Sample vitrification using Vitrobot Mark IV

General considerations:

Be careful when handling liquid nitrogen or things in contact with it. Wear closed-toe shoes, long pants and appropriate PPT (goggles or face shield, gloves, lab coat).

All tools that are near or contact vitrified grids need to be cooled down at liquid nitrogen temperature (past the Leidenfrost point; wait for several minutes after 'boiling' stops) to avoid ice devitrification.

Note: Pictures in this protocol were made in the absence of cryogens (to maintain good visibility) and without the filter paper installed on the blotting pads (to avoid wasting the paper). First, practice the steps in the protocol without the cryogens.

Overview

The purpose of this protocol is to prepare vitrified grids of a biological sample (proteins, cells, etc., "frozen in time" as they existed in solution at the time of the freezing, without structural alterations due to the freezing process) using a TF Vitrobot Mark IV (Fig. 1a). The Vitrobot has a digital interface (**DI**) in which you can change vitrification conditions (e.g., temperature, blot time, blot force, relative humidity, etc), a chamber (**C**) that contains the blotting pads (**BP**) and is accessible on both sides for sample application (the place where the sample is pipeted on the grid and then "thinned out" by blotting), and a cup assembly (**VC**) with two main compartments – a central one where ethane/propane is liquified for rapid sample freezing (**VC_c**), and a peripheral one (**VC_p**) for liquid nitrogen (used to cool the ethane and keep cold the prepared vitrified grids in the grid box). The grid is moved in different areas of the Vitrobot (e.g., Vitrobot chamber, Vitrobot ethane cup, etc) by a plunger (**P**) on which the Vitrobot tweezers (**VT**, which hold securely the grid), are reversibly attached. The relative humidity in (**C**) is generated with a humidifier (**H**), using distilled water. The process of grid vitrification involves the following steps:
1) the grid is secured on (**VT**), which in turn are fixed on (**P**) (done by user)
2) the (**P**) holding the (**VT**) moves up in the **(C)** (done by the Vitrobot)
3) the (**VC**) containing liquified ethane/propane (**VC_c)** and liquid nitrogen in (**VC_p**) along with a grid box is positioned on its Vitrobot platform by the user and then raised by the Vitrobot close to (**C**),

Figure 1. (a) **Vitrobot overview**. (b) Vitrobot cup disassembled (left) and assembled (right) (c) Vitrobot tweezers. (d) Humidifier.

4) the plunger positions the grid (done by Vitrobot) so that the sample can be applied through the side ports of (**C**) using a pipet (done by user),
5) the plunger moves the grid between the blotting pads which then move in and blot the excess liquid from the grid (using the blot parameters set up by the user) (done by the Vitrobot)
6) the plunger moves the grid from (**C**) into the liquid ethane (**VC_c**) and then the Vitrobot moves together (**P**) and (**VC**) so that the grid is kept in liquid ethane (done by Vitrobot)
7) the user detaches the (**VT**) from the plunger and moves the grid from the liquid ethane (**VC_c**) to the liquid nitrogen compartment (**VC_p**), where the grid is placed in a slot in the grid box.
Vitrified grids can then be clipped to be loaded in a cryo-TEM for collection of 2D images/movies (as desired for single particle analysis, tomography or imaging for sample quality control).

I. Preparation

I1. Make sure you have the following (see Figs 2 & 3):
- grids
- grid holder block (1) for glow-discharging
- fine-tip tweezers for grid handling (2)
- Vitrobot tweezers (**VT**)
- grid boxes with lids (4)
- tools for opening/closing grid box lids (5)
- Vitrobot blotting paper (6)
- sample to be vitrified
- Vitrobot cup (**VC**)
- Vitrobot ethane cup (**VEC**)
- Vitrobot spider for cooling the ethane cup (**VSp**)
- Vitrobot box holder (**VB**)
- Vitrobot anti-contamination shield (**VSh**)
- distilled water
- heating block or hair dryer to warm up tools if needed
- 10-20 ul pipet and pipet tips
- liquid nitrogen in a dewar (4L)

Figure 2. Tools for vitrification.

