HANDBOOK OF
Cryo-Preparation Methods for Electron Microscopy

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Chapter 4

CONTROLLED VITRIFICATION

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GENERAL INTRODUCTION

Fundamental research within the scope of cell, structural biology and nanotechnology is increasingly focusing on unraveling interactive biological and biochemical processes and pathways at the macromolecular level. For this, high resolution transmission electron microscopy (TEM) is indispensable. Of paramount importance is the three-dimensional visualization of macromolecular structures and molecular machines in their native hydrated state. Their physical fixation within ultra-thin vitrified ice layers is the crucial starting point.

This chapter describes the essentials of controlled vitrification, a crucial step for cryo-observation in particular and a starting point for various cryo-preparative methods in general.

Cryo-observation of vitrified samples allows the ultrastructural study of macromolecules, molecular assemblies and cells in their natural (= hydrated) environment (see Chapters 3, 7 – 10, 17). 3-D reconstructions can be obtained from vitrified specimens by applying careful microscopy and data analysis. 3-D data are based on single particle analysis (SPA; resolution better than 1 nm) or tomography (resolution better than 3 nm). Controlled vitrification is the starting point for these 3-D studies and has the potential advantage of accurate timing of the vitrification process (1 msec precision), thus enabling time resolved studies.

Prior to vitrification, samples are vulnerable and sensitive to environmental conditions. Control over humidity and temperature of the environment is the cornerstone of sample preparation in a critical vitrification procedure,\(^1\), as will be outlined in this chapter.

Practical procedures will be illustrated using the Vitrobot™ as an instrument for computer-controlled vitrification.

**Figure 4.1**: Modern cryo-electron microscope: (FEI Titan) tailored for 3-D cryo-EM by tomography and single particle analysis.
1. PRINCIPLES OF CONTROLLED VITRIFICATION

1.1. Effect of Environmental Conditions

- For cryo-electron microscopy, samples (macromolecules, molecular assemblies, virus particles, bacteria etc.) are suspended in buffer and cells cultured on a flat support.

- A three microliter droplet is applied to a hydrophilic grid (e.g., glow discharged).

- By blotting away most of the liquid, a 100 nm thick film is formed which comprises about 1/5000 of the applied volume.

- The thin specimen that forms is shot into melting ethane.

- The thin specimen is vulnerable to environmental conditions as heat and mass exchange occur quickly because the surface to volume ratio is rather extreme.

- A high-entry velocity in ethane is required to optimize heat exchange to vitrify the specimen. To obtain such velocities over a short traveling distance (about 7 cm) requires an acceleration of ca. 3 times the gravitational force.

- The fragility at a nanometer scale contrasts with the robustness at the millimeter scale where the grid is subjected to blot forces and acceleration forces.

- Depending on the thickness of the specimen, the temperature decreases to about the dew point in seconds or split seconds. The temperature difference thus established between specimen and environment depends on environmental humidity.

- A thin specimen (typically around 0.1 μm), as used for cryo-EM, will thus attain its dew point temperature within 0.1 sec. The temperature of a thin film in equilibrium will be lower than the temperature of the environment unless the environment is saturated with water vapor.

Figure 4.2 Dew point effect and thickness. Image shows the relation between time and temperature with a thin specimen at 40°C and 40% relative humidity (rH). (Reproduced with permission.)
Depending on the temperature and the environmental humidity, a temperature gradient will be established. This results in a heat influx toward the specimen and in further evaporation of water. The evaporation velocity is independent of the thickness of the specimen. Evaporation is calculated according to the kinetic theory of gas and assumes evaporation from a free spanning thin film (evaporation from two flat surfaces).

Figure 4.3 Evaporation velocity versus temperature and humidity. (Reproduced with permission.)

