21

A New Microbiopsy System Enables Rapid Preparation of Tissue for High-Pressure Freezing

Dimitri Vanhecke, Peter Eggli, Werner Graber, and Daniel Studer

Summary

A microbiopsy system was developed to overcome long sampling times for tissues before they are cryo-fixed by high-pressure freezing. A commercially available biopsy gun was adapted to the needs of small-organ excisions, and biopsy needles were modified to allow small samples (0.6 mm × 1.2 mm × 0.3 mm) to be taken. Specimen platelets with a central slot of the same dimensions as the biopsy are used. A self-made transfer device (in the meantime optimized by Leica-Microsystems [Vienna, Austria]) coordinates the transfer of the excised sample from the biopsy needle into the platelet slot and the subsequent loading in a specimen holder, which is then introduced into a high-pressure freezer (Leica EM PACT; Leica Microsystems, Vienna, Austria). Thirty seconds preparation time is needed from excision until high-pressure freezing. Brain, liver, kidney and muscle excisions of anesthetised rats are shown to be well frozen.

Key Words: Cryo-fixation; electron microscopy; fast sampling; freeze substitution; high-pressure freezing; microbiopsy system; rat tissue

1. Introduction

Chemical fixatives (e.g., aldehydes and osmium tetroxide) have negative effects on the native structure and antigenicity of biological specimens. They withdraw osmotic gradients during fixation and, therefore, affect ultrastructure *(1,2)*. The search for alternative fixation methods started soon after the commercialization of electron microscopy *(3,4)*. Cryo-fixation, or physical fixation, turned out to be the best alternative, and numerous cryofixation techniques were developed based on vitrification of the specimen (reviewed in **ref.** *5*). Using ambient or high-pressure cryotechniques, the fixation occurs within milliseconds (usually 0.1–100 ms, depending on the size of the sample), which is much faster than chemical fixation. The major drawback of ambient cryo-fixation methods is the small fixation depth; under optimal physical conditions at ambient pressure,

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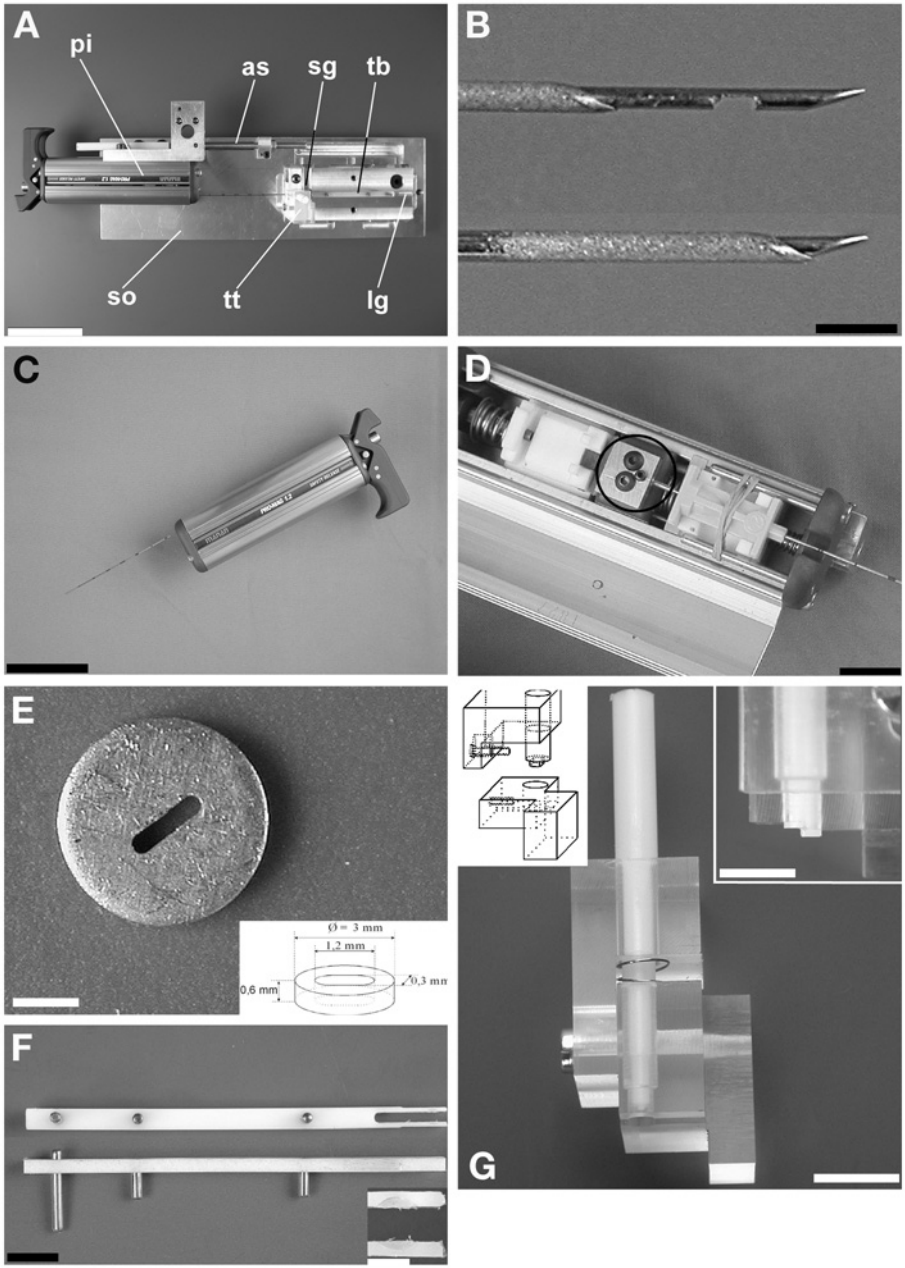

Fig. 1. Overview of the microbiopsy tools involved. **(A)** Overview of the microbiopsy transfer station. One can see the raised part, called the "tissue transfer bench" (tb), with a long axis groove (lg), and the transfer tool (tt) mounted in the short axis groove (sg). The remainder of the microbiopsy transfer station is called the "socket" (so) and provides

the fixation depth rarely exceeds 20 µm *(5–8)*. High-pressure freezing at about 2000 bar, however, allows a 10-fold increase of vitrification depths *(7,8)*.