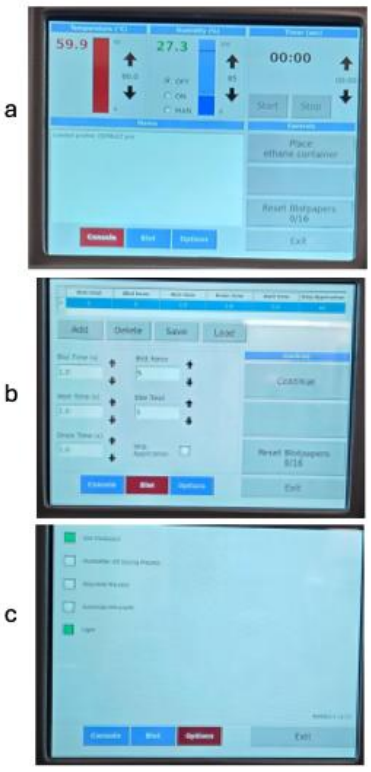
Figure 3. Vitrobot cup components (left) and its assembly (right)

I2. Prepare the Vitrobot:

<u>I2.1. Attach and prepare the humidifier</u> <u>(Fig. 4).</u>
I2.1.1. Connect the power cord coming from the bottom part of the Vitrobot (see Fig. 4c, white arrow) to the humidifier (**H**) by lining up the red dots (Fig. 4a, white arrows) on the humidifier and power cord.
I2.1.2. Turn the humidifier vertically so that the written label is towards you (Fig. 4b), then push (**H**) upwards in the bottom opening in (**C**) and rotate left (**H**) to seal it on (**C**) (Fig.4 b & c).

I2.1.3. Fill a 60-ml syringe with distilled water. Connect the syringe to the humidifier tube at the bottom (red arrow) and fill the (**H**) with water by pushing the syringe plunger. When the syringe is empty, while still connected to the plastic tube, pull the syringe plunger to create under-vacuum in the syringe and thus eliminate any trapped air from (**H**).

Figure 5. The windows in (DI): (a) " Console", (b) "Blot" and (c) "Options".



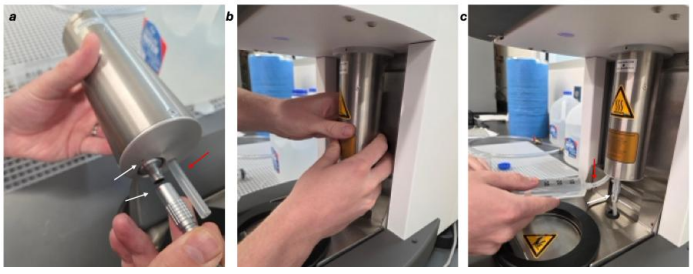
<u>I2.2. Prime the Vitrobot</u>.

I2.2.1. Turn on the Vitrobot by flipping the switch in the back (bottom-right). The screen **(DI**) will turn on, and the Vitrobot plunger will move in position. You can select on the screen three windows (they appear at the bottom of (**DI**), see Fig. 5): "**Console**" (in which you can specify values for temperature and humidity and can advance the vitrification step, for example plunger position or movement of the Vitrobot cup platform, Fig. 5a), "**Blot**" (in which you can specify blotting parameters, including blot time and force, drain and wait times, etc., Fig. 5b), and "**Options**" (in which you can turn on the light in the Vitrobot chamber, select to use the pedal to advance the vitrification steps, etc., Fig. 5c).

I2.2.2. Using the stylus or your finger, select on the (**DI**) windows (Fig. 5) the desired parameters (temperature, humidity, blotting conditions, operational preferences – use of footpedal, etc.). For example, 4 °C, 100% humidity (in "**Console**"), 4 s blot time, blot force 0, drain time 0 s, wait time 0 s (in "**Blot**"), check 'Use footpedal' and 'Skip grid Transfer'(in "**Options**").

Note: The necessary parameters to set at this point are the temperature and humidity ("**Console**") because it takes some time for the Vitrobot chamber to reach the set values. Blot parameters ("**Blot**") and vitrification preferences ("**Options**") can be set up just before starting the vitrification.

Figure 4. Humidifier installation: power cord connection (a), sealing of humidifier at the bottom of Vitrobot chamber (b) & filling of humidifier with water (c) through the plastic tube (red arrow in (a) & (c)).



I2.2.3. Wearing gloves, fix the Vitrobot blotting paper (6 in Fig. 2) using the white circular rings (e.g., see Fig. 7f, at the bottom left of the blotting pads) onto the Vitrobot blotting pads.

Note: some people prefer to do this step right before starting the vitrification to avoid filter paper getting affected by the high humidity, other people prefer to let the filter paper in the chosen conditions to equilibrate for 20-30 mins.