Preparation at 20°C and 40% rH (typical environmental conditions in our laboratory) will result in the evaporation of water at a rate of 40 nm/sec). A thin film of 100 nm thus losing water will have an increasing solute (and particle) concentration. Liposomes, permeable to water, are exposed to an increasing salt concentration and will lose internal volume to maintain the same osmotic pressure internally as externally. The internal volume may decrease by some 50% during “open air” preparation of a thin film (concomitant with a two-fold increase of the salt/solute concentration). The loss of internal volume is accompanied by a change in shape — spherical vesicles turn into concentric vesicles connected through an “orifice” that keeps the bilayer continuous.

Figure 4.4 Osmotic effects of lab environment. Effect of evaporation on the internal volume of vesicles. (Reproduced with permission.)
• **DLVO theory** (Derjaguin-Landau-Verwey-Overbeek): Molecular distance related to attraction/repulsion. Relation depending on salt concentration. Balance between van der Waals attraction and electrostatic repulsion.

An increase in salt concentration has not only osmotic effects, but also an effect on molecular interactions (DLVO theory). The balance between electrostatic repulsion and attraction forces is modulated by the salt concentration (and valence of the ions involved). For the study of macromolecular assemblies, molecular machines and cells, it is essential to maintain the “original” salt concentration. This can only be achieved in an environment saturated with a solvent (e.g., water).

### 1.2. Process Control prior to Vitrification

• Although vitrification of a specimen is a rather simple and straightforward 3-step process (apply, blot, and plunge), every step requires careful attention.

An increase in salt concentration has not only osmotic effects, but also an effect on molecular interactions (DLVO theory). The balance between electrostatic repulsion and attraction forces is modulated by the salt concentration (and valence of the ions involved). For the study of macromolecular assemblies, molecular machines and cells, it is essential to maintain the “original” salt concentration. This can only be achieved in an environment saturated with a solvent (e.g., water).

#### 1.2.1. Thin film formation upon blotting

• Preparing a thin vitrified object from bulk material is an important step for cryo-EM. The sample can be a solution/suspension (macromolecules, macromolecular assemblies, or particles) (see Chapters 3, 7 - 10, 17) or small, thin cells (see Chapter 12). A convenient (small) volume of liquid is used to obtain a “representative” thin film in which the object is suspended. Producing the thin film in a controlled and, therefore, reproducible manner is an asset of the Vitrobot™ technology.

An applied volume of 3 µL would form a cylinder of about 0.5 mm on a diameter of 3 mm carrier grid and upon blotting the thickness is reduced to a thin film some 100 nm thick.

• The blotting action is defined by time and pressure and has to be optimized for each specimen under investigation.

The reproducible production of a thin film is thus an important processing step for cryo-EM.

• A gentle and controlled movement of the blot pads toward the grid was found to be important as well as the final pressure they exert onto the grid in the stationary phase (maximum contact/pressure).

An optimal thin film is formed when it is continuous over the whole grid and thick enough to suspend the specimen in a pseudomonolayer.

The movement of the blot pads is symmetric and perpendicular to the grid surface.
• The liquid is removed from one side by gently pressing the blotting paper. The blot pad on the other side is used as backing to prevent damage by lateral or bending forces.

• Both the blot pressure and blotting time can be varied and selected by the user interface.

• Blotting is done with filter paper.

> For viscous solutions, the blotting time can be increased and the pressure should be higher than for more liquid samples.

> The absorbing properties of the filter paper are important as they also influence the blotting time and pressure.

> Other types and brands of paper than those provided with the Vitrobot™ can be used, but the optimum blotting parameters have to be established.

> Note: The blotting paper may release components, e.g., \( \text{Ca}^{2+} \), as contaminants that can influence the structure of the specimen.

1.2.2. Vitrification in melting ethane

• For vitrification of aqueous solutions, a high freezing velocity \( (10^4 \text{°C/sec}) \) is the key and a coolant with high thermal conductivity is necessary. In addition, the specimen is plunged into the coolant at a high velocity.

• Plunging blotted specimens into melting ethane (liquid between \(-88\) and \(-172\)°C) became the standard for vitrification following the monumental first observations on vitrified, aqueous solutions/suspensions by Dubochet at al.\(^5,6\) (see Chapters 1, 3, 7).

