Cryo-electron Microscopy of Vitreous Sections

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Chapter 10

Cryo-electron Microscopy of Vitreous Sections

Petr Chlanda and Martin Sachse

Abstract

More than 30 years ago two groups independently reported the vitrification of pure water, which was until then regarded as impossible without a cryoprotectant [1, 2]. This opened the opportunity to cryo-electron microscopy (cryo-EM) to observe biological samples at nanometer scale, close to their native state. However, poor electron penetration through biological samples sets the limit for sample thickness to less than the average size of the mammalian cell. In order to image bulky specimens at the cell or tissue level in transmission electron microscopy (TEM), a sample has to be either thinned by focused ion beam or mechanically sectioned. The latter technique, *C*ryo-*E*lectron *M*icroscopy *o*f *Vi*treous *S*ection (CEMOVIS), employs cryo-ultramicrotomy to produce sections with thicknesses of 40–100 μm of vitreous biological material suitable for cryo-EM. CEMOVIS consists of trimming and sectioning a sample with a diamond knife, placing and attaching the section onto an electron microscopy grid, transferring the grid to the cryo-electron microscope and imaging. All steps must be carried on below devitrification temperature to obtain successful results. In this chapter we provide a step-by-step guide to produce and image vitreous sections of a biological sample.

Key words Cryo-ultramicrotomy, Cryo-transfer, Cryo-transmission electron microscopy, Trimming, Sectioning, Vitrification

1 Introduction

Conventional electron microscopy of cells and tissue, which consists of chemical fixation, dehydration at room temperature, and resin embedding, suffers from several artifacts such as material aggregation and extraction. In cryo-EM these artifacts are avoided, and the sample can be imaged close to a native state. However, resolution in cryo-TEM as in conventional TEM is limited by the imaging setup of the microscope itself and by sample thickness. Cryo-electron tomography (cryo-ET) can be applied to study plunge-frozen whole cells but it is restricted to a thin (<500 nm) periphery of the cell whereas the central area remains inaccessible. Cryo-ultramicrotomy technique—CEMOVIS, developed by Jacques Dubochet and coworkers, enables a scrutiny of the cells and tissue by cryo-EM [3–5]. In this technique a life specimen

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(i.e., cell suspension or tissue with a thickness up to 200 μm) is vitrified by high pressure freezing, directly sectioned in the cryo-ultramicrotome and subsequently imaged in a cryo-electron microscope. Since CEMOVIS does not necessarily involve sample purification, fixation, and no dehydration is required; it is an ultimate and reference technique for structural cell biology. However, CEMOVIS comes at a price. It is not an absolutely artifact-free technique: sections are impaired with mechanical artifacts as compression, knife marks and crevasses [6]. In fact, precise sectioning of vitreous biological samples is technically difficult, and it would not be possible without long-standing efforts in microtome and diamond knife development.

CEMOVIS can be combined with cryo-electron tomography (cryo-ET) in a technique refereed as *T*omography *o*f *Vi*treous *S*ections (TOVIS) [7–9]. This opens up the possibility to retrieve three-dimensional and high-resolution information from vitreous sections. However, section undulations and incomplete attachment to the grid support result in large eucentric height differences on a small area; hence, the tilt-series collection and alignment is challenging. A recently developed technique to attach sections to the grid by electrostatic charge rather than by mechanical force improves section attachment to the grid and makes TOVIS more feasible [10]. In spite of sectioning artifacts the macromolecules within the vitreous section have preserved molecular structure as tested by electron diffraction [11, 12]. Cryo-ET, together with subtomogram averaging, provides structural details of intracellular material with a molecular resolution. Subtomograms can be extracted from tomograms of vitreous sections and a structure obtained from X-ray crystallography can be fitted into the electron density of an average subtomogram [13].

Although CEMOVIS is a priori incompatible with immunolabeling, vitreous sections can be imaged in the cryo-fluorescent microscope utilizing endogenously expressing fluorescent fusion protein. The fluorescent image can then be correlated with cryo-electron micrograph in order to obtain the location of fluorescently tagged protein within the cell [14].

The current technical limitation of CEMOVIS is sectioning artifacts. The size of the crevasses increases with the increasing thickness of the section and a search for a region of interest in the electron microscope with a thickness higher than 100 nm is difficult due to the high electron density of crevasses [6]. Thus, a future technical challenge is to further minimize sectioning artifacts by using specialized diamond knives, for example, an oscillating knife manufactured by Diatome [15] to produce thick sections (300 nm), which can be used for cryo-ET. Another challenge is to localize proteins in vitreous sections with high precision using correlative light electron microscopy strategically applied for Lowicryl sections [16].

CEMOVIS can be applied to answer both cell and structural biology questions, and with cryo-electron tomography and correlative light electron microscopy, it opens an avenue to a structural cell biology in which the macromolecule structure can be studied in the native environment of a cell.

2 Materials

2.1 Equipment

1. Cryo-microtome either UC6/7 equipped with FC6/7 cryo-chamber (Leica Microsystems, Vienna, Austria) (*see* Fig. 1c, d, e) or PT-X/XL equipped with CR-X cryo-chamber (RMC, Tucson, AR) (*see* Fig. 1a, b).
2. Static line ionizer (Diatome AG, Biel, Switzerland) or Leica EM crion (AC/DC version) (Simco, Lochem, The Netherlands) (*see* Fig. 8a-d).
3. Cryo-diamond knife for sectioning (35° angle) and cryo-diamond knife for trimming with 45° trimming angle (Diatome AG) (*see* Fig. 4c).
4. Single Tilt Liquid Nitrogen Cryo-Transfer Holder, Model 626 (Gatan, Pleasanton, CA) (*see* Fig. 9a).
5. Gatan 655 Dry Pumping Station (Gatan).
6. Gatan SmartSet Cold Stage Controller (*see* Gatan) (*see* Fig. 9a).
7. TEM CM120 (Philips, Eindhoven, The Netherlands).

2.2 Tools

1. Eye lash glued with nail polish on wooden stick (*see* Fig. 2a).
2. Containers for transfer (e.g., lid of 50 ml falcon tube).
3. Fine forceps for handling grids (*see* Fig. 2b).
4. Cryo-tools (Leica Microsystems) (*see* Fig. 2c, d).
5. Cryo-gridbox (*see* Fig. 2e).
6. TEM grids (1000 mesh copper grids with carbon film, or C-flat grids, or quantifoil 7U/7U grids with an additional layer of carbon film).
7. Cryo-glue (mixture of ethanol and 2-propanol in a 1:3 ratio).
8. Dextran (35–45 kDa) in PBS or in another solution.

2.3 Samples

Unicellular organism can be frozen either in copper tubes (outer diameter 650 μm, inner diameter 300 μm) using the Leica EM-pact/EM-Pact2 (Leica microsystems) or in gold tubes (*see* Fig. 3b, c) (outer diameter 300 μm, inner diameter 200 μm; Goodfellow Corporation, Oakdale, CA) with the Baltec HPM010 (Abra-fluid, Widnau, Switzerland). The addition of cryoprotectant in the surrounding medium for the sample is mandatory here to vitrify the