I3. Prepare the grids, boxes and sample

Note: While handling grids at room temperature, do not wear gloves (static electricity can make the grids move).
I3.1. Prepare the grids for glow-discharging. With fine-tip tweezers (2), take out the desired number of grids from a grid box and place them with the carbon side up (or shiny, metallic face down) on the seats in the grid holder block (1 in Fig. 2).
I3.2. Note the grid boxes (number, color, etc.) for storing your vitrified grids and place them in the Vitrobot grid box holder (**VB**, Fig. 1) without their lids.
I3.3. Have the sample handy, and prepare the pipette for the desired volume (3-4 ul).

I4. Prepare the glow-discharger:

- **Write how to change the head of the coater**

If needed, change the parameters (e.g., pressure, current, polarity, glow-discharge time) as desired, by pressing next and changing the corresponding settings. For carbon grids, typical settings are: pressure 0.25 mBar, current 20 mA, glow time 30 s and hold time 10 s.

II. Grid freezing

II1. Prepare the Vitrobot cup for vitrification:
II1.1. Assemble the Vitrobot cup (Figure 3, right):
II1.1.1. Put the ethane cup (**VEC**) in the central compartment of (**VC)** (**VC_c**).
II1.1.2. Sit the box holder (**VB**) containing the desired grid boxes (without lids) in (**VC_p)**.
II1.1.3. Position the cooling spider (**VSp**) on top of (**VEC**) (**VSp** is used for cooling faster **VEC**).
II1.1.4. Put the anticontamination shield (**Vsh**) in (**VC_p**) (this will create an atmosphere of liquid nitrogen vapor above **VEC**, helping with the grid transfer between (**VEC)** and (**VC_p**)).
II1.2. Make liquid ethane/propane in VEC:
II1.2.1. Pour liquid nitrogen into (**VC**), including (**VEC**), to cool it down. As nitrogen boils vigorously and evaporates, keep pouring liquid nitrogen in (**VC_p**) until the cooling spider (**VSp**) and box holder (**VB**) are completely cooled and the liquid nitrogen in (**VEC**) has completely evaporated.
II1.2.2. Turn on the large valve of the ethane/propane cylinder (**do not touch the regulator**, it has been set at the right pressure by staff) and insert the tube tip into (**VEC**) until it touches the bottom. Slowly open the close-by, small valve that controls the flowrate of the ethane and adjust the flowrate as the ethane liquifies (you'll hear a hissing sound when ethane liquifies).
II1.2.3. Once the liquid ethane level reaches the top of (**VEC**), reduce the ethane flow and take the tip out. Open completely the ethane small valve to clean out the ethane tube of any moisture, then close both the small and large valves of the ethane tank and replenish (**VC_p**) with liquid nitrogen.
II1.2.4. Take out the cooling spider (**VSp**) with the large tweezers. Now the Vitrobot cup is ready to be used for grid vitrification.

II2. Glow-discharge the grids
Note: Glow-discharging the grids makes the carbon film hydrophilic so that the sample can spread uniformly on the grid. Glow-discharged grids should be used within ~ 30 mins.

- Ensure the glow-discharger is ready (see I4)
- After the end of the glow discharge, wait for the pressure in the chamber to reach atmospheric pressure, then carefully rotate the transparent cylinder to ensure the pressure difference between the inside and outside has equilibrated. Remove the cylinder to access the grid block and take it out. The grids are ready for vitrification.

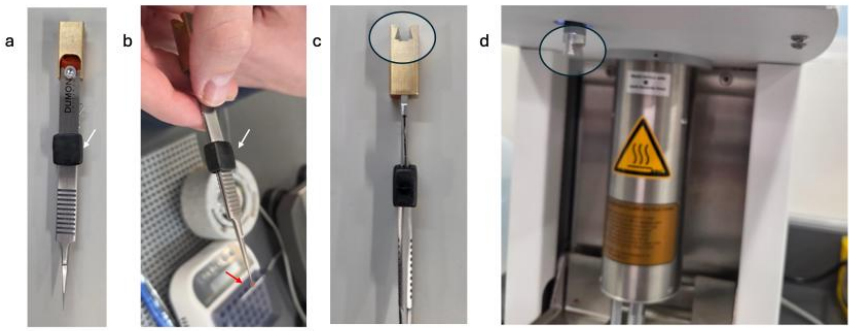
II3. Grid vitrification

Notes:

- Activation of steps during the vitrification cycle can be done by pressing on the foot pedal (if it was selected in the "**Options**" window) or by pressing with the stylus (or your finger) on the desired action in the "Controls" panel (present in "**Console**" and "**Blot**" windows). The next step in the vitrification process appears at the top of the "Controls" panel.
- Before starting, ensure that (**C**) has reached the desired temperature and humidity (you set up in I2.2.2).