• Ethane residues may protect the specimen during transfer, which evaporates in the airlock and vacuum of the microscope.

• The excess liquid ethane on the grid can be limited by performing two manipulations:

> Other hydrocarbons seem to be less suited, e.g., liquid methane \((-161\) and \(-183\)°C). However, methane is liquid over a limited temperature range compared to liquid nitrogen \((-196\) and \(-210\)°C) or liquid propane \((-42\) and \(-187\)°C), which leaves residues on the specimen.

> Most of the excess of liquid ethane originates from the liquid inbetween the tweezers’ tips or from the fact that the grid is removed from the ethane too abruptly.
1. Squeeze the tweezers’ tips together while removing the grid from ethane.
2. Slowly lift the grid from the solution through the ethane surface while observing how the liquid film detaches from the grid.

ู่ The ethane will form a meniscus of the liquid from the solution to the grid that detaches perfectly when the movements are done slowly.

1.2.3. Set up the temperature conditions for vitrification

• The Vitrobot is designed to vitrify samples from temperatures between 4°C and 60°C.
• The temperature and humidity prior to vitrification should be set using the user interface of the Vitrobot.
• The bulk of the sample is normally at the same temperature as the chamber of the Vitrobot and, therefore, the sample solutions should be thermally equilibrated as well.
• Special care and attention should be given to the thermal equilibration of the tweezers that are holding the specimen grid.
• We strongly advise preheating the tweezers before they enter the chamber.

ู่ A Peltier element is chosen for heating or cooling the instrument with appropriate accuracy.
ู่ When the conditions chosen deviate from ambient conditions, time has to be allotted for equilibration; 5 minutes at 25°C, 45 min at 4°C or 55°C are typical figures.
ู่ The chamber of the Vitrobot could be used for equilibration or any incubator or water bath.
ู่ At a high relative humidity, a slight difference in temperature of the tweezers and the environment may result in condensation of water on the tweezers. The condensed water may flow down on the grid and dilute the solution.
ู่ Slightly overheating keeps you on the safe side.

1.2.4. Set up humidity conditions for vitrification

• The ultrasonic humidifier has to be filled with distilled water or demineralized water following the instructions of the manual.

ู่ Note that switching on the humidifier has an effect on the temperature as evaporation of water extracts heat. Although the software critically adjusts heat input and water evaporation, it can take some time to reach equilibrium temperature (> 40°C) and humidity (> 95% rH).

1.2.5. Condensing ethane

• The ethane container should be filled up to the rim to prevent precooling of the sample in cold gas before it enters the liquid ethane.

ู่ Note: Liquid ethane can cause severe burns and gaseous ethane may explode. Always work in a fume hood. Follow the safety rules of the manufacturer.
1.2.6. Checklist

- Control the temperature of ethane, which has to be about –172°C.

- Control (or load) all parameters in the user interface of the Vitrobot.

- Check the tweezers of the Vitrobot.

- Sample and pipette should be ready for application and eventually brought to the same temperature as the chamber.

- Replace blotting paper to ensure reproducible working conditions. Upon every blotting action, the blot-pads are turned to expose a fresh area of filter paper (16 blot actions to make a full turn).

Temperature can be checked with a thermocouple or by visual inspection. The coexistence of liquid and solid ethane indicates that ethane is at its melting point (see Chapter 3, Figures 3.12, 3.13).

Are the set temperature and humidity values already attained and stable?

Are they clean and at the same temperature as the chamber?

Liquid nitrogen, cryo-storage box and all necessary tools and utensils should be at hand.

The instrument keeps a record of the number of blotting actions and gives a message (“replace blot papers”) when a full turn is made.

Blotting papers are mounted on the foam pads with a clamping ring around the central hole.

2. SUMMARY OF THE DIFFERENT STEPS

Figure 4.5 Glow discharge grids.
Figure 4.6 Condense ethane (aluminum spindle placed on top of the chamber).