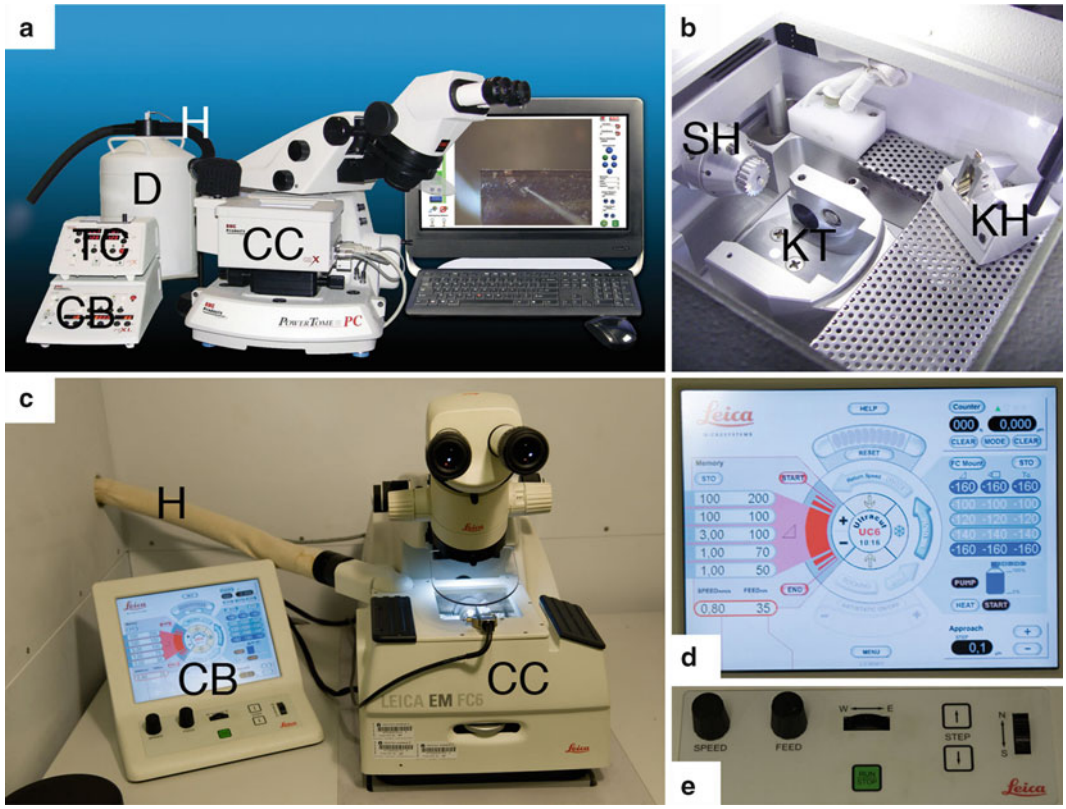

Fig. 1 Overview of the two commercially available cryo-microtome systems. (**a**) RMC PC microtome equipped with a crX cryo chamber (CC). The lower control board (CB) regulates the microtome settings whereas the upper control board (TC) sets the temperature in the cryo chamber. A hose (H) connects the dewar (D) with the pump to the cryo chamber. (**b**) View into the cryo chamber with the sample holder (SH) and knife holder (KH) to be inserted into the knife table (KT). (**c**) A Leica UC6 microtome equipped with a FC6 cryo-chamber (CC), which is connected via a hose (H) to the pump and the dewar with liquid nitrogen (not shown). Left of the microtome is the control unit (CB), which consists of a touch screen and a part for mechanical settings. (**d**) Touch screen of the UC6. On the right side of the screen the settings for the speed and nominal feed of sectioning can be selected with four stored settings. On the left side is the temperature control with four stored settings. In the centre the three different light sources are regulated as well as settings of the cutting window. The lower part allows for regulating the strength of the ionizer/crion system. The upper part shows the progress of the arm of the microtome and its reset function. (**e**) Lower part of the control unit for the mechanical settings. The speed for sectioning is adjusted with the most left button. The second left button allows setting the nominal feed for sectioning. With the central wheel the knife table is moved in an east-west direction. The wheel on the right moves the knife table in a north–south direction for the approach to the sample. A stepwise approach can be done with the two buttons next to the wheel. The central green button starts and stops the automatic sectioning

surrounding medium as well. For this we use 20 % high molecular weight dextran (35–45 kDa) in PBS or in another solution, if required. Tissue can be frozen in the aluminum/brass planchette holders (*see* Fig. 3a) with a depth of 200 μm using the HPM010.

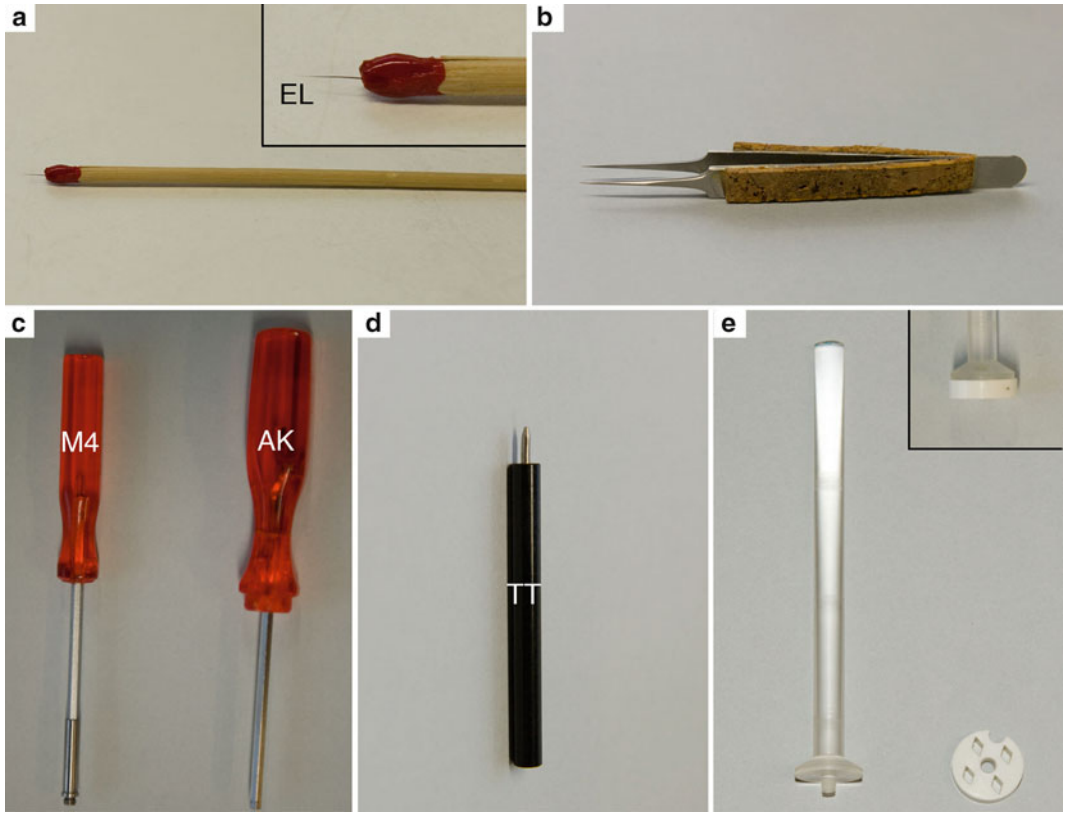

Fig. 2 Tools for manipulation inside the cryo-chamber. (**a**) Eyelash (EL) mounted on a wooden stick with nail polish. The insert shows a higher magnification. (**b**) Fine forceps (Dumont No 5) for the handling of the grids in the cryo-chamber. Cork plates were glued with epoxy resin to the side of the forceps to isolate the forceps and to avoid burning of the fingers during longer manipulations. (**c**) On the *left* the key with the M4 thread (M4) to lift out knives or the knife holder from the cryo-chamber. On the *right* is the Allen key (AK). (**d**) The tightening tool (TT) to fix the sample in the sample holder, knife in the knife holder and to adjust the clearance angel on the knife holder. (**e**) Homemade cryo-grid box. The long handle of the lid is convenient for manipulation in the cryo-chamber of the microtome

3 Methods

3.1 Cryo-Sectioning

3.1.1 Preparation of the Cryo-Chamber

1. Fill the dewar of the microtome with liquid nitrogen (LN_2) and insert the pump slowly. Connect the pump with the hose and start cooling down the cryo-chamber to −140 °C.
2. Insert the sample holder (*see* Fig. 3g) into the microtome arm and tighten the torque limited screw with the Allen key (*see* Fig. 5a).
3. Before placing the knife holder (*see* Fig. 4a) in the cryo-chamber set the clearance angle to a value of 6° (*see* Fig. 4b), insert the trimming knife to the left position of the knife holder and fix it using the tightening tools (*see* Fig. 4c, d).