The fast fixation performed by cryo-fixation is only profitable when post-mortem autolytic processes, which start immediately after cell death, are kept to a minimum. Therefore, the time between tissue excision and cryo-fixation is crucial and should be as short as possible *(9)*. For this reason, a microbiopsy system for the Leica EM PACT high-pressure freezing machine *(10)* was developed. The entire procedure from excision until freezing lasts about 30 s. The microbiopsy system presented herein is available from Leica Microsystems (Vienna, Austria).

2. Materials

1. Microbiopsy system (*see* **Fig. 1A**).
2. Biopsy needles (Manan, Northbrook, IL) (*see* **Fig. 1B**).
3. Biopsy gun (Pro-mag 1.2 Manan biopsy gun, Manan, Northbrook, IL) (*see* **Fig. 1C,D**).
4. Biopsy platelets (*see* **Fig. 1E**).
5. Platelet transporter (*see* **Fig. 1F**).
6. Tissue transfer tool (*see* **Fig. 1G**).
7. Anesthetised rat.
8. Leica EM PACT high-pressure freezing machine (Leica Microsystems, Vienna, Austria).
9. Styrofoam box (10 cm × 5 cm × 5 cm); sb in **Fig. 2A**.
10. Transport container (1-L cryogenic Dewar); cd in **Fig. 2A**.
11. Torque wrench (a torque force of 35 cNm is required) (*see* **Fig. 2A**).
12. Pod of flat specimen holder system of Leica EM PACT high-pressure freezing machine (Leica Microsystems, Vienna, Austria); pd in **Fig. 2A–D**.

Fig. 1. *(Continued)* a location for the biopsy pistol (pi), which is aligned for biopsy transfer with a screw (as). (Bar=5 cm.) **(B)** The relative positions of the inner and the outer needles before tissue excision (top picture) and after outer-needle release (bottom picture). (Bar=2 mm.) **(C)** The Pro-mag 1.2 Manan biopsy gun. (Bar=5 cm.) **(D)** Detail of the inner mechanism of the modified Pro-mag 1.2 biopsy gun. In the circle, the modification is shown: the inner needle is mounted in a fixed position. (Bar=2 cm.) **(E)** Depiction of the biopsy platelet, with indications of its dimensions (schematic). (Bar=1 mm.) **(F)** The platelet transporter depicted as seen when mounted on the tissue transfer bench (top) and in side view (bottom), revealing the small rod at the rear end used for the perfect positioning of the transporter. A detail of the platelet clamp is shown in the inset. (Bar=1 cm; inset bar=0.25 cm.) **(G)** The tissue transfer tool shows the ridge at the rod ending (right inset). This ridge is needed to transfer the tissue. An assembly scheme of the tissue transfer tool is depicted (left inset), providing an easier three dimensional view. (Bar=1 cm.)

