Covering grids with the plastic film

1. Fill the water container to the top with distilled water.

2. Scrape the edges of the plastic film to facilitate floating off the film upon entry into water.

3. Breathe on the slide and gently introduce it into the water container, designated side up, at a 30° angle with the water (the plastic should detach from the slide and float on the water surface).

4. Delicately arrange copper grids dull side up on the plastic film (avoid the edges of the plastic).

The plastic film/grids should have adhered to the underside of the filter paper.

Before the filter paper has an opportunity to slowly begin to sink into the water, securely grab a portion without grids under it and meticulously pull the filter paper out of the water as if peeling the skin off a fruit.

5. To retrieve the grids, overlay filter paper onto them.

6. Dry flat for 3–4 hours (overnight is even better) with the grids facing up (the shiny side should be up with the plastic over it).
4.1.5. Busting the holes

1. Fill the acetone chamber with acetone, replace grate once this is done.

2. Take one grid off the filter paper and place it on a microscope slide; this is the pretreated grid.

3. Place the filter paper with the grids in the acetone chamber for one minute.

4. After removing the filter paper with the grids from the chamber, transfer a representative grid from the filter paper to the microscope slide containing the pretreated grid.

5. Compare the two grids: The representative grid should have thinner plastic between the bubbles/holes (i.e., bigger holes in the representative grid than the bubbles in the pretreated grid) compared to the pretreated grid.

Figure 3.1. Good homemade holey carbon (the big holes have an approximate diameter of 5 to 6 \( \mu \text{m} \)).

4.1.6. Finishing up

1. Coat the grids with carbon using the carbon discharge protocol (see Harris\(^4\)). When done, you should see a little bit of carbon deposited underneath the grids (showing that the holes have popped).

The thickness of the carbon may vary depending on your own needs. Typically a 10 to 20 nm layer is a good thickness for holey carbon, but thicker layers may work too, depending on the application and the sample.
2. To dissolve the remaining plastic from the grids, remove the grids from the filter paper and put them onto the grate in the acetone chamber, dull side up, overnight.

3. Eventually, use a sputter-coater to deposit a very thin layer of gold/palladium (1–5 nm).

4. To make sure you removed the plastic completely, wash the grids with ethyl-acetate and rinse with water right before use.

**ALTERNATIVE METHOD**

1. Use precleaned glass slides or clean them with ethanol or methanol (90%) and Kimwipes (delicate task wipers) beforehand.

2. In a small glass beaker, prepare a 0.5% Formvar solution in chloroform, e.g., 0.25 g in 50 mL chloroform (use agitation + cover; it takes a while to dissolve).

3. Add about 0.5 mL of 50% glycerol with a Pasteur pipette on the surface of the Formvar/chloroform solution. You can adjust the volume of glycerol added to get more or fewer holes (higher ratio of glycerol/Formvar solution gives smaller holes).

4. Use ultrasonic treatment to make an emulsion of glycerol droplets in the Formvar.

5. Dip an ultrasonicator tip in mix, approximately 1 inch deep, sonicate 1 minute at maximum power (50 W; 20-30 kHz). Quickly dip the glass slides, one by one, vertically in this emulsion, blot the sides and let them dry vertically (you can use, for example, a big beaker with filter paper at the bottom). You can prepare ~20 slides each time. Make sure to dip the glass slides immediately after the ultrasonic treatment.

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**Materials and chemicals for the alternative method:**
- Beaker 250 mL
- Filter paper, wiping paper (e.g., Whatman grade 1)
- Forceps
- Glass microscope slides (clean)
- Glass pipette with rubber bulb
- Glow-discharge apparatus
- Kimwipes wipers
- Optical microscope
- Pasteur pipette
- Ultrasonicator with tip
- 90% acetone
- 99% chloroform
- 90% ethanol and methanol
- 99% ethyl acetate
- Formvar solution (plastic); Ladd Research Williston, Vermont
- 50% glycerol
- Water (distilled, nanopure, etc)

**Vary the glycerol/Formvar ratio in the solution and sonication times to adjust hole number and size.**
6. Check the size of the holes with an optical microscope. You can adjust the number of glycerol drops and the sonication time to obtain the hole number and hole size you want.