II3.1. Pick up a grid from the grid block (after glow-discharging) with the (**VT**) and secure the tweezers by sliding the black rubber stopper until the first or second notch (Fig. 6b). Lightly tap the tweezers to ensure the grid is held well. ***Note***: Moving up the black stopper allows release of the grid, moving the stopper in the 1st or 2d notch (**VT**) keeps the grid on (**VT**).

Figure 6. Vitrobot tweezers (**VT**) before (a) and after (b) picking up the grid (red arrow). Notice the position of the black stopper (white arrows) used to secure the grid. The head of (**VT**) (c) is complementary in shape to the Vitrobot plunger head (d), so that you can slide (**VT**) back and forth at the end of the plunger.



II3.2. In the "Controls" panel, press on 'Place new grid'. The plunger (**P**) will come down through and out of (**C**) so the tweezers with the grid can be mounted at the bottom of (**P**).

II3.3. Slide the plunger adaptor of (**VT**) (Fig. 6c) onto the tip of (**P**) (Fig. 6d) so that the (**VT**) is centered on (**P**) tip, ensuring that the carbon side of the grid is towards the side for sample application (right, if right-handed) (Fig. 7a).
II3.4 In "Controls" panel press on 'Continue'. The (**VT**) will be pulled into the (**C**) and the chamber bottom white shutter closes covering the exit hole for (**P**) (Fig. 7b).
II3.5. Put the (**VC**) onto the circular support underneath (**C**) (Fig. 7c) and press 'Place ethane cup' and 'Continue' in "Controls" panel. The cup will be raised until it touches (**C**) (Fig. 7d).
II3.6. In "Controls" press on 'Start process'. (**P**) will position (**VT**) so that the sample can be applied to the grid (Fig. 7e). With a pipette, take 3-4 ul of sample and guide the pipette tip through the small side door of (**C**) until it is very close to the grid. Without touching the grid, release a bit of the solution until it touches the grid, then release all the liquid (Fig. 7f).
II3.7. Remove the pipette, close the side door, and activate 'Continue'. The Vitrobot will place the grid between the blotting pads and will blot the grid as specified in the program (I2.2.2) (Fig. 7g). Then, it will plunge the grid into the Vitrobot cup underneath, and the plunger and Vitrobot cup will move down together (Fig. 7h).
II3.8. Slide out (**VT**) from (**P**) carefully, keeping the grid centered in (**VEC**) and ensuring the grid does not hit (**VEC**). Move aside (so you have better visibility) the (**VC**) together with (**VT**) maintaining the tweezers centered in the (**VEC**) so that the grid is always in ethane (Fig. 7i).
II3.9. Swiftly, move the grid from (**VEC**) to (**VC_p**), in liquid nitrogen, close to your grid box (Fig. 7j). Slide the black rubber up while keeping the tweezers closed on the grid with your hand (this will allow you to position the grid in the grid box and release it there). Position the grid on top of the

Figure 7. Illustration of the grid vitrification process.

desired slot in the grid box and carefully release the grid by opening your grip of the tweezers (Fig. 7k).
II3.10. Repeat the grid vitrification cycle (II3.1-9) with the rest of the planned grids.
II3.11. After a grid box gets full, cool down a box lid attached to a box opening tool (5 in Fig. 2) in (**VC_p**), away from the grid box, (or cool it in a nearby liquid nitrogen container) and carefully close the box by rotating the tool and lid clockwise. Then, release the grid box into liquid nitrogen storage (within **VC_p** or somewhere else) by pushing the button on the lid tool.

III. Additional Notes

Movies showing the procedures:

For Vitrobot preparation & grid vitrification:
https://www.youtube.com/watch?v=QML_KMQbOMc
https://www.youtube.com/watch?v=gfY4b-bCLg8

For further details refer to the Vitrobot Mark IV manual.

- For viscous solutions, blot time and pressure can be increased. With low viscosity sample, if ice is too thick, increase the number of blotting actions. With high viscosity samples, increase the blotting time before increasing the number of blotting actions.
- Time for equilibration: 5 mins for 25 C, 45 mins at 4 C or 55 C. Sample solutions should be thermally equilibrated. At high relative humidity, small difference in temperature of tweezers and environment -> condensation of water on tweezers, which may reach the grid diluting the sample. Preheat the tweezers before they enter the vitrobot chamber.
- Initial thickness is dictated by blot time; further thinning occurs during draining process (e.g., drain time 10 s).