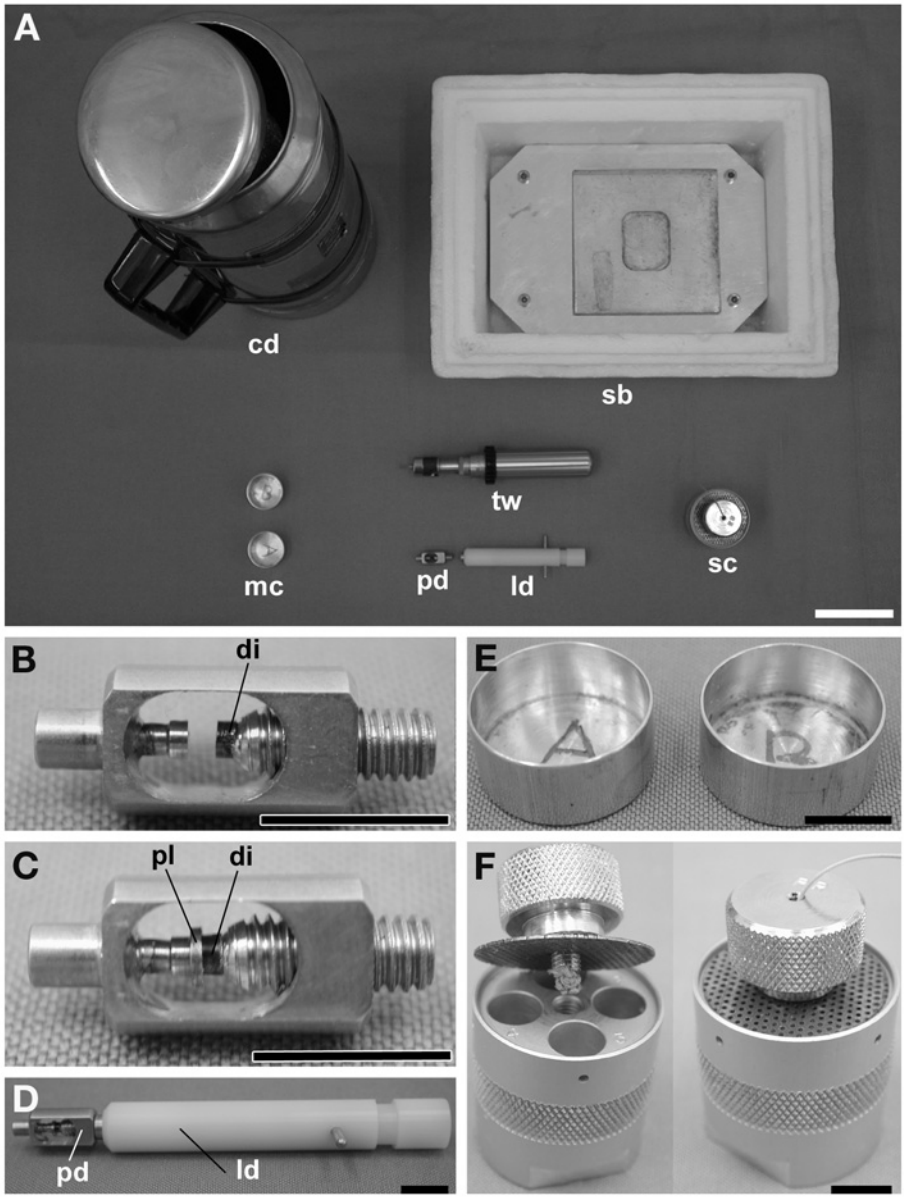

Fig. 2. Overview of the cryo-tools involved. **(A)** High-pressure frozen samples are transferred from the Leica EM PACT high-pressure freezing machine in small metal containers (mc) to a styrofoam box (sb), both filled with liquid nitrogen. Submersed in the liquid nitrogen, the platelets are transferred from the metal containers to a homemade storage container (sc). The storage containers can be held temporarily in a cryogenic Dewar (cd) for a few hours before they are transferred to liquid nitrogen storage or,

13. Numbered small metal containers (diameter: 2 cm; height: 0.5 cm); mc in **Fig. 2E**.
14. Homemade storage containers ($\phi = 2.5$ cm, $h = 5$ cm) for holding samples in liquid nitrogen; sc in **Fig. 2A,F**.
15. Approximately 20 L of liquid nitrogen (LN_2).
16. Forceps.
17. Stereomicroscope.
18. Rat anestheticum mixture: 1 part Prequillan (10 mg/mL acepromazinum [Fatro S.p.A., Ozzano Emilia, Bologna, Italy]), 4 parts 0.9% NaCl, 5 parts Xylapan (20 mg/mL xylazinum), and 10 parts Narketan (100 mg/mL Ketaminum; both from Chassot AG, Belp, Switzerland).
19. Fluothane, 0.5 mL/L volume (Astrazeneca, Wedel, Germany)

3. Methods

3.1. Anesthesia of the Rats (see *Note 1*)

The rats were relaxed by enclosing the cage with a plastic bag containing two paper tissues soaked with Fluothane for 5 min. Deep anesthesia was achieved by injection of 120–140 µL of anesthetic mixture per 100 g body weight.

3.2. Preparation of the Microbiopsy Transfer Station

The platelet transporter (*see* **Fig. 1F**) controls the perfect positioning of the platelet (*see* **Note 2**) in the transfer bench. The transporter can be positioned at three different locations on the transfer bench (**Fig. 3A**, positions A, B, C) and holds the biopsy platelet in a clamp.

1. Install the platelet transporter in the groove of the long axis (lg in **Fig. 1A**) of the tissue transfer bench.
2. Place the platelet transporter (*see* **Fig. 1F**) in position A (most retracted position) (*see* **Fig. 3A**).
3. Load a biopsy platelet in the clamp of the platelet transporter (*see* **Fig. 3**).
4. The slot of the biopsy platelet must be parallel with the direction of movement of the platelet transporter.

Fig. 2. *(Continued)* alternatively, samples can continue immediately with subsequent treatments. The pod (pd) of the flat specimen holder system of the Leica EM PACT high-pressure freezing machine with a loading device (ld) and a torque wrench (tw) are depicted. **(B)** The pod of the flat specimen holder system of the Leica EM PACT is shown. **(C)** The diamond (di) seals the biopsy platelet (pl). **(D)** Pod (pd) fixed to the loading device (ld) is shown. **(E)** Small metal containers are used to transfer platelets from the liquid nitrogen bath of the Leica EM PACT to a liquid-nitrogen-filled styrofoam box. **(F)** Open and closed homemade storage containers. (Bar in A = 5 cm; bars in B–F = 1 cm.)

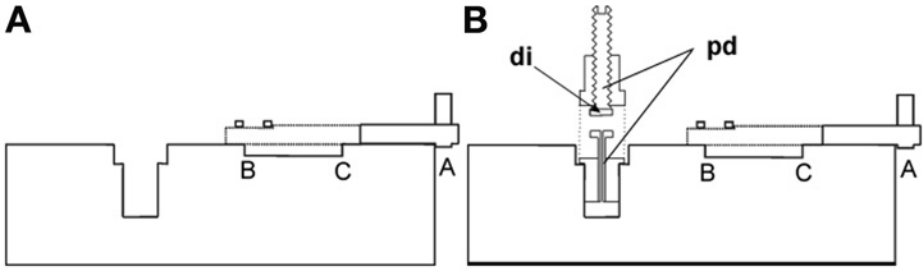

Fig. 3. Schematic representations of the tissue transfer bench before transfer. **(A)** A side-view scheme of the tissue transfer bench, with the platelet transporter in the fully retracted position (position A) and the biopsy platelet in the clamp. **(B)** A side-view scheme of the Leica EM PACT pod (pd), with indication of the diamond (di).