Figure 4.7 Replace filter papers.

Figure 4.8 Mount the grids onto tweezers.
Figure 4.9 Set vitrification parameters.

Figure 4.10 Apply the sample to grid.

Figure 4.11 Blot the sample.

Figure 4.12 Vitrify the sample.
3. MATERIALS/PRODUCTS/SAMPLES

3.1. Materials

- **Vitrobot**

- **Cryo-holder and transfer system**
  - The vitrified grid is mounted in a cryo-holder and then inserted into a cryo-TEM.
  - Alternatively, grids may be loaded into the special cartridge of a Tecnai Polara EM.

- **Electron microscope with low-dose imaging capabilities and low-dose software**
  - Cryo-samples are very sensitive to radiation damage caused by electrons.
  - The Low Dose SW suite has been developed to minimize the electron dose during searching, focusing and image acquisition of the specimen.
- Image acquisition
  - For single particle analysis (see Chapter 7), the Leginon SW suite allows automated particle picking and acquisition in the most optimal areas of vitrification.
  - For cryo-tomography (see Chapter 12), FEI’s Xplore 3D and Inspect 3D may be used for acquisition and reconstruction of low-dose tomograms.

- Image reconstruction
  - Various reconstruction packages exist for particle averaging and/or tomographic reconstruction analysis, e.g., Spider, eMan, Imagic (see Chapters, 7, 12, 24).

### 3.2. Products

- Grids
  - Quantifoil R2/2 (Quantifoil GmbH, Jena, Germany) or Lacey carbon film grids (various suppliers) are used for vitrification.
  - A typical mesh size is 300.
  - Grids need to be glow-discharged in order to make them hydrophilic.

- Grid box
  - Circular grid boxes that fit in the metal grid box holder of the ethane container are used. The grid boxes are provided with the Vitrobot (FEI Company), but can be easily self-made from a square grid box.

- Liquid nitrogen
  - Liquid nitrogen is used for precooling the container and for condensation of the coolant (e.g., propane or ethane).

- Ethane
  - Ethane with a purity > 99.9%.
  - Liquid ethane enables a very fast cooling rate (dT> 105K) in order to generate an amorphous ice layer.
  - **Note:** Liquid ethane can cause severe burns and gaseous ethane may explode. Always work in a fume hood. Follow the safety rules of the manufacturer.

- Filter paper
  - Provided with the instrument, Schleicher & Schuell 595 or Whatman/Schleicher & Schuell 597; both Ø 55 mm.
  - Any other filter paper may be used, but has to be tested for its blotting properties.

- Homemade punch
  - Used to prepare blotting paper by punching a hole in standard Ø 5 cm filter papers.
3.3. Samples

- Various aqueous suspensions: Concentration range may vary depending on original concentration/density.
- Isolated cells.

- E.g., proteins, protein complexes, viruses, bacteria or soft matter chemical substances, such as synthetic polymers.
- Cells in suspension or cultured as monolayers on carbon coated grids.

4. PROTOCOLS

1. Glow discharging the grids

   - Glow discharge helps to spread the applied solute and prevents the liquid film from breaking into smaller (micro) droplets after blotting.
   - Upon glow discharge, a grid will remain hydrophilic for several hours.
   - In extreme cases, the material may adhere to the support film instead of spanning the holes in solution.
   - Aging of the grids may reduce this tendency.

   **Figure 4.15** Bombardment of the grid with ionized air.

   The surface of the support film should be hydrophilic. A droplet applied should spread out and behave as one entity transforming into a continuous thin film.

2. Filling the humidifier.

   - Prior to activation of the Vitrobot, the humidifier must be filled with 60 mL distilled water through the plastic tube at the bottom of the apparatus using a syringe (see Figures 4.16, 4.17).

   **NOTE 1:** Be aware to fill the humidifier beaker with sufficient distilled water prior to enabling the ultrasonic humidification.