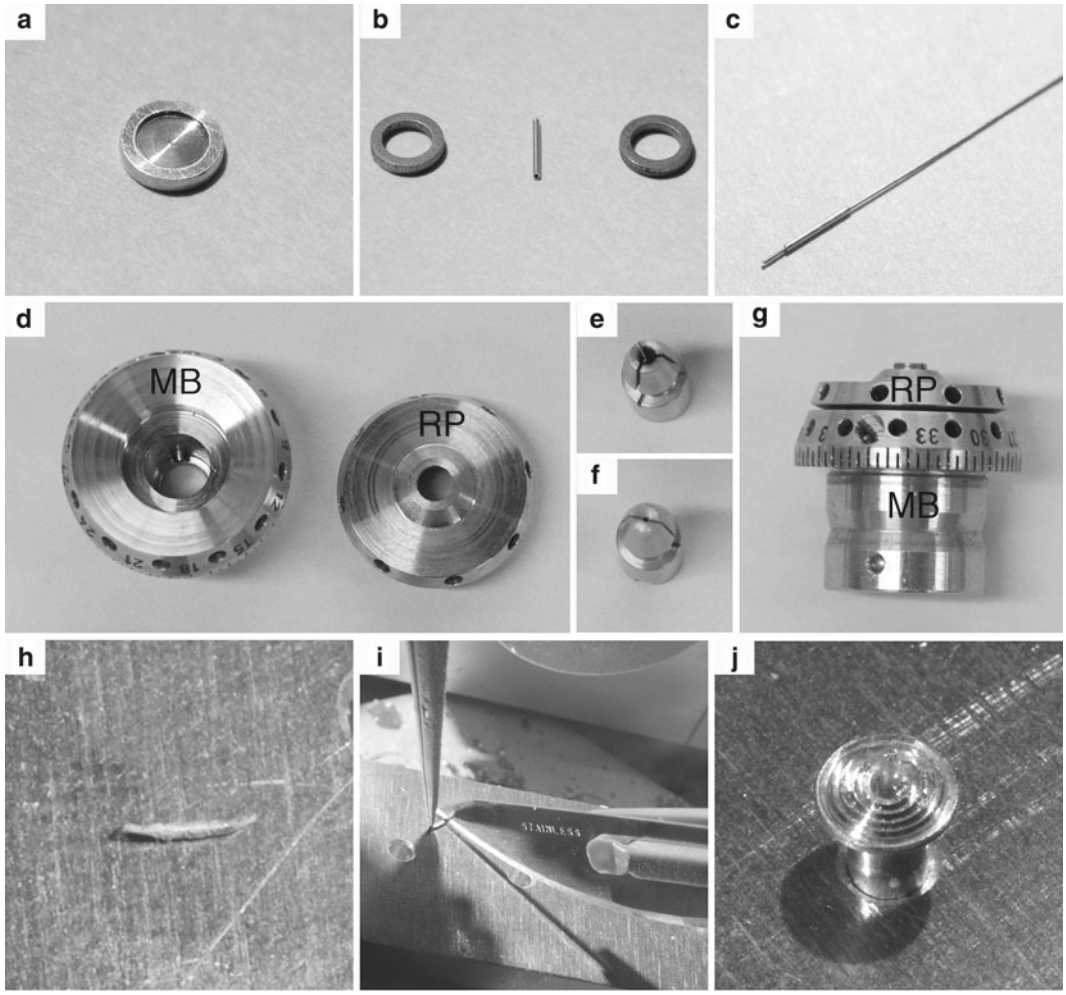

Fig. 3 Sample holders for the HPM 010. (**a**) An aluminum planchette with a 200 μm-deep cavity to freeze tissue samples. (**b**) Gold tube for freezing of samples in suspension. After loading, the tube is clamped between the two steel rings and loaded into the sample holder of the HPM 010. (**c**) Gold tube with a steel piston inserted for loading of the sample. (**d**) The main body (MB) and the flat round plate (RP) of the sample holder. (**e**) The sample clamp for the pins of the sample holder. (**f**) The sample clamp for the copper tubes of the EM pact system. (**g**) Side view of the assembled sample holder, which shows the 360° scale on the MB. (**h**) Gold tube after freezing with the two ends clamped by the steel rings. (**i**) With a precooled scalpel the clamped ends are cut away in the cryo-chamber. (**j**) Cut tube mounted on a pin with cryo-glue

4. Lock the position of the knife table by a lever on the side of the cryo-chamber to avoid any undesirable movement, which can damage knives during mounting in the knife holder.
5. To transport the knife holder with the knives into the cryo-chamber use a key with a M4 thread, which can be screwed into the knife holder. To make more room for mounting the specimen, lock the knife table with knives in the backmost position inside the cryo-chamber. Allow the knives to cool down (5–10 min). The chamber must be cooled down to at least −140 °C before inserting the samples to prevent their devitrification.

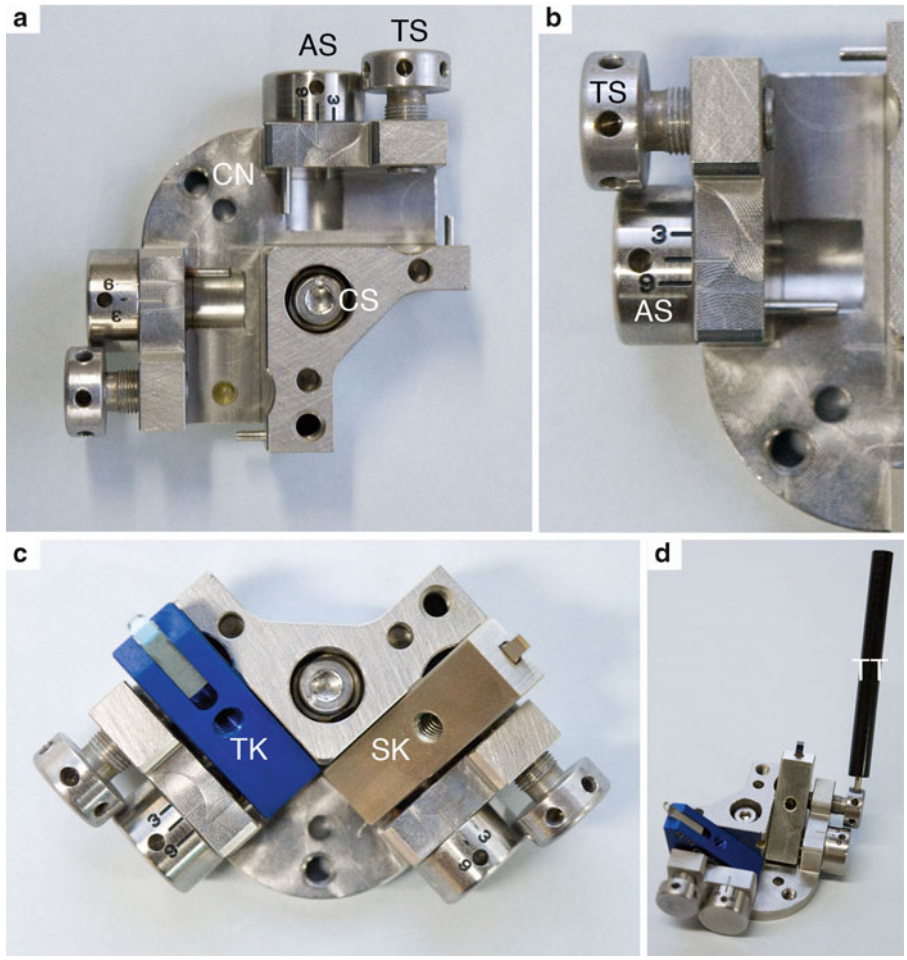

Fig. 4 Knife holder and knives. (**a**) Top view of the knife holder with the two lanes for knives with 90° angle in respect to each other. On the outside of each lane is one screw (TS) to tighten the knife and a second (AS) with a grade to adjust the clearance angle of the knife. The central screw (CS) of the knife holder is to fix the knife holder to the knife table in the cryo-chamber with the Allen key. (**b**) Detail of the TS and CS. The connection (CN) allows inserting and removing the knife holder with the M4 key. (**c**) Knife holder with trimming knife (TK) and sectioning knife (SK) mounted. (**d**) The tightening tool allows fixing the knives and adjusting the clearance angle within the cryo-chamber

3.1.2 Mounting the Specimen

The sample holder of the Leica UC6/FC6 consists of three parts: (1) the main body (*see* Fig. 3d) that is inserted into the arm of the microtome, (2) the sample clamp inserted into the center of the main body (*see* Fig. 3e, f), and (3) the flat round plate screwed on the main body (*see* Fig. 3g). Turning the flat round plate clockwise against the main body will tighten the central sample clamp and fix the sample. There are two types of the sample clamp differing in the hole diameter. The sample clamp with a wide hole diameter (*see* Fig. 3e) is used for samples frozen in gold tubes or for tissue samples. The sample clamp with a narrow diameter (*see* Fig. 3f) is used for samples frozen in copper tubes using Leica EM-pact.

Once the temperature in the cryo-chamber is stabilized, take out the samples from the LN_2 and transfer them into the cryo-chamber using a transfer container. *For all of the following steps it is important that all forceps and other materials that come into contact with the sample, or are used to manipulate the sample, should be pre-cooled to avoid devitrification of the sample.*

Copper tubes. Insert the copper tube into the sample clamp placed inside the sample holder. Tighten the inserted copper tube using the tools for the sample holder of the cryo-microtome (*see* **Note 1**).