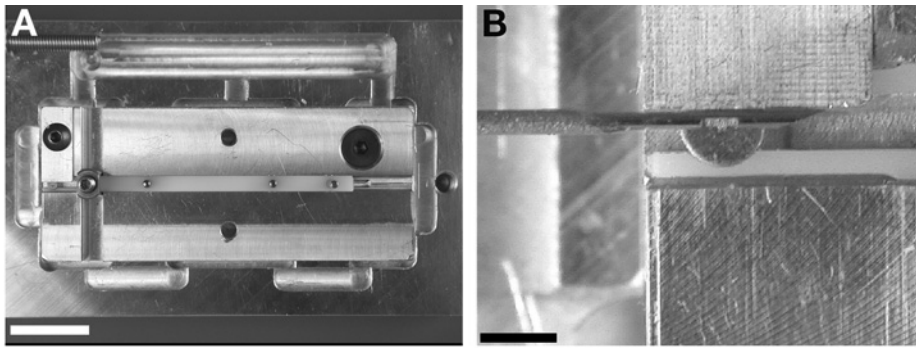

Fig. 4. Schematic representations of the tissue transfer bench before transfer. **(A)** The tissue transfer bench with the platelet transporter in position B is shown. (Bar = 2 cm.) **(B)** A detail of the tissue transfer bench, revealing the platelet transporter clamp with platelet. The perfect match between the needle notch (still empty) and the empty platelet slot is obvious (*see also* **Fig. 5B**). (Bar = 0.25 cm.)

5. Install the pod of flat specimen holder system (*see* **Note 3** and **Fig. 3B**) in the central opening (where the two grooves cross).
6. Open the screw of the pod (*see* **Fig. 3B**).
7. Move the platelet transporter, with the platelet in its clamp, in the direction of the pod, to position B. The biopsy platelet and the two arm rests of the clamp go through the pod (*see* **Fig. 4A**).
8. Insert the biopsy gun (*see* **Note 4**), without excised tissue, in the socket to check if the needle notch is perfectly aligned above the slot of the biopsy platelet (*see* **Fig. 4B**). If this is not the case, adjust the position of the gun using the long screw (as in **Fig. 1A**) by the side of the socket (also in **Fig. 1A**). This needs to be done only once per needle.

With the biopsy platelet installed properly, the gun accurately aligned in the socket, and the platelet transporter in position B, the notch of the biopsy needle appears to be exactly above the biopsy platelet slot (*see* **Fig. 4B**). Excised tissue can now be transferred from the needle notch (*see* **Note 5**) to the biopsy platelet.

3.3. Excision of the Biopsy

It is critical for tissue preservation that from the moment the sample is excised, all steps should be executed as quickly as possible.

1. Retract the outer tube of the gun by pulling on the lever at the back of the gun.
2. Position the needle notch at the place of interest in the tissue by inserting the needle in the organ of the anesthetised rat. The tissue fills the needle notch.
3. Release the cutting tube by pushing the button at the rear of the gun.
4. The tissue in the notch is excised.
5. Quickly remove the biopsy needle from the tissue and retract the outer needle once more by pulling the lever. The needle notch, with its content, is now exposed.
6. Quickly dip the needle tip in 1-hexadecene. This will avoid drying of the sample.
7. Place the biopsy gun in the socket of the microbiopsy transfer station.

3.4. Transfer of Tissue

The transfer of the tissue from the needle notch to the platelet slot is carried out with the help of the tissue transfer tool (*see* **Note 6**). The use of a stereoscopic microscope during this step makes the transfer very easy.

1. Install the tissue transfer tool in the short axis groove (sg in **Fig. 1A**) of the tissue transfer bench. This tool is properly installed when the small ridge fixes the gun needle against the bench (*see* **Fig. 5A**). The tissue transfer tool is then positioned precisely above the tissue in the needle notch (*see* **Fig. 5A,B**).
2. Press down the rod of the tissue transfer tool. The piece of tissue is transferred through the biopsy needle notch into the slot of the biopsy platelet (*see* **Fig. 5C**).
3. Remove the tissue transfer tool by sliding it into the groove. To and fro movements should be avoided because there is a risk of withdrawing the tissue piece from the platelet slot.
4. The biopsy platelet is now filled with tissue (*see* **Fig. 6A**).
5. Place the platelet transporter in position C. The platelet is located exactly under the diamond of the pod (*see* **Figs. 5D** and **6B**).
6. Close the pod screw with a torque wrench (*see* **Figs. 2A** and **7A**). Apply a force of 35 cNm.
7. Remove the platelet transporter; the clamp will detach easily.
8. Finally, fix the pod to the loading device (ld in **Fig. 2A,D**) of the Leica EM PACT high-pressure freezing machine (*see* **Fig. 7B**).

From now on, all further steps involve methods explained elsewhere. Only brief descriptions will be given.