   **NOTE 2:** The humidifier has to operate on distilled water.

   **Figure 4.16** Filling the syringe.
3. Starting up the Vitrobot.

4. Defining the Vitrobot user interface.

- The Vitrobot User Interface consists of two pages: The Console and the Options screen (see Figures 4.19, 4.20). A variety of vitrification parameters can be set in both pages.

\[\text{It is important that a “vacuum” is created inside the syringe (by “pulling” the piston) to remove the “air” from the humidifier.}\]

\[\text{The humidifier is bayonet-attached to the bottom of the climate chamber. By turning and pulling, the humidifier can be removed.}\]

Figure 4.17 Filling the humidifier.

\[\text{Before starting, make sure all cables and wires are properly connected including the pressurized air link.}\]

\[\text{Activate the pressurized air switch and make sure pressurized air flows into the Vitrobot.}\]

\[\text{The Vitrobot is switched on with the hard lock switch on the backside of the machine.}\]

Figure 4.18. Switching on the Vitrobot.

\[\text{After activating the hard lock switch, the embedded computer, with MS Windows® operating system, automatically starts up. The start-up page appears shortly followed by the Vitrobot User Interface (see Figure 4.19).}\]

\[\text{On the console screen, the temperature can be set to any value between 4° and 60°C with the + and – buttons. The actual temperature read-out is displayed in red (21.8).}\]

\[\text{The humidity — displayed in blue (41.1) — in the climate chamber can be set with the + and – buttons.}\]

\[\text{Enable the humidity switchbox to start the evaporation (On/Off switch).}\]

\[\text{The light in the climate chamber can be switched on.}\]

\[\text{A chronometer records the experimental time. Once a specific time is set, the chronometer counts down displaying a counterclockwise movement.}\]
• In the Options screen, additional vitrification parameters can be set.

• The semiautomatic grid transfer (i.e., the automatic movement of the grid from the liquid ethane/propane toward the grid box in the liquid nitrogen atmosphere) is default activated, but can be deactivated by checking the skip box.

• Parameters that affect the blotting process are the number of blottings (blot total), the time of each individual blot (blot time) and the position of the grid between the blot pads (blot offset).

☞ In the Controls box on the right, the sequence of the complete vitrification process (Place New Grid, Start Process, etc.) or to Exit is displayed and operated.

☞ After 16 sequential blottings, the “reset blot paper” button becomes red pointing out that the blot papers need to be replaced.

☞ The Memo box on the left side of the interface functions as an event logger. All major actions and warnings are displayed.

Figure 4.19. The Console page.

☞ E.g., the time that the grid is dipped into the vial (plunge time), the relaxation time before blotting (wait time) and the level of the liquid in the vial (in case of dipping).

☞ Alternatively, manual application of a drop of suspension onto the grid through the right or left side entry port can be selected.

☞ In the Miscellaneous box, the use of a foot pedal switch (instead of the mouse) and the possibility to switch off the humidifier during manual application and plunge-freezing can be selected.

Figure 4.20 The Options page.

☞ In addition, all essential freezing parameters can be saved (Save) or loaded (Load) depending on the type of sample or experiment.

☞ If the “auto raise ethane lift” option is checked, the coolant container will be lifted toward the bottom of the climate chamber simultaneously with the uplift of the tweezers (this can also be done in separate actions).

☞ Blot offset determines the force with which the excess fluid is removed from the grid.

☞ Another factor of significance is the drain time, the time between blotting and plunge-freezing.
• One of the features in the Mark III Vitrobot is the option to do repetitive sample application onto the grid and subsequent blotting prior to plunge-freezing.

- To activate this function, press ADD and define the application parameters for the first substance that is to be applied on the grid.

- In the Processes section, the Process ID number, including subsequent parameters, are displayed.

- By pressing ADD again, the application parameters for the second substance can be defined and displayed in Processes as Process ID 2.

- Up to 20 application cycles can be added in this way (1, 2, 3, 4 to 20) – Figure 4.23.