7. When the slides are dry, cut the edges of the holey membrane on one side and float it on the surface of distilled water. Cover with copper grids and pick them up with a piece of filter paper (or whatever works best). Let them dry on filter paper. Soak the entire sheet in methanol for 30 minutes to get rid of the glycerol to form the holes, and then let them dry.

8. Evaporate carbon onto the grids, which are covered with the holey plastic membrane (as described before).

9. Remove the Formvar by soaking the grids in chloroform right before use. Let them dry on filter paper.

10. Eventually use a sputter-coater in order to deposit a thin layer of gold/palladium (1–5 nm).

11. Wash the grids with full-strength ethyl-acetate (pure) and then with water right before use.

4.2. Plunge-Freezing Grids

4.2.1. Glow-discharge treatment

Glow-discharge treatment of the grids right before use can help to obtain a good spreading of the sample across the holes in the carbon support. Apply right before use because the charging effect of the carbon film is only temporary (lasting time may depend on the apparatus used). It also cleans the carbon support surface. Glow-discharging time may depend on the apparatus you are currently using in your lab. Please refer to the user’s manual of the equipment for further operating instructions.
4.2.2. Preparing the cryogen/vitrification device

1. In the meantime, prepare the vitrification system. Prepare the material you are going to need on the bench (see Figure 3.2), near the vitrification device, as timing is important in the next few steps.

2. Make sure the Styrofoam box is placed on the vitrification device at the right place (see Figure 3.3).

Figure 3.2 Forceps, EM-grade tweezers, Petri dish, Whatman filter paper, EM grids, Parafilm and, of course, the sample are displayed on the bench.

Figure 3.3 Placement of the Styrofoam box.
3. In order to make your life easier, it is useful to have a direct light coming from behind, typically from a desk lamp (see Figure 3.4).

![Figure 3.4 Direct lightening coming from behind is advisable.]

4. If you are using a commercially available vitrification device (e.g., the Vitrobot, see Figure 3.5 and Chapter 4), please refer to the user’s manual for further instructions.

5. The small metal cup (typically aluminum) that will contain the cryogen should also be placed in the box; keep the aluminum cup in the center where the tweezers will fall later (see Figure 3.6). Test before use.

![Figure 3.5 Vitrobot vitrification device.]

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6. Prepare the gridbox and place it in the area that will be filled with liquid nitrogen (see Figure 3.7).

7. The plastic grid storage box should also be placed underneath the liquid nitrogen level; make sure it stays on the bottom by using a metal holder. Vitrified samples will be placed in this storage box.

8. Make sure you wear lab-certified protection eyewear BEFORE you start using ethane (see Figure 3.8).

9. Use your tweezers to pickup a grid (see Figure 3.9).

10. Fill the whole box with liquid nitrogen, do it slowly, no nitrogen should splash into the aluminum cup that will contain ethane (see Figure 3.10).
11. Start filling the ethane cup (see Figure 3.11) and wait for the liquid ethane to reach the right temperature (see Figure 3.12).

12. Typically, one would wait until the ethane starts to become solid and then add some more ethane to the cup to fill it to the top (see Figure 3.13).

The freezing point is a good way to make sure the vitrification temperature is reached (liquid nitrogen will be at about \(-192^\circ\text{C}\)).
By repeating this several times the ethane remains liquid, at the right temperature to vitrify biological samples.

**Figure 3.11** Filling cup with ethane.

**Figure 3.12** Liquid ethane reaching the right temperature.

**Figure 3.13** When ethane starts to become solid, more is added to fill the cup.

### 4.2.3. Pipetting the sample on the freshly prepared grid

1. Typically 3–5 μL of the sample solution are applied to the freshly glow-discharged EM grid (see **Figure 3.9**).

2. Mount the tweezers, holding the grid, in the vitrification device (see **Figure 3.14**).
4.2.4. Removing excess liquid

1. This is the key step. Use filter paper in order to remove excess liquid of the suspension on the grid. Typical blotting time is 2–3 seconds at room temperature and in a relatively dry (< 60% relative humidity) environment (see Figure 3.15)

2. The blotting time is also dependent on the concentration of the protein solution and the presence of lipids or detergents in the buffer.