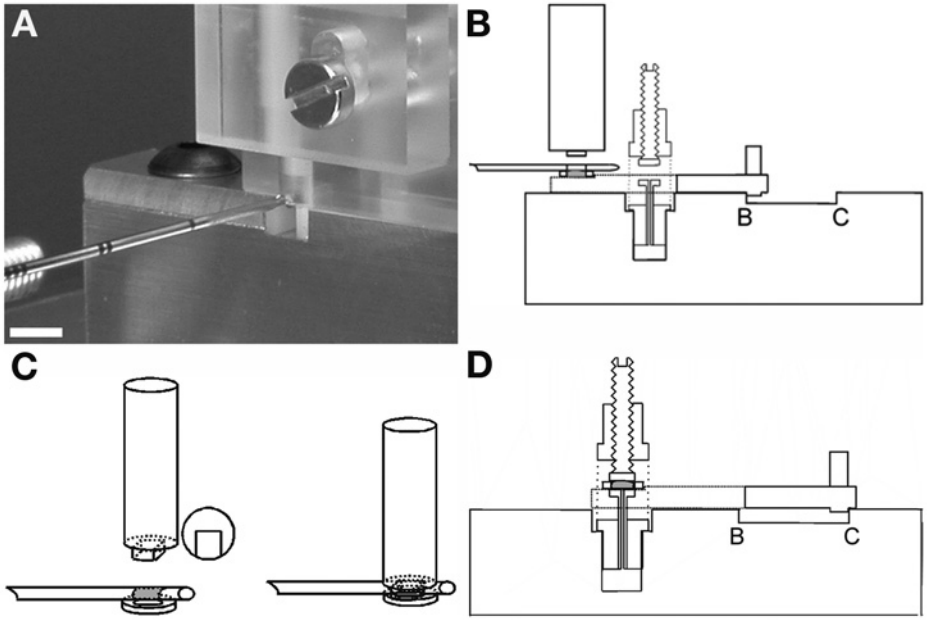

Fig. 5. **(A)** A detail of the tissue transfer bench, just prior to the tissue transfer. The tissue transfer tool is correctly positioned on the tissue transfer bench. The needle is pushed against the ridge. (Bar = 0.5 cm.) **(B)** A side-view scheme of the tissue transfer bench after the sample was transferred from the biopsy needle notch into the slot of the biopsy platelet. For simplicity, only the rod is depicted. **(C)** A schematic representation of the function of the tissue transfer tool. **(D)** A side-view schematic of the tissue transfer bench with the platelet transporter in position C. The biopsy platelet is now correctly aligned and fixed into the pod of the Leica EM PACT.

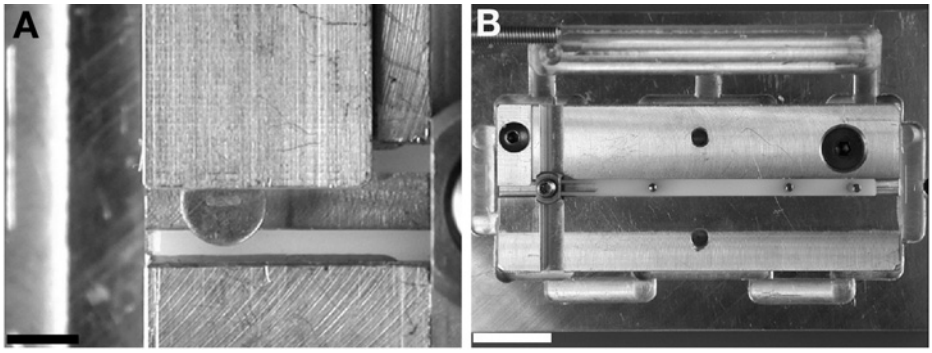

Fig. 6. **(A)** Muscle tissue in the platelet slot after the transfer. (Bar = 0.25 cm.) **(B)** The transporter in position C, ready to be fixed into the Leica EM PACT flat holder pod. (Bar = 2 cm.)

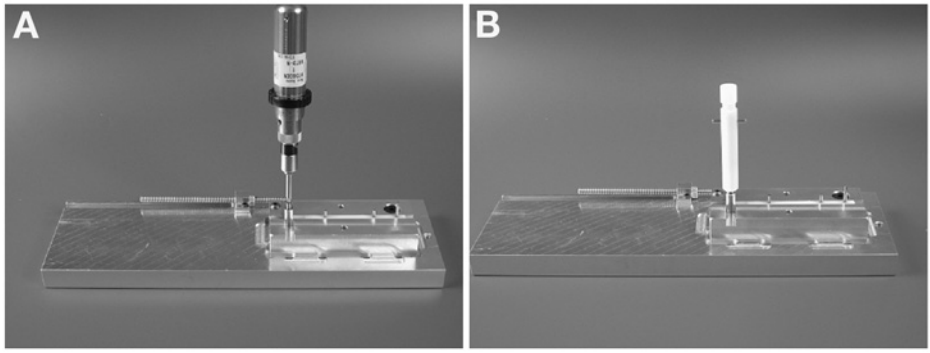

Fig. 7. **(A)** Closing the pod with the torque screw (torque: 35 cNm). **(B)** Fixing the pod into the loading device (L) of the Leica EM PACT.

3.5. Cryo-Fixation in the Leica EM PACT High-Pressure Freezing Machine

Once the tissue is cryo-fixed by high-pressure freezing, handle the tissue with care when using forceps (*see* **Note 7**). The manipulations are described in detail in **ref.** ***10***.

1. Rotate the pod in the loading device until the flat surfaces of the pod are perpendicular to the metal rods of the loading device.
2. Position the loading device in the Leica EM PACT loading arm.
3. Close the arm manually.
4. Push the "LOCK" button on the touch screen of the "preparation" menu.
5. Push the "START" button to freeze.
6. The sample is high-pressure-frozen and stored in the LN_2 bath.
7. Retract the platelets from the pod by loosening of the pod screw and collect them in the small containers (diameter: 1 cm; height: 0.5 cm).

The small containers (*see* **Fig. 2A,E**) are used to transport the samples from the LN_2 bath of the Leica EM PACT freezing machine to a styrofoam box (10 cm × 5 cm × 5 cm; *see* **Fig. 2A**), which is filled with LN_2. In the styrofoam box, the samples are transferred from the small containers to a closable storage container (*see* **Fig. 2A,F**). A small cryogenic Dewar (*see* **Fig. 2A**) is used to transport the containers to a large LN_2 storage system.

3.6. Follow-Up Methods

3.6.1. Freeze-Substitution

Freeze-substitution was carried out in an AFS freeze-substitution system (Leica Microsystems, Vienna, Austria) using Eppendorf tubes filled with the substitution medium. To avoid poor substitution because of 1-hexadecene (acetone

does not dissolve 1-hexadecene at low temperatures), samples surrounded with it were cleaned by softly scratching the samples with forceps in liquid nitrogen.