Gold tubes. Samples frozen in the gold tubes need to be mounted on pins. First cut away the clamped ends of the gold tube using a precooled scalpel (*see* Fig. 3i). Place the pins in either a homemade metal block with appropriate holes or in the outer holes of the knife holder of the Leica UC6/FC6. Next place a drop of cryo-glue onto the precooled pin and mount the gold tubes such that a small part is sticking out of the glue (*see* Fig. 3h). To harden the cryo-glue, cool down the chamber to −160 °C, subsequently placing the pin with the mounted gold tube into the sample holder and tighten as described above.

Planchettes. Carefully remove the frozen tissue from the planchette using a precooled scalpel and tweezers and then mount the tissue with cryo-glue on a pin (*see* **Note 2**).

3.1.3 Trimming

Proper trimming of the block is crucial to obtain good cryo-sections. The aim is to trim a squared, flat-topped pyramid (*see* Fig. 5d). If the sample is in either a copper or gold tube, both metals are soft enough that they can be trimmed away without damaging the diamond trimming knife. This is different for materials such as aluminum or brass, which contain impurities and can damage the trimming knife. In this case we advise the use of two trimming knives: one dedicated for the removal of the metal and the second for the trimming of the block.

1. Set the position of the sample holder to 0°. The 360° scale on the sample holder allows for precise 90° rotation.
2. The angle of the knife in respect to the sample can be set by a wheel located on the front of the FC6/7 cryo-box. In the middle position (0°) the wheel locks with a click. We recommend choosing this position to start trimming the blockface of the sample.
3. Put the ionizer/Crion in place.
4. To approach the trimming knife to the sample use the light from underneath, which allows for a better perception of the distance between the sample surface and the knife edge.
5. Set the trimming speed at the maximum for the microtome (100 mm/s) and the nominal feed between 50 and 500 nm. For fragile or cryo-glued samples we recommend setting the feed at

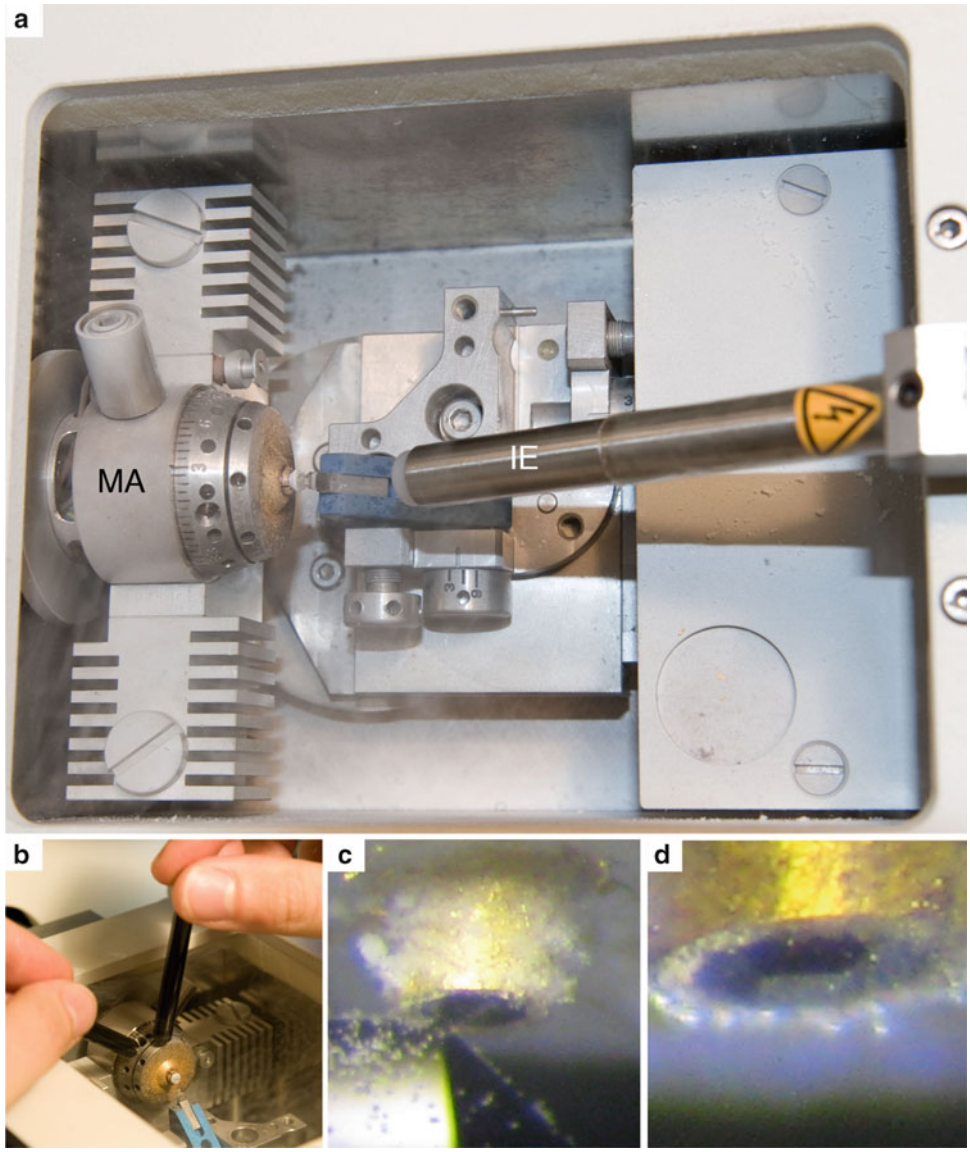

Fig. 5 Trimming of the sample. (**a**) Top view of the cryo-chamber ready for trimming. The sample is fixed in the sample holder and the trimming knife is close to the gold tube to trim the surface. The ionizer (IE) is mounted with its tip pointing towards the knife edge. (**b**) One tightening tool holds the central part of the sample holder. With the second tightening tool the disk of the sample holder is turned clockwise until the sample is locked. (**c**) The left side of the block is trimmed with the right side of the trimming knife. (**d**) Trimmed sample

50 nm, whereas for clamped copper tubes up to a 500 nm feed can be selected. Setting the feed higher than 500 nm might damage the knife or the sample. While trimming, the ionizer is set to maximum for ion production to facilitate removal of trimmed material from the knife edge (*see* **Note 3**).

6. Continue trimming until the surface of the sample is smooth. The surface of a well-vitrified sample normally appears black and shiny as viewed through the binocular using the top-light. Samples with a surface displaying a white and milky appearance should be sorted out at this point since this indicates ice formation during freezing.
7. To trim the first side of the pyramid, move the knife table first back and then to the left, in parallel to the sample surface until the right outer edge of the trimming knife is at the starting point (*see* Fig. 5c). With the light source projecting from underneath, a reflection of the knife appears on the trimmed surface of the sample. This reflection indicates the distance between the knife edge and the sample and helps with determining the manual approach of the knife before starting the trimming of the side.
8. After trimming 20–30 μm deep into the sample, move the knife back and right until the left outer point of the trimming knife is in the correct position for the second side of the pyramid. The aim is to trim the pyramid with a width of not more than 100 μm (*see* **Note 4**). The inner diameter of the tubes (copper tubes = 300 μm, gold tubes = 200 μm) gives an internal reference. Alternatively, the FC6 allows measuring the width of the sample using the central wheel (west-east) on the control unit (*see* Fig. 1e).
9. Unlock the sample holder with the Allen key and turn it 90°. The third and fourth sides of the pyramid are trimmed as described above.

3.1.4 Thin Sectioning and Transfer Using the Cryo-Tools

Technical and environmental conditions. Obtaining good frozen and hydrated sections depends on a number of different conditions such as a starting with a properly vitrified sample and using a sharp diamond knife with good gliding properties. Electrostatic charging effects formed during the cutting often result in decreased gliding or even an attachment of the ribbon to the knife edge. These effects can be overcome by using an ionizer or crion device. The ionizer produces a shower of positive and negative ions that minimizes the interaction between the knife surface and the section; thus increasing gliding properties. Additionally, environmental factors have an impact. Most important are the humidity and temperature in the room. High humidity results in ice crystal formation, which accumulates in the chamber and on the vitreous sections. During imaging electrons cannot penetrate the ice crystals deposited on the section; thus part of the information is lost. In addition the humidity influences the functioning of the ionizer. It is desirable to section at 20 °C with a relative humidity below 30 %. This can be achieved by placing the cryo-ultramicrotome either into a room with controlled humidity and an air-drying device or into a glove box, which is commercially available as cryosphere

by Leica microsystems (Vienna, Austria). Another important point is to wear clothes made out of cotton and not made of wool or synthetic since this material can produce static electricity and will interfere with sectioning and handling of the sections.