Figure 3.14 Tweezers mounted in vitrification device.

© E.g., Whatman type I.

 resultat The backlight should help one to see a “halo” forming on the filter paper; release the plunging mechanism when the halo starts to disappear.

 E.g., Whatman type I.

 resultat If you are using a temperature and humidity controlled chamber, blotting times could be as long as 10 seconds.

 resultat You can use blotting paper that has been preexposed to water (or buffer) by dipping or spraying (whichever is more convenient).

Figure 3.15 Using filter paper to remove excess liquid on the grid.
4.2.5. Vitrification

The cryogen usually used for vitrification is liquid ethane (in some labs liquid propane is used). Immediately after blotting the grid (previous step), the sample is plunged into the cryogen (see Figure 3.16).

- You can also set up a homemade system to blow humid air at 37°C right onto the grid (typically from behind the grid) while you apply the blotting paper to the front side.
- Please refer to the lab equipment’s user’s manual if you are using commercially available vitrification devices.

- Make sure the sample-releasing mechanism works efficiently, as the cooling process must be fast enough in order to guarantee vitrification.
- Cubic and hexagonal ice will be observed if the cooling process was too slow, or if the sample was warmed up to a temperature above −135°C.

Figure 3.16 Sample plunged into cryogen.

4.2.6. Specimen mounting and transfer

1. After plunging, keep the grid in liquid ethane and transfer it quickly to liquid nitrogen, move it underneath the nitrogen level to the storage area (see Figure 3.17).

- The grid must always be kept at liquid nitrogen temperature after vitrification.

Figure 3.17 Transferring the grid to the storage area.
2. Use the precooled screwdriver to close the grid box firmly (see Figure 3.18).

The grid can be stored in a Dewar under liquid nitrogen, or directly mounted on the cryo-specimen holder and, subsequently, observed at liquid nitrogen (or liquid helium) temperature in the cryo-electron microscope.

Figure 3.18 Closing the grid box with a screwdriver.

5. ADVANTAGES/DISADVANTAGES

5.1. Advantages

- Preservation
  Unstained, frozen-hydrated biological macromolecules are prepared in a fully hydrated environment, e.g., water or physiological buffer. Therefore, they are observed in a near-native state.

- Artifact-free
  Unstained, frozen-hydrated biological macromolecules, prepared and observed using the method described here are fully suspended in an aqueous environment. Provided that the specimen is fully embedded in the vitreous layer, flattening and stain-related artifacts, often observed with conventional air-dried, negatively stained samples, are thus avoided.

- The electron microscope must be equipped with an anticontaminator in the column and a low-dose beam-deflection unit to avoid beam damage when the specimen is observed at liquid nitrogen temperature.

- The low-dose exposure for unstained, frozen-hydrated biological specimens is usually below 15 electrons/Å².

- Because the protein density is very close to that of vitreous water, the amplitude contrast arising from scattering is very low. Thus, object visibility in cryo-EM is mainly obtained by defocusing (increasing phase contrast).

- Defocusing alters the contrast transfer function (CTF) of the electron microscope, usually leading to loss of resolution in the data.
5.2. Disadvantages

- Beam damage
  Unstained, frozen-hydrated biological complexes are highly sensitive to the electron beam. Low-dose exposure techniques are required to avoid beam-induced damage.

- Low signal-to-noise ratio (contrast)
  Because of the low-dose mode required to image unstained protein complexes, the final visibility of the object (more precisely signal-to-noise ratio) is very low.

- A field-emission gun (FEG) electron source and computer-assisted CTF correction are usually required to obtain medium to high resolution results.

- FEG-equipped microscopes operating at 200 to 300 kV are nowadays the standard in many EM labs where single-particle cryo-EM and cryo-electron tomography are routinely employed.

6. REFERENCES