A substitution medium of 2% osmium tetroxide in acetone (calcium chloride dried) was applied *(10)* for 26 h at 183 K, 8 h at 213 K, and 8 h at 243 K. Temperature rises of 2°C/h were used, resulting in two temperature slopes of 15 h. Afterward, the biopsy platelets were removed and the samples put on ice (273 K) in dry acetone for 1 h. The removal of biopsy platelets was carried out by holding the platelet with forceps and pushing the sample out of the slot with a piston of appropriate size.

3.6.2. Embedding

Samples were embedded in Epon 812 stepwise (12 h in 30% Epon in acetone at 277 K, 12 h in 70% Epon in acetone at 277 K, and 12 h in 100% Epon at room temperature). Embedding was completed by polymerization for 3 d at 333 K using fresh resin.

3.6.3. Electron Microscope Preparation

The Epon blocks were sectioned with diamond knives (Diatome, Biel, Switzerland) using an ultramicrotome (UCT; Leica Microsystems, Vienna, Austria). Ultrathin (50 nm) sections were collected on 150-mesh grids and contrasted with uranyl acetate and lead citrate.

3.7. Results

Brain, muscle, liver, and kidney biopsies were taken from anesthetised rats. Untrained, but well-informed, persons were able to perform all steps (from excising until the tissue is cryo-fixed by high-pressure freezing) in a time period of about 30 s.

The samples were well frozen, and structural preservation was excellent, as shown in **Figs. 8–13**.

3.8. Discussion

A newly developed microbiopsy system allows very quick excision of soft animal tissue for high-pressure freezing. Moreover, it allows excision of small organs (e.g., rat kidney). It was pointed out *(9)* for mouse brain tissue that sampling times of 30 s result in a different morphological preservation compared to longer sampling times, reflecting artifact formation during longer preparations. The system presented allows cryo-fixation of animal tissues within 30-s time periods. The 30 s from excision to cryo-immobilization are still a long time period for certain samples (e.g., brain). However, to date, it is the best approach for preparing bulk tissue samples for cryo-fixation by high-pressure freezing. The good quality of the micrographs is proof that the system works.

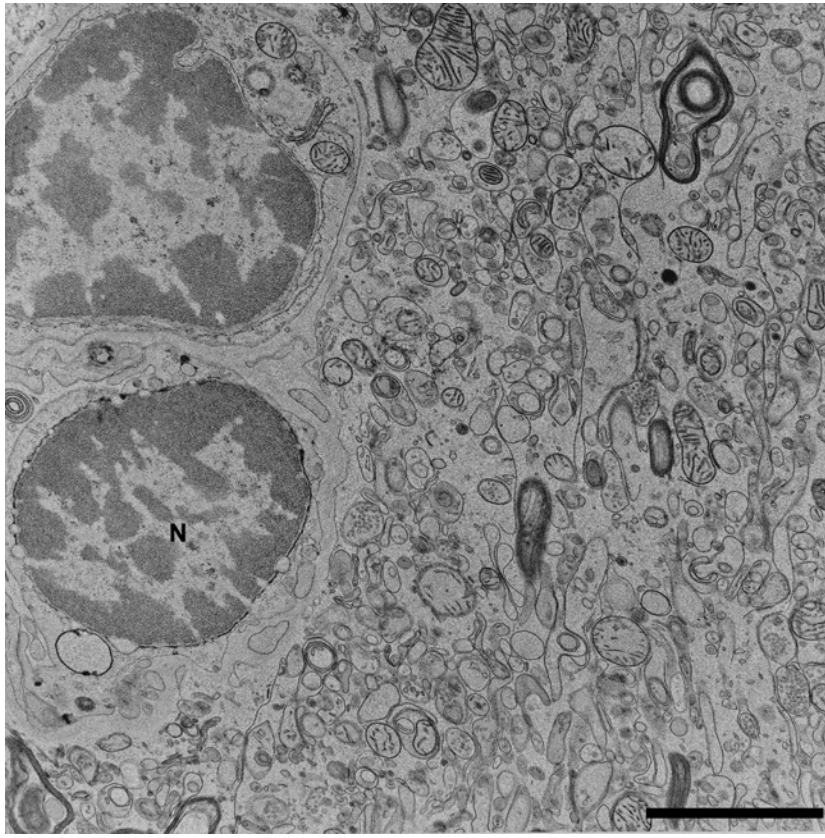

Fig. 8. Electron micrograph overview of a brain biopsy. Two cells with nuclei (N) are shown. (Bar = 2 µm.)

4. Notes

1. Although anesthesia can cause alterations on a cellular level, we favor the use of these drugs over the excision from both dead and conscious animals. Because autolytic reactions commence immediately after death, the excised tissue will not represent the native state. On the other hand, taking biopsies from conscious animals demands an expertise that is not available in every lab. Moreover, from the viewpoint of animal ethics, it is better to anesthetize the animals before taking any biopsies.
2. Biopsy platelets (*see* **Fig. 1E**) are the link between the microbiopsy system and the Leica EM PACT high-pressure freezing machine (Leica Microsystems, Vienna, Austria).The excised tissue in the needle notch fits exactly in the slot of the biopsy platelet, and the platelet is easy to mount in the flat specimen holder system of the Leica EM PACT.