**Figure 4.21** Repetitive Sample Application Processes.

- INS allows for inserting application parameters at a specific position in the sequence of events that has been defined (1, 2, 3, 4 to 20).

- By pressing DEL, selected application parameters can be removed from the sequence list.

- Besides setting the proper parameter conditions (*see* Section 4.4.), the LED lights are switched on, the pneumatic pressure control must be active and the blotting papers attached to the blot pads by using the white clipping rings (*see* Figure 4.23).

**Figure 4.22** Mounting the filter papers.

- A glow-discharged grid is attached to the tweezers. Make sure that the black clamping ring is fixed in such a way that the grid does not fall off in vertical position.

**Figure 4.23**
Picking up the glow-discharged grid.
Mount the tweezers onto the connection groove in the central axis. To do this, first select the ‘Place New Grid’ button in the Vitrobot User Interface to put the central axis into the loading position.

7. Preparation and lifting of the coolant container.

- Prior to setting the coolant container in the proper position for vitrification, it needs to be precooled and filled with liquid ethane.

Figure 4.24 Mounting onto the connection groove of the plunge axis.

The tweezers with grid are subsequently lifted into the climate chamber by selecting the Start Process button in the User Interface, or alternatively, use the foot pedal switch.

The outer ring of the container must be filled with liquid nitrogen. Cooling is a two-step process to be carried out in a fume hood; first, the peripheral reservoir will attain liquid nitrogen temperature; then the central part having a higher heat capacity will cool down. Vigorous boiling (‘Leidenfrost effect’) followed by a “calm” equilibrium indicates that the metal parts have attained liquid nitrogen temperature.

The central cup can be precooled with liquid nitrogen before filling with ethane or propane.

Figure 4.25 Cooling down the container.

Note: Wait for complete evaporation of the remaining liquid nitrogen in the central part.

To improve heat exchange/cooling of the condensing ethane, an aluminum “spindle” is placed on top of the cup. The spindle is a temporary heat conductor between the liquid nitrogen and the liquid ethane and should be positioned during condensing ethane and further cooling of ethane down to −172°C.

Figure 4.26 Filling the container with ethane.
When the central cup is at liquid nitrogen temperature, ethane or propane can be condensed. With appropriate pressure reduction, a gentle stream of gas is brought into contact with the cold metal surface where it condenses into a liquid. When enough ethane is condensed, the gas flow can be adjusted (slightly increased) to prevent clogging in the feed line (visual inspection will then tell that solid ethane is formed).

The ethane container should be filled up to the brim to prevent precooing of the sample in cold gas before it enters the liquid ethane.

Immediately after the appearance of the halo of solid ethane, the metal spindle must be removed.

The spindle may interfere by creating a cold gas atmosphere (about –160°C) that may precool the specimen before it enters the liquid coolant.

Thawing the frozen ethane between spindle and ethane cup, by placing a bolt on the spindle for 10 seconds, is a more careful way to remove the spindle.

Figure 4.27 Removal of the spindle.

When the ethane/propane container is ready for vitrification, it is placed on the platform ring under the Vitrobot. Then the foot pedal switch or the Continue button in the User Interface must be pressed in order to raise the container toward the bottom of the climate chamber.

Figure 4.28 Placing the container onto the platform ring.

For manual application of the sample, select Manual Application in the Process parameters of the Options page.

Figure 4.29 Setting the application parameters.
• The liquid sample can be manually applied to the grid through the left- and/or right-hand side entry port using a pipette.

When the foot pedal switch is pushed or the mouse bottom (Continue to proceed) is pressed, the tweezers slightly lower to allow the application of suspension through the side-entry port using a pipette. As a consequence, the suspension is applied on one side of the grid only. The advantage of this method is that only small volumes of the sample (typically 3 µL) are used.

Figure 4.30 Manual sample application.

• Excess suspension must be removed from the grid prior to plunge-freezing.