1. *Mounting the knife*. Insert the cutting knife into the correct position of the knife holder (*see* Fig. 4c). Move the knife holder back and unlock it using the Allen key. Move the arm of the microtome to the uppermost position to ensure that the trimmed sample cannot be touched while turning the knife holder. Turn the knife holder 90° counter clockwise so that the diamond knife faces the sample. If necessary, adjust the clearance angle to 6° and fix the knife using the tightening tools.
2. Mount the cryo-tool platform onto the knife holder with the two knives. The cryo-tool platform consists of a pressing system and a track for the grid holder. The pressing system pliers are made of two opposing metal pieces with a layer of polished ceramic facing each other (*see* Fig. 6a). The pressing tools can be lifted and lowered like a tollgate, which allows moving the grid holder close to the knife. Allow both the knife and the cryo-tool platform to cool down (approx. 15 min) (*see* **Note 5**).
3. *Placing a grid into a grid holder*. Open the upper part of the pressing system. Place a grid in the middle of the lower part with a small portion of the grid extending over the border of the pressing system (*see* Fig. 6a). Press on the grid holder using a forceps to open it and move it towards the grid on the pressing system (*see* Fig. 6b). When it is opposed to the pressing system, close the grid holder around the part of the grid that extends over the border of the pressing system and move it back. Let the grid cool down for 2 min. Open the lower part of the pressing system and move the grid close to the knife edge directly in front of the sample (*see* Fig. 6c and **Note 6**).
4. *Approaching the sample with a knife*. Switch the bottom light on and slowly approach the block face with the knife until the reflection of the knife is visible on the block face. The reflection should be parallel with the lower edge of the block and should not vary at any position when moving the block down. Set the cutting window on the control panel and do the fine, stepwise approach until the reflection on the block face of the sample is close to disappearing.
5. *Set up of sectioning conditions*. Set the cutting speed, the strength of the ionizer and the nominal feed of the microtome arm (section thickness). The first two settings must be determined empirically for each sample. In general, sections glide more easily on the knife with faster sectioning. But sectioning with a fast cutting speed requires a certain amount of skill and fragile samples break more easily. It is therefore advisable to begin with a speed of

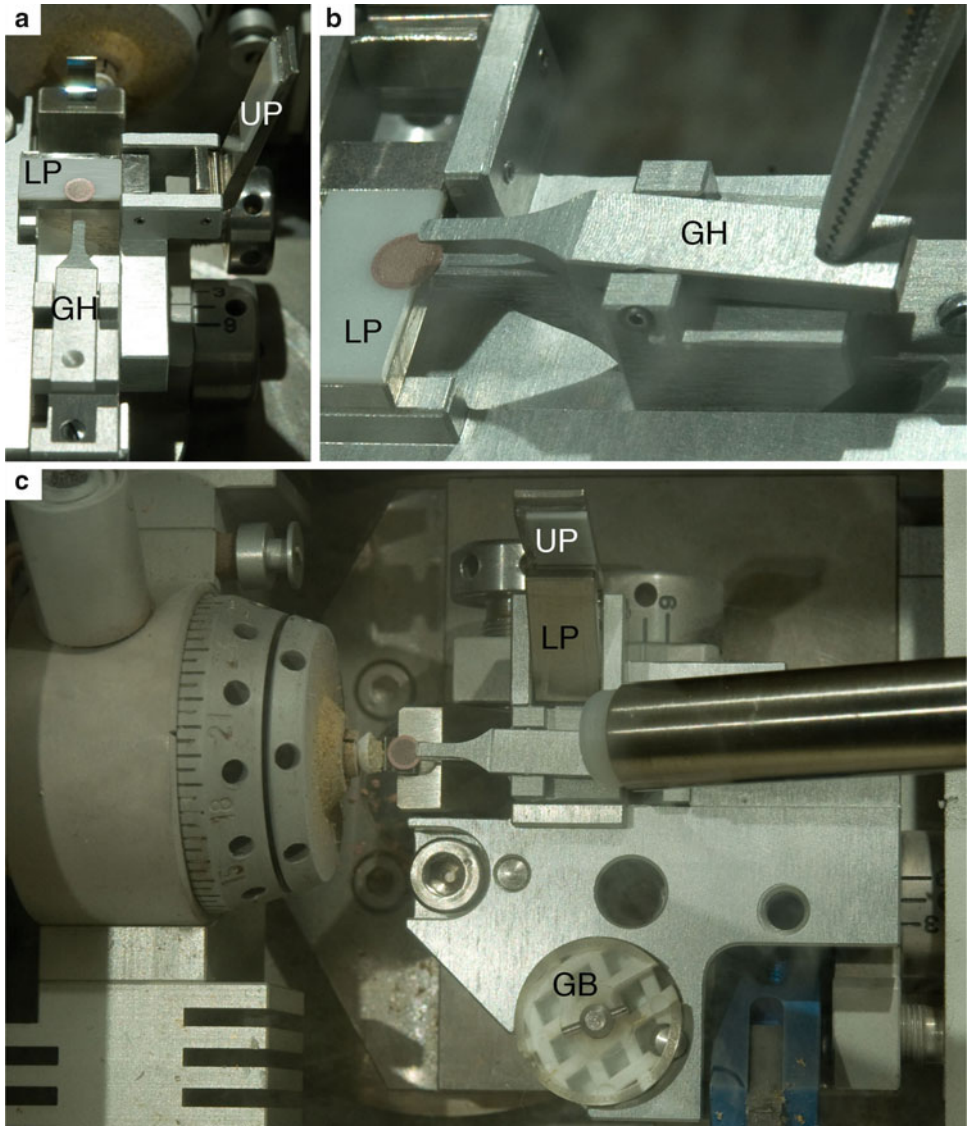

Fig. 6 Loading of the grid with the cryo-tools. (**a**) The upper part (UP) of the pressing platform is opened and a grid is deposited on the lower part (LP) of the open pressing platform. (**b**) The grid holder (GH) is opened by a forceps and moved close to the grid before it is closed to hold the grid. (**c**) Top view of the cryo-chamber ready to section. Note the lower part of the pressing platform is now also moved to the open position, which allows moving the grid holder with the grid close to the knife. The tip of ionizer (IE) points to the edge of the knife. GB: cryo-grid box

1 mm/s and adjust according to the gliding behavior of the sections and personal skill. The strength of the ionizer depends on the setting of the control panel and on the distance of the electrode to the knife edge. If the ionizer produces too much charge the sections will fly away from the knife edge. If the charge is too low the sections will not stretch or will stick to the knife edge. We recommend starting with maximum power to avoid