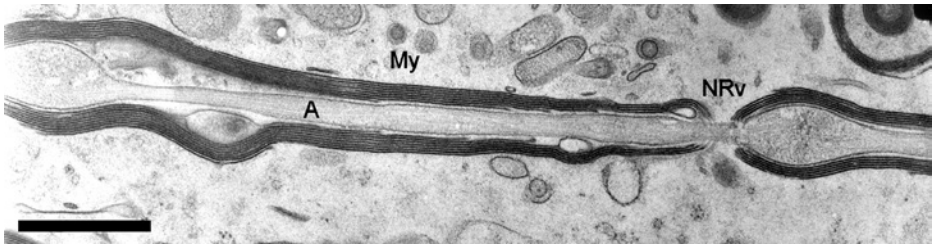

Fig. 9. Longitudinal section through an axon in rat brain, revealing the darker-stained myelin sheath (My) wrapped around the axon (A). A node of Ranvier (NRv) can be seen at the interruption in the myelin sheath. (Bar = 500 nm.)

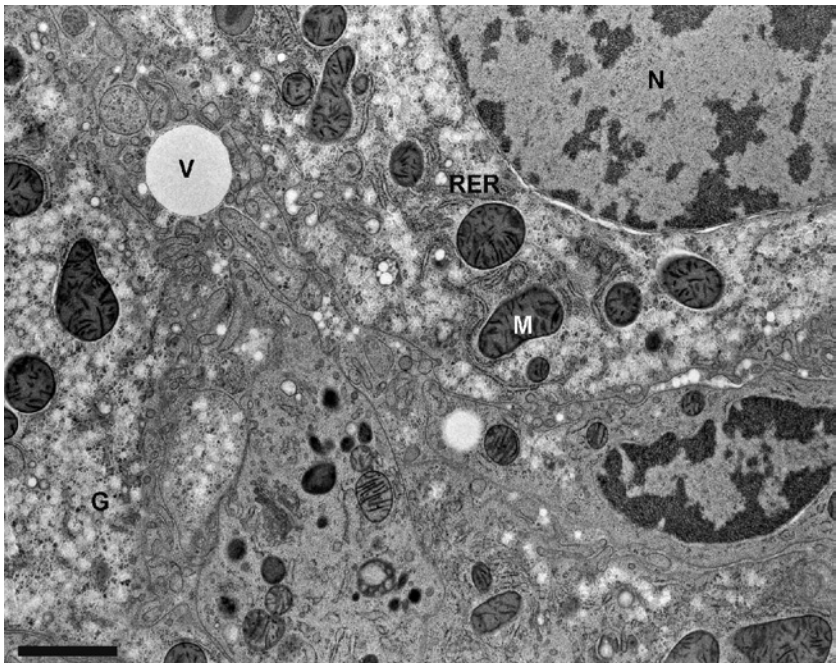

Fig. 10. Cross-section through rat liver. Mitochondria (M) are dark stained and glycogen (G) and vesicles (V) are apparent. Rough endoplasmic reticulum (RER) is present around the nucleus (N). (Bar = 2 μm.)

3. The pod consists of an alloy body with a central opening and an apical screw. On the tip of the screw, a diamond is mounted on a ball joint. The biopsy platelet has to be placed between a small table and the diamond screw. High pressure will be applied to the biopsy platelet slot via a small outlet in the center of the table. By closing the screw properly with a torque wrench (35 cNm applied), the biopsy

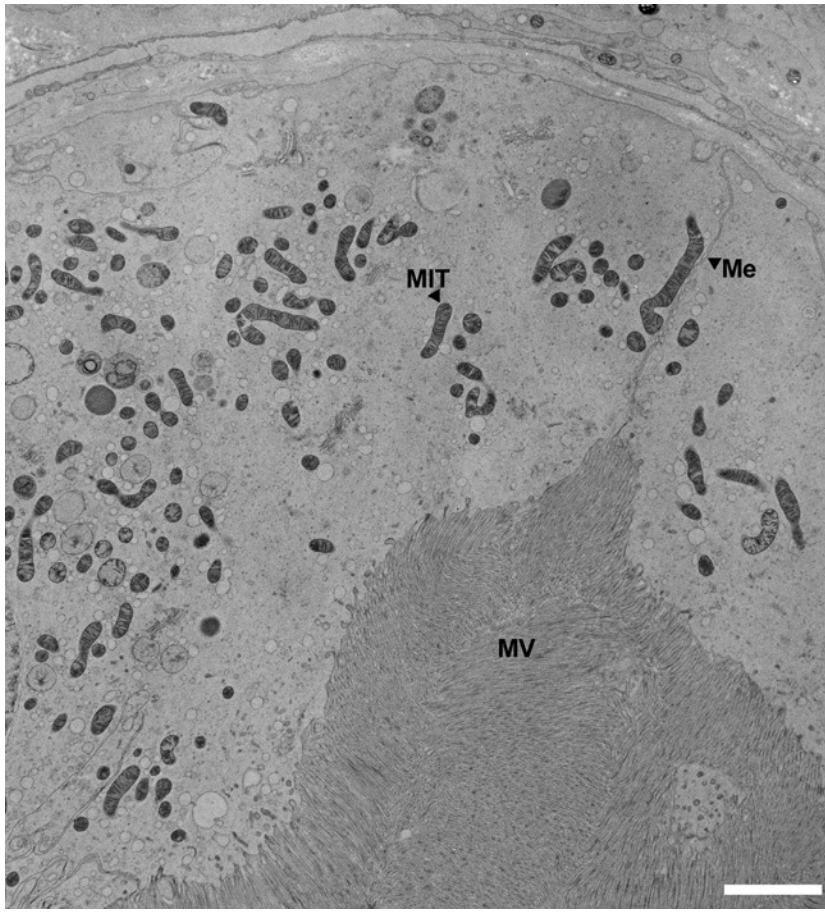

Fig. 11. Rat kidney overview: part of a cross-section through a tubule. In the parietal cytoplasm, darkly stained mitochondria (MIT) are present. A cell membrane (Me) borders two neighboring cells. The central area of the tubule is filled with cellular protrusions, microvilli (MV). (Bar = 4 μm.)

platelet slot is tightly sealed with the diamond and can withstand the more than 2000 bar pressure during the high-pressure freezing process. It is important that the biopsy platelet is accurately centered in the pod. If not, pressure losses will occur during freezing and high pressure freezing will fail.