Select Continue in the user interface or use the foot pedal. This activates a slight uptake of the grid toward the correct position between the blot pads and a subsequent blotting of the grid. After each blot, the blot pads undergo a slight rotation to ensure a clean, new area of filter paper for the next blotting.

Figure 4.31 The blotting process.

• The blotting procedure is immediately followed by injection of the tweezers with the grid into the liquid ethane or propane. The only delay between blotting and plunge-freezing is determined by the drain time that can be set in the Options page and the time required for removing the shutter from the hole in the climate chamber.

The actual plunging mechanism is mediated by pneumatics at the central axis combined with the gravitational force.
10. Transfer of the vitrified sample.
- After vitrification, the frozen grid must be transferred into a storage box or mounted into the cryo-holder.

☞ After plunge-freezing, both the liquid coolant container and the tweezers with the grid are automatically and simultaneously lowered while keeping the grid in liquid ethane. This prevents contamination and rewarming of the freshly frozen sample.

☞ When the “semiautomated grid transfer” is active, the grid is automatically transferred from the liquid ethane into the liquid nitrogen.

☞ The tweezers must be carefully disconnected from the central axis prior to positioning the grid into the grid box.

Figure 4.32 Removal of the tweezers.

☞ To make the grid transfer more convenient, the coolant container may be lifted from the support ring and positioned next to the Vitrobot.

☞ The excess of liquid ethane on the grid can be limited by squeezing the tweezers’ tips together and lifting the grid slowly from the ethane surface while observing how the liquid film detaches from the grid.

☞ The anticontamination ring, which floats on the liquid nitrogen, creates a cold gas atmosphere, which facilitates the transfer and minimizes possible ice contamination on and rewarming of the grid.

Figure 4.33 Transfer of the grid from the liquid ethane into the liquid nitrogen.

☞ The outer ring contains a circular storage grid box for four grids underneath a layer of liquid nitrogen.
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11. Shutting down/switching off the Vitrobot

- When pressing the Exit button in the interface, two questions appear:
  - Pair of tweezers removed? Confirm.
  - Pair of tweezers not removed? Confirm.

- After shutting down the Vitrobot PC, the remaining water in the humidifier should be removed. Pull the metal ring connector downward to disconnect the electronic cable from the humidifier.

\[\text{NOTE: To prevent bacterial growth in the preheated water of the humidifier, it is advisable to drain the water supply at the end of each working day.}\]

\[\text{After the grids have been transferred into the grid box, the box is sealed with a special screw and stored in a Dewar with liquid nitrogen.}\]
\[\text{The transfer of the grid box into the Dewar should be done swiftly to minimize the risk of rewarming.}\]

Figure 4.34 Loading the grid into the grid box.

Figure 4.35 Switching off message.
Twist and pull the humidifier to unleash the bayonet connection.

Emptying the humidifier is performed in two steps:
- Drain the humidifier to empty the central reservoir.
- Reconnect the syringe to the plastic tube at the bottom and remove the water from the outside reservoir.

**Figure 4.36** Emptying the humidifier.

## 5. ADVANTAGES/DISADVANTAGES

### 5.1. Advantages

- Fully automated and reproducible vitrification of aqueous suspensions.

- High vitrification quality through controlled environment.

- The liquid coolant container with an anticontamination device.

- The transfer from the vitrification medium into the liquid nitrogen has been automated.

- Easy to Use.

- All essential vitrification parameters — temperature, relative humidity, number of blottings, blotting pressure and drain time — can be programmed for each individual application and stored.

- High sample throughput.

- The Vitrobot provides a controlled environment, preventing cooling of the specimen and concentration of solutes due to evaporation before freezing.

- These artifacts are inevitable when using conventional freezing apparatus.

- More efficient “after-freezing” handling.

- More constant and high yield sample output.

- Cryo-fixation has become easier with the newly designed and software controlled Vitrobot User Interface.
5.2. Disadvantages

- The Vitrobot is designed for vitrification of aqueous suspensions only.