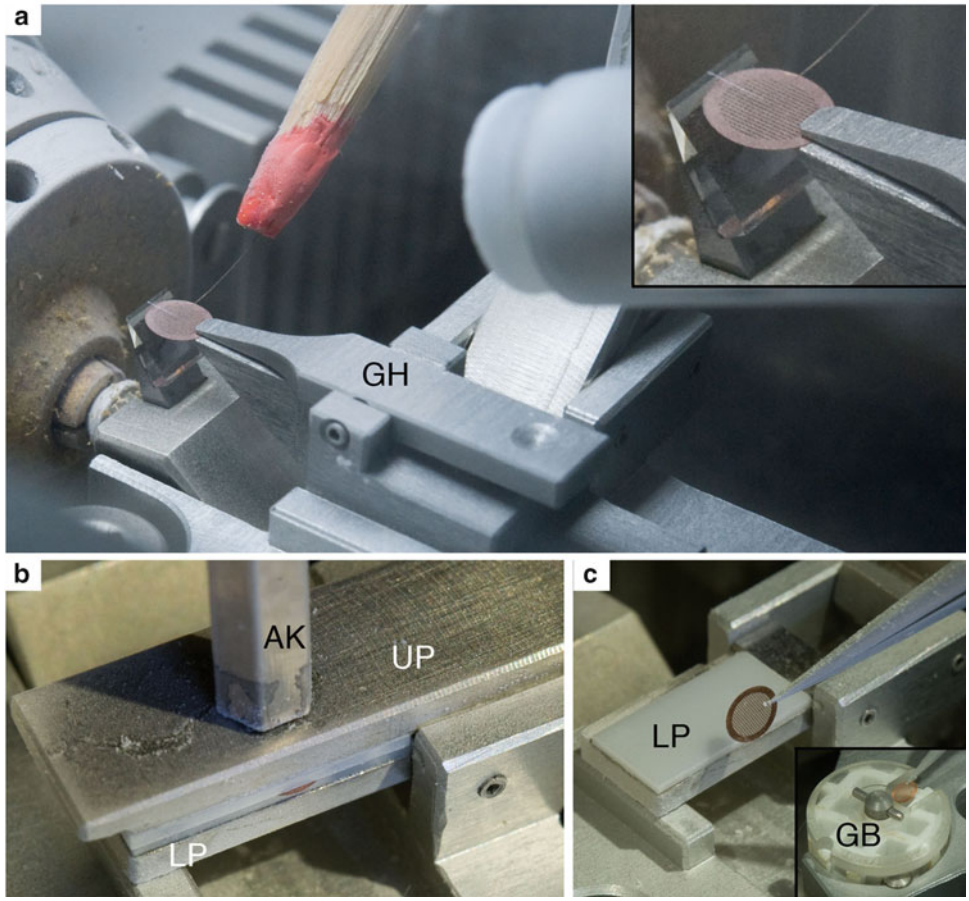

Fig. 7 Sectioning and transfer of the sections. (**a**) To the first section of the forming ribbon the eyelash is attached and with it the ribbon is pulled over the grid, which is positioned by the grid holder (GH) close to the knife. The *insert* shows a higher magnification. (**b**) The grid with the ribbon on it is pressed between the two porcelain parts of the pressing tool. To apply force the precooled Allen key (AK) is used. Note that the whole grid is not in the pressing tool. The border of the grid out of the pressing tool makes pick up of the grid with the forceps after pressing easier. (**c**) With the precooled forceps the grid is transferred from the lower part (LP) of the pressing tool into the cryo-grid box (GB) next to the pressing tools (*insert*)

contamination of the knife by sticking sections and then reduce the setting or increase the distance if the sections fly away.

6. *Sectioning and transfer of the sections to the grid*. Start the automatic sectioning and have the eyelash ready to hold the forming section. Under good conditions the sections will form a ribbon and the ribbon will easily slide off the knife edge. Hold the front of the ribbon above the grid from underneath using the eyelash (*see* Fig. 7a). When the ribbon is long enough stop automatic sectioning, turn off the ionizer, slightly pull the ribbon and place it on the grid. Detach the ribbon from the knife edge with the eyelash. *See* Table 1 that addresses frequent sectioning difficulties (*see* **Note 7**).

Table 1
Troubleshooting table for CEMOVIS

Problem	Possible causes	Corrective action
Ribbon is not straight and bends towards one side	Unevenly trimmed or damaged pyramid Section remains or ice crystals on the knife edge	Re-trim the pyramid Clean the knife edge with an eyelash, eventually change position on the knife
Ribbon flies away from the edge	Ionizer power too high	Lower the power of the ionizer, increase the distance of the electrode tip from the knife edge
Ribbon stays attached at the knife edge	Cutting speed too high/slow Ionizer power too low Knife too dirty	Change cutting speed Clean electrode tip and position it 1–2 cm from the edge of the knife, increase the power of the ionizer Clean the knife
Sections are too compressed	The pyramid is too large Section remains or ice crystals on the knife edge, dull knife edge	Trim the pyramid to decrease width, lower the cutting speed Clean the knife edge with an eyelash, eventually change position on the knife. If the problem persists consider re-sharpening the knife
Sections do not attach to the grid	Ceramic surface of the pressing tool is dirty Charging power too low, electrode misaligned or too far from the knife edge	Clean the ceramic surfaces of the pressing tool with water Increase the charge output on crion, correct the position of the electrode, clean the electrode tip

7. *Attaching the ribbon to the grid using the pressing system*. Once the ribbon(s) is on the grid, move the grid holder to the back-most position in the cryo-chamber. Put down the lower part of the pressing system and move the grid holder close to it so that the grid is lying on the ceramic surface. Subsequently open the grid holder to release the grid and move the grid holder back. Close the pressing system by moving down the upper part to sandwich the grid between the two ceramic surfaces. With the precooled Allen key, press the sandwich to ensure the adhesion of the ribbon(s) to the grid (*see* Fig. 7b and **Note 8**).
8. *Grid transfer to the grid-box*. After the pressing, open the cryo-grid box and lift the upper part of the pressing system. Using a precooled pair of forceps, take the grid and place it into the cryo-grid box (*see* Fig. 7c). Close the cryo-grid box to avoid ice-contamination.

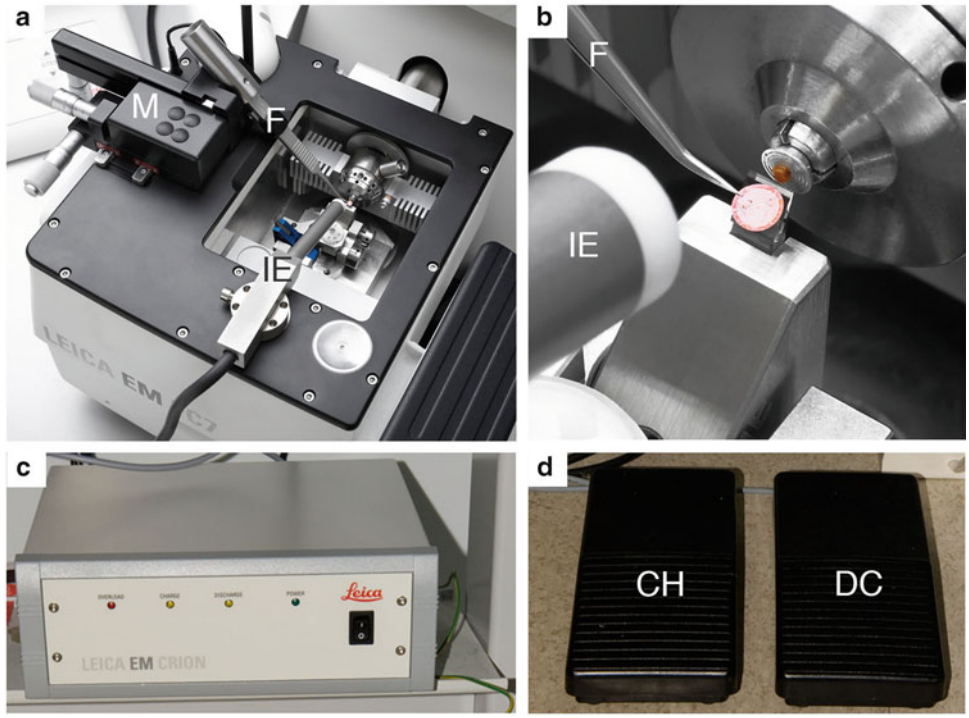

Fig. 8 Ribbon attachment to the grid using Crion device. (**a**) FC7 cryo-chamber equipped with manipulator (M) with three gauges allowing for precise control of the forceps (F) holding a grid. (**b**) The electrode (IE) is facing the grid with 1–2 cm distance. The grid is in close proximity to a knife edge. (**c**) The crion box with the on/off switch on the right and four LEDs, which signal the state of the crion (power, discharge, charge, and overcharge, from right to left, respectively). (**d**) The two foot-pedals to active/inactivate the charge (CH) and discharge (DC) mode of the crion

3.1.5 Thin Sectioning and Transfer Using the Crion Charging/ Discharging System and Micromanipulator

The Crion electrostatic apparatus consists of a high voltage generator, an electrode, and two foot-pedals controlling the output and control box. It produces either both positively charged and negatively charged ions (discharge mode) or an excess of ions of one polarity (charge mode). In the discharge mode the device functions identically to the ionizer, which is to facilitate smooth gliding of the ribbon from the knife edge. Charge mode is used to attach the ribbon to the grid. A micromanipulator allows for grounded forceps holding a grid to achieve close proximity to the knife edge. Attachment by using the charging system is more efficient than that by mechanical pressing and does not result in disruption of section integrity [10] (*see* **Note 9**).

1. Place a grid into the grounded forceps and use the micromanipulator to approach the knife edge. The electrode tip should be 1–2 cm distant from the knife edge (see Fig. 8 and Note 10).
2. Select the discharge mode by pressing the discharge foot-pedal once (discharge LED indicator is on). Start automatic sectioning and hold the front of the ribbon above the grid

from underneath using the eyelash. If necessary, adjust the output voltage value on the touch screen panel such that the ribbon is sliding from the edge of the knife.