4. The biopsy gun is a modified version of a Pro-mag 1.2 Manan biopsy gun (Manan, Northbrook, IL). When triggering and releasing the original Pro-mag 1.2 Manan biopsy gun (*see* **Fig. 1C,D**)**,** the inner needle is inserted into the tissue, immediately followed by the outer needle. Because of the extrusion of the inner needle, difficulties arise when one wants to obtain a biopsy from a particular, well-defined area or when small organs (e.g., mouse kidney) are subjected to excision. The modification

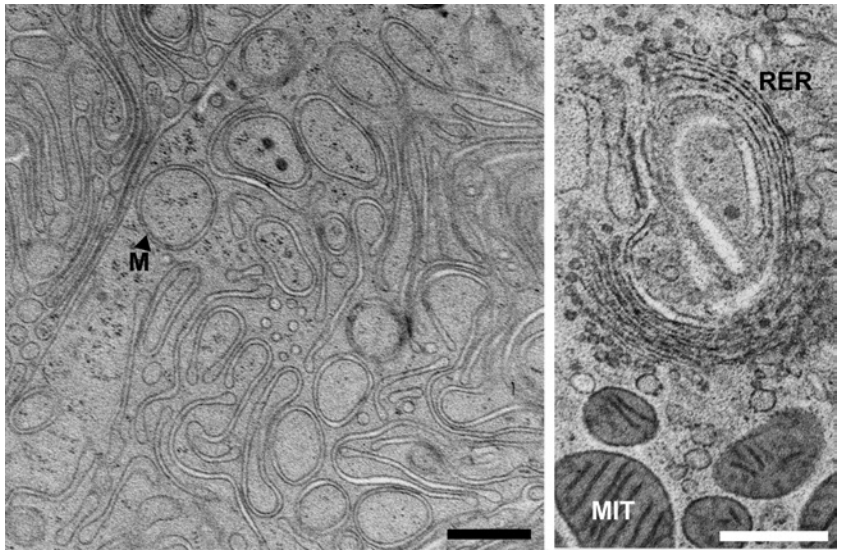

Fig. 12. Rat kidney ultrastructure. In the left panel, a section through the filter apparatus with smoothly outlined membranes (M); in the right panel, rough endoplasmic reticulum (RER) with surrounding mitochondria (MIT). (Bars = 500 nm.)

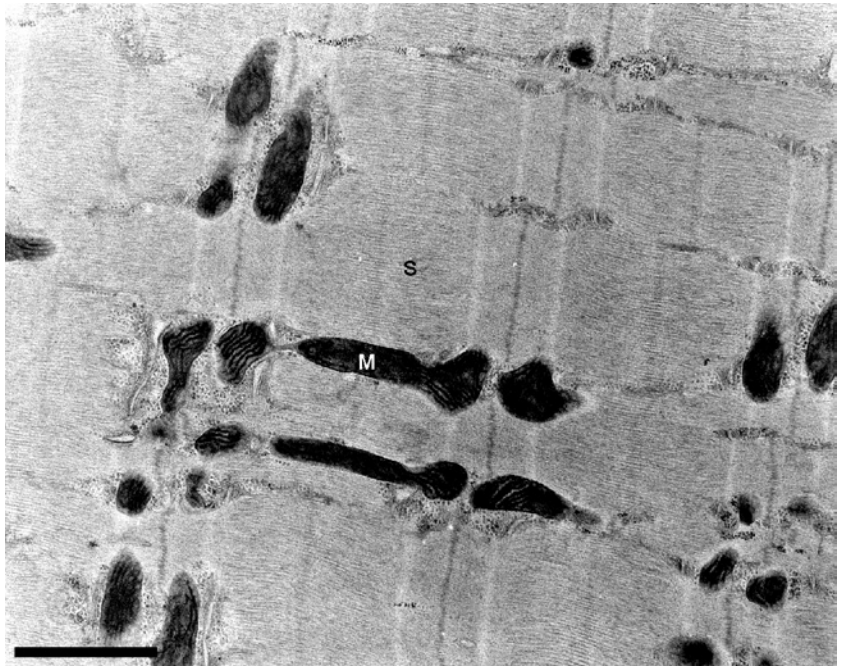

Fig. 13. Rat muscle ultrastructure shown in longitudinal section. The striped appearance of the sarcomeres (S) can be observed. The mitochondria (M) are well preserved and darkly stained. (Bar = 1 μm.)

we applied consists of fixing the inner needle to the gun (*see* **Fig. 1D**). The needle is inserted in the tissue; the notch positioned at the place of interest is filled with tissue. When releasing the cutting mechanism, only the outer needle moves forward and dissects the piece of tissue filling the notch.

5. The modified biopsy needles (*see* **Fig. 1B**) are based on Manan 20Ga.TWx8cm (Manan, Northbrook, IL) needles. The modified needles consist of an inner needle (diameter of 0.6 mm) with a notch and are surrounded by a tube with a sharp edge. The notch is 1.2 mm in length and half of the needle diameter deep.
6. The commercially available tissue transfer tool as developed by Leica differs from the device described here, but both tools are based on the same principle. The body of the tool ensures the position of a rod or ledge exactly above the needle notch. With a vertical movement, the tissue is pushed out from the needle directly into the platelet slot.
7. Forceps should always be precooled when handling cryo-fixed samples. When frozen samples are handled with warm (room temperature) tweezers, recrystallization of ice could occur, resulting in severe deterioration of the sample. Holding the tips of the forceps in liquid nitrogen until boiling stops (approx 20 s) is sufficient.

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