- Monolayer cell cultures can also be vitrified.
- In special cases, material suspended in organic solutes can be vitrified as well.
- Ethane, however, cannot be used as coolant because it is a “lipid solvent” and it often dissolves organic solutes even at low temperatures.
- Decalin and acetone are examples of organic solutes that can be vitrified upon cooling in (the inert) liquid nitrogen, but not all organic solutes will vitrify under these conditions (see Chapter 17).

6. WHY AND WHEN TO USE A SPECIFIC METHOD

6.1. Preparation of a Suspension

- Use of the standard vitrification technique (see Section 4.2) that avoids adsorption, staining and dehydration artifacts.

- Best structural preservation.
- Most suitable to recover excellent structural information upon image analysis.
- Most suitable to detect functional conformational changes and to analyse specimen dynamics.

Figure 4.37 Cowpea mosaic virus (CPMV) embedded in a thin layer of plunge-frozen water.

- Recommended starting conditions for suspensions of macromolecules (2 mg/mL) in aqueous buffers; blotting time of two seconds, one single blotting operation at 100% humidity, a temperature of 21°C. Setting blot offset at 0 mm is also a good point to start.
- Depending on the observed results, the parameters can be changed accordingly.
- In case the ice layer is too thick, change the blotting time to three seconds.
If ice is too thin or no sample is left, change the blotting time to one second. Also, the blot offset, i.e., blotting pressure on the grid, can be adjusted. Going up in offset (positive offset values) will leave more sample on the carbon film because the pressure on the grid will be lower. Going down (negative offset values) will remove more of the sample.

Figure 4.38 Herpes virus capsids imaged in a patch of vitreous ice.

6.2. Preparation of a Viscous Sample

6.2.1. Gels

For a sample with higher viscosity than water, longer blotting time (e.g., five seconds) is advised. A sample containing long fibers like DNA will have a gel-like behavior and most often needs to be blotted longer.

Figure 4.39 Shampoo at 25,000×.

6.2.2. Creams

For even more viscous material, an extra number of blots may be helpful. Creams are difficult to prepare in a film thin enough to be penetrated with electrons. Good results have been obtained with one second blotting time and three blots. Sometimes it is useful to use 300 mesh or 400 mesh bare grids to obtain a large area of thin material in the center of a grid square.

Figure 4.40 Handcream at 25 000×.
6.3. Preparation of Cells

6.3.1. Bacteria

A suspension of *Escherichia coli* bacteria can be vitrified under similar conditions as a diluted suspension of macromolecules.

![Bacterium in a patch of a frozen sample.](image)

**Figure 4.41** Bacterium in a patch of a frozen sample.

6.3.2. Cells growing on an EM grid

Growing cells directly on the EM-grid covered with a thin carbon film is a fast way of observing cellular structures.

This is, however, only possible in thin parts of the cell, e.g., at its circumference or in filopodiae.

![Rat liver endothelial cell, isolated and cultured on an EM grid and frozen in liquid ethane.](image)

**Figure 4.42** Rat liver endothelial cell, isolated and cultured on an EM grid and frozen in liquid ethane.
7. OBSERVED RESULTS

ียว Single particle image reconstruction of a set of 700 Cowpea mosaic virus (CPMV) images.
ียว A typical 3-D map of 700 views has a resolution of 2 nm. Clear structural features can be observed.

Figure 4.43 CPMV from 700 views.

мышл Vitrified microtubules imaged using low-dose mode.

Figure 4.44 Microtubules are reconstructed by single particle reconstruction.
T4-phage GP23 proteins form tubes of different diameters. Tomograms of these tubes provide more information on their organization.

Figure 4.45 Tubes from GP23 of T4-phage.

Polymer-layered silicate (PLS) nanocomposites were subjected to plunge-freezing and low-dose cryo-electron tomography.

The field of nanomaterials is a fast developing area where the unique properties of inorganic-layered silicates are investigated.

Figure 4.46 Laponite clay-enriched nanoparticles by cryo-electron tomography and reconstruction (see Chapter 12).
8. REFERENCES