3. When the ribbon is long enough, stop automatic sectioning and press the discharge foot-pedal to stop discharging. Slightly pull the ribbon, bring it close to the grid and press the charge foot-pedal until the ribbon jumps onto the surface of the grid. Place the grid with the ribbon into the grid-box (*see* **Note 11**).

3.2 Transfer to the Microscope

3.2.1 Preparation of Gatan Cryo-Transfer Workstation and Cryo-Holder

1. Prior to cryo-transfer, attach the cryo-holder to the Cold Stage Controller and Gatan 655 Dry Pumping Station (*see* Note 12). Choose to run a warm-up cycle on the Gatan SmartSet Cold Stage Controller and pump the cryo-holder until the vacuum in the dewar reaches the range of 10–5 Torr (*see* Note 13).
2. Insert the cryo-holder into the cryo-transfer workstation and connect the cable of the Smartset (*see* Fig. 9a) to the cryo-holder to monitor the temperature.
3. Simultaneously start filling both the cryo-holder dewar and the cryo-transfer workstation with LN2 until the cryo-holder reaches a temperature below –190 °C (approx 10 min). Keep the cover of the chamber closed when not refilling LN_2 to avoid ice contamination.

3.2.2 Grid Loading into the Cryo-Holder

When loading the grid try to avoid direct breathing into the chamber to minimize ice contamination on the grid.

1. Place a cryo-grid box into the workstation chamber (*see* Fig. 9b) of the cryo-transfer workstation and cool down the insulated pair of forceps, clipper-ring tool and grid box screwdriver (*see* Fig. 9c).
2. Open the shutter by pulling the sliding knob at the back of the dewar (Fig. 9a, arrow, 9d).
3. Remove excess LN2 from the cryo-chamber by tilting the cryo-transfer workstation and cryo-holder until the level of the LN2 is just below the grid slot of the cryo-holder (*see* **Note 14**).
4. Use the clipper-ring tool to remove the clipper-ring placed in the slot for the grid. Unscrew the lid of the cryo-grid box with the grid box screwdriver tool. Keep the clipper-ring attached to the clipper-ring tool cooling in the chamber.
5. Use insulated forceps to transfer the grid from the box to the grid-slot in the holder and place the clipper-ring on the top of the grid to secure the grid (*see* Fig. 9f, g).
6. Close the cryo-shutter and refill LN2 into the chamber of the cryo-transfer workstation.

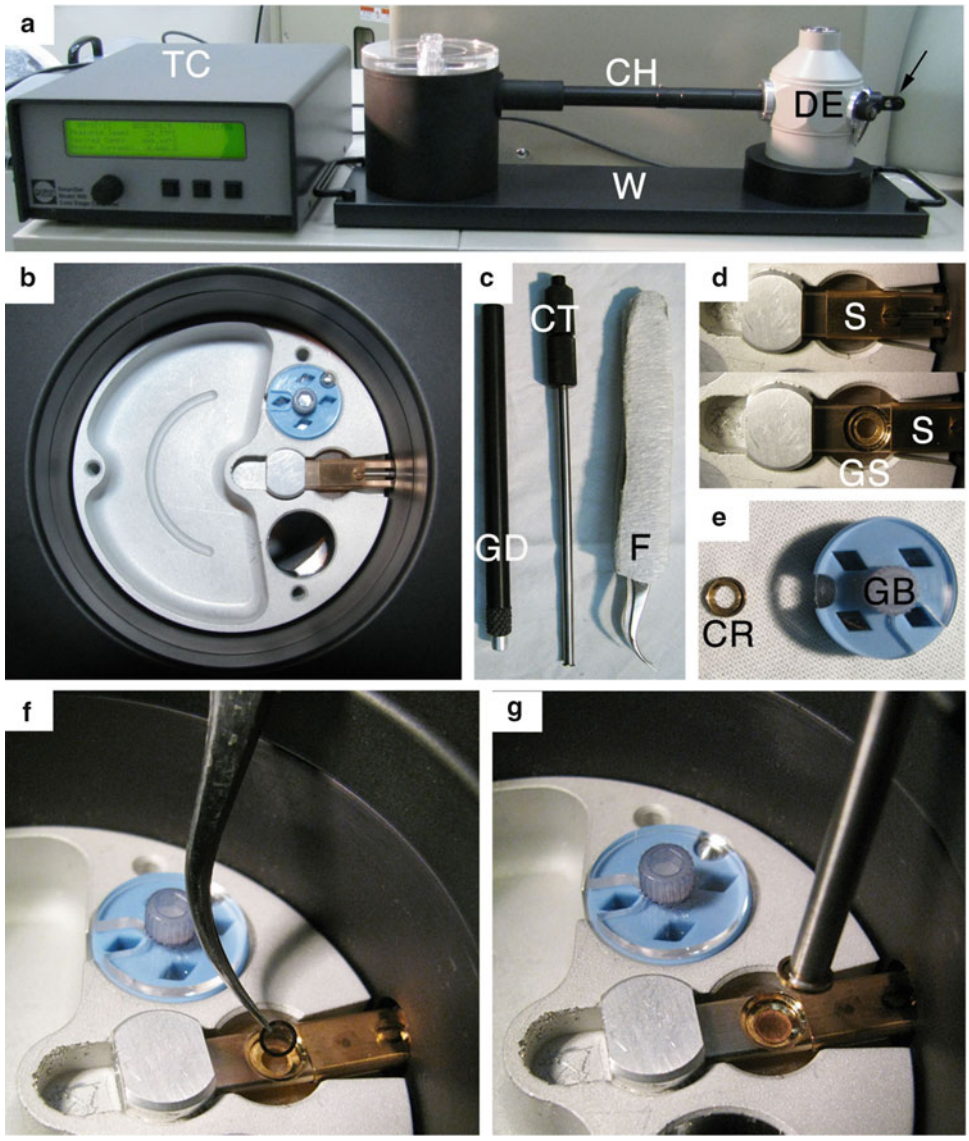

Fig. 9 Loading a grid into a cryo-holder. (**a**) Cryo-holder (CH) inserted in the cryo-transfer workstation. The control for shutter opening is located in the back of the dewar (DE) (*arrow*). Temperature control device (TC) allows monitoring of the temperature during the transfer and warming up of the holder prior to pumping. (**b**) Cryo-chamber from the top with cryo-holder inserted and cryo-grid box (GB). (**c**) Tools (*left–right*): Grid box screwdriver (GD), clipper-ring tool (CT), and forceps (F). (**d**) Cryo-holder with open and closed shutter (S). (**e**) Clipper ring (CR) and cryo-grid box (GB). (**f**) Removing the grid from a grid box and placing it into the cryo-holder. (**g**) Securing the grid by clipper-ring using clipper-ring tool

3.2.3 Gatan Transfer to the Microscope

1. Remove the cryo-holder from the cryo-transfer workstation and swiftly and carefully insert it into the microscope. Rotate the holder using the right hand to fully insert the holder into the airlock (Refer to the manufacturer's instructions to insert the specimen holder into the microscope). While turning the

holder, collect LN2 spilling from the dewar into a plastic beaker using your left hand.

2. Once the evacuation of the airlock is accomplished, complete the insertion of the cryo-holder, fill the dewar with LN_2 and close it with a dewar lid (*see* **Note 15**).

3.3 Cryo-Transmission Electron Microscopy

3.3.1 Low Dose Imaging

Vitreous sections are susceptible to beam damage and thus low-dose mode has to be applied. A microscope with a beam blanker and a high-sensitivity camera should be used. For imaging of thin sections (50 nm) a low accelerating voltage (120 kV) microscope is beneficial for higher contrast. Cryo-transmission electron tomography (cryo-ET) requires higher accelerating voltage (200–300 kV) depending on section thickness. Low-dose imaging should be conducted at intermediate spot-size [4–6] and consists of three independent settings: search, focus and record. Search settings should be set to low magnification and high defocus (−80 μm) to gain on contrast while searching for region of interest (ROI). Focus setting allows for the focus adjustment to return to a desired value prior record. Focus value depends on the purpose of the imaging: at closer focus the higher resolution information is preserved at the expense of contrast. Focus adjustment is intentionally done on an area 2–3 μm distant from ROI. Adjust focus until the image with the lowest contrast is obtained, reset the focus value to zero and then set the focus to a desired value (i.e., −2 μm). Acquire a record image in record settings such that total dose is about 5–20 $e^-/Å^2$.

For cryo-ET, gold or quantum dot fiducial markers diluted in isopentane can be deposited on vitreous sections as described by Masich et al. [17] and Gruska et al. [14] or, alternatively, fiducial markers can be applied on the grid prior to sectioning [10]. Attention has to be paid particularly for the section attachment to the carbon layer since a poor attachment results in section movement during tilt-series acquisition. Thus, attachment using electrostatic charging is recommended.

3.3.2 Interpretation and Artifacts

Vitreous sections differ from plastic sections; vitrification, beam damage and sectioning artifacts have to be kept in mind when it comes to image interpretation [18]. Vitrification should be verified prior to imaging by electron diffraction. The diffraction pattern of vitreous sample shows smooth rings (*see* Fig. 10d). Exceeding cumulative dose by about 100 $e^-/Å^2$ usually leads to smoothing out of the sectioning artifacts, such as crevasses and knife marks (*see* Fig. 10b, c) and contrast enhancement [19]. Although such images are more appealing, the structural information of biological material is altered. Further increase in electron dose leads to formation of electron-lucent hydrogen bubbles [20]. One should also bear in mind that structures such as membrane-bound organelles are compressed in the direction of sectioning (*see* Fig. 10c). Direction of sectioning can be determined from the direction of knife marks

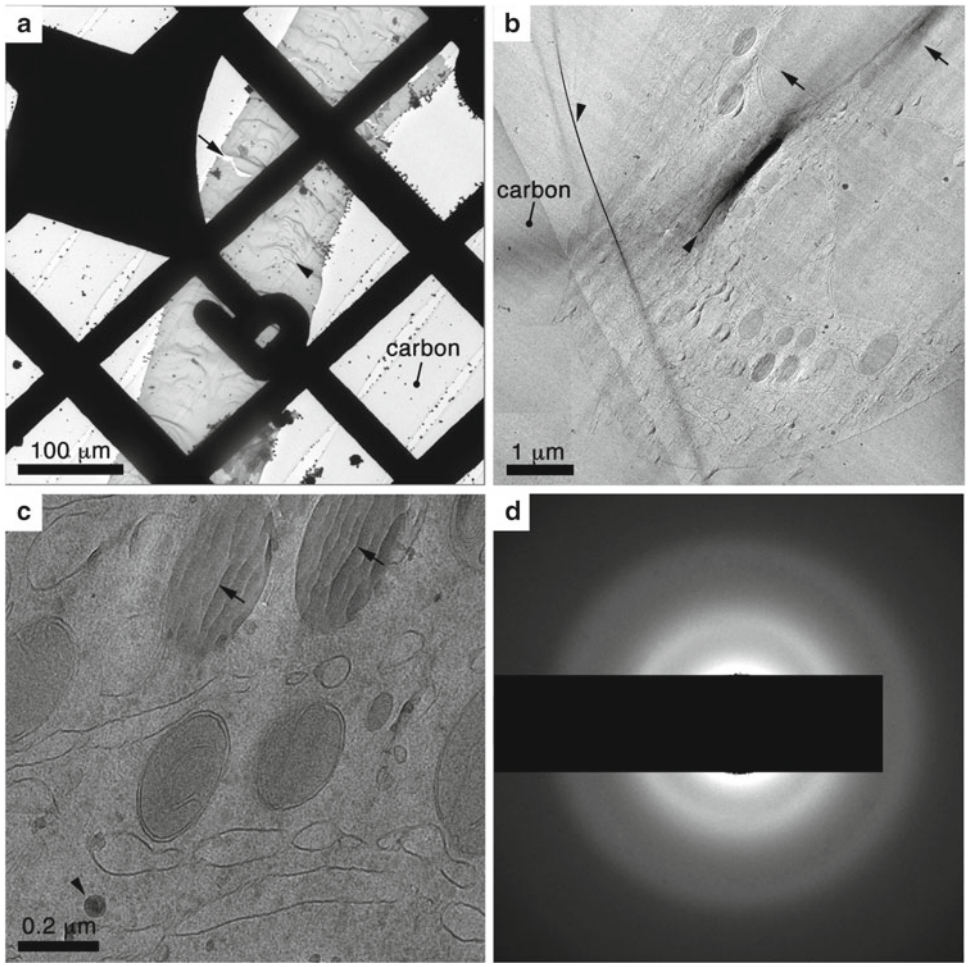

Fig. 10 Imaging vitreous sections. (**a**) A ribbon with nominal thickness (60 nm) attached by pressing system to the H2 Copper (200 mesh) finder grid coated with carbon. Apparent cracks (*arrow*) and folds (*arrowhead*) are visible on the ribbon. (**b**) A vitreous section of HeLa cell with visible knife marks (*arrows*) and folds (*arrowheads*). (**c**) A magnified view of (**b**) showing the cytoplasm and organelles inside the HeLa cell. Crevasses are visible inside the lipid droplets, which are compressed in the direction of sectioning (*arrows*). (**d**) A diffraction pattern of section imaged with an inserted beam stopper to test vitrification. The smooth airy discs indicate that the section is vitreous

and the shape of the crevasses. The level of compression (40 % for sections with nominal thickness, ~70 nm) decreases with the section thickness since mechanical stress results in crevasses [6].

4 Notes

1. To provide stability while trimming and sectioning only a small portion of the tube should be outside the clamp.
2. Pins with mounted gold tubes or tissue can either be immediately sectioned or stored in LN_2. However, repeated temperature

cycles between –160 °C and liquid nitrogen temperature will induce small cracks in the cryo-glue. After several cycles it is necessary to remount the sample at –140 °C using a new pin and fresh cryo-glue.

3. During longer usage of the ionizer/crion electrode, ice crystals might deposit on it and impair function. Ice crystals should be removed either with a fine tissue or by turning the ionizer off and removing it from the cryo-chamber, followed by warming and drying it.
4. With the 45° trimming diamond the angle of the sides will be 45° and when sectioning into the pyramid the sample becomes wider. Using a 20° trimming knife results in an angle of 70° and thus a more fragile pyramid with a narrower base.
5. If work is conducted in an environment with low humidity, both knives and the platform can be mounted before cooling down the cryo-chamber. Without humidity control, introduction of the knife and the platform before trimming will result in ice contamination on both.
6. Grids with either a continuous or holey carbon layer should be used. Carbon film is important for successful attachment and thus bare grids cannot be used. Since images taken in the holes have higher signal-to-noise ratio, films with large holes are recommended. Carbon film is in general more fragile than formvar film; particularly grids with low mash numbers and large holes have to be manipulated with extra care. Commercially available C-flat grids [21] with 20 nm thick, holey carbon film with superior flatness are, from our experience, better than quantifoil grids [22] of which carbon film shows slight wrinkles and rough edges of the holes. Quantifoil offers a large set of patterns and dimensions including carbon films with squared holes and large areas without carbon (7×7 μm mesh). To increase adhesion of the section and stability during imaging we put a layer of carbon on top of the 7×7 μm mesh of quantifoil. Glow discharging of the grid does not seem to have a consistent influence on the attachment of the ribbon to the grid.
7. The aim is to transfer the ribbon from the knife surface and place it on the grid as flat as possible. This is influenced by the charge of the grid. In our experience coating the grids with an additional carbon on the day of the sectioning improves the attachment of the ribbon to the grid. If it is not possible to obtain a long enough ribbon for the above described immediate transfer, it is also possible to guide the forming ribbon down the surface of the knife. Stop the sectioning and the ionizer and detach the eyelash from the first section of the ribbon. Next, use the eyelash to detach the ribbon from the knife edge and transfer it onto the nearby grid. This approach bears the risk for the

ribbon to fly away or curl. Once a ribbon is curled it is impossible to spread it flat on a grid and it needs to be discarded.

8. It is important that the surfaces of the pressing tool are clean. The ribbon will stick to the ice-contaminated or dirty ceramic surface rather than to the grid.
9. Crion output voltage is in the range of tenths of kV and thus the device must be properly grounded and operated according to the manufacturer's instructions.
10. The grid should be in close proximity to a knife edge but not touching the diamond to avoid grounding through a knife and to maximize the charging effect.
11. To test whether the ribbon is well attached to the grid, activate the discharge mode by pressing the discharge foot-pedal and set value to full power. A well-attached ribbon should stay on the grid.
12. A movie demonstrating cryo-transfer using a Gatan 626 holder can be found on the Gatan website (http://www.gatan.com/).
13. When the cryo-holder is used for the first time or not used for an extended period (more than 1 month), run a bake-out cycle controlled by SmartSet while pumping.
14. Loading the grid in N_2 vapors is easier than in LN_2.
15. Do not fill the dewar completely and let the holder stabilize for 10 min to avoid vigorous LN_2 boiling, which causes vibration that may interfere with imaging.

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