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"*IN VIVO* CRYOTECHNIQUE" FOR PARADIGM SHIFT TO "LIVING MORPHOLOGY" OF ANIMAL ORGANS

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*The morphological study has been one of the major approaches in medical and biological fields. For the last century, the conventional chemical fixation and alcohol dehydration were commonly used as an easy preparation method, but it was frequently pointed out that they usually yield many structural artifacts during their preparation processes. Although both conventional quick-freezing and high-pressure freezing methods, by which animal tissues are resected and frozen for physical fixation, can reduce such structural artifacts, the tissues have to be removed from living animal organs for the freezing. Therefore, such specimens are inevitably exposed to noxious stresses of anoxia and ischemia, exhibiting only dead morphological states of animal tissues without blood circulation. To the contrary, our "*in vivo *cryotechnique", by which all cells and tissues in animal bodies are cryofixed* in vivo*, can prevent such artifacts of resected specimens. By means of the cryotechnique, it is now possible to reveal the* in vivo *morphology of cells and tissues in living animal organs. Actually, it has been already applied to several animal organs, such as kidney, liver, intestine, cerebellum, eye ball, blood vessel, and joint cartilage, and brought new morphological findings, reflecting their physiological significance, which had been difficult to demonstrate by the conventional preparation methods. Moreover, its application to immunohistochemistry has also revealed more precise immunolocalizations of dynamically changing molecules in living animal organs, easily translocated by ischemic stresses and anoxia caused during the tissue resection. The "*in vivo *cryotechnique" allows us to perform novel morphological investigations of "living" morphological states, and develops new medical and biological fields with "living morphology" during this 21st century.*
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INTRODUCTION

The morphological study had been one of the indispensable approaches to understand the physiological and pathological features of living animals for the last century. Although substantial progress of research techniques in the molecular biology has been realized to establish new biological fields during the last decade (1,2), morphological techniques were also necessary for more precise understanding in the field, because structures of cells and tissues reflect some functional aspects of living animal organs. In such morphology, the electron microscopy, developed in the middle 20th century, has yielded the enormous progress of ultrastructural analyses, and it is now indispensable in the morphological field with many applications (3). Other progress in light microscopic approaches was also remarkable during the last decades, and

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new fluorescence technologies have enabled us to get dynamic images of signal molecules and their molecular interactions in living cells (4,5). However, each technique always has its own merit and demerit, and so it would be necessary to understand such technical features for choosing appropriate techniques in various morphological experiments.

Since 1995, we have been developing the "*in vivo* cryotechnique" to clarify functioning morphology of living animal organs, and reported new findings of dynamically changing structures and molecular immunolocalization in cells and tissues by both electron and light microscopy (6-16). Although this cryotechnique has some unique merits for the morphological analyses of living animal organs, there has been no systematic report about its comparison with other specimen preparation methods until now. Therefore, the purpose of this review is to describe some significant merits of the "*in vivo* cryotechnique", and review the new findings obtained by the cryotechnique.

PROBLEMS OF CONVENTIONAL PREPARATION METHODS

Although new technologies have greatly expanded our ability to examine structures of molecules, cells and tissues, they are still technically limited at the present time (5,17). In most morphological studies of animal organs, therefore, their preparation procedures are usually composed of chemical fixation, dehydration in organic solvents, embedding in paraffin wax or epoxy resin, sectioning, and dye or metal staining steps, followed by observation with a light or electron microscope (18). It has been known that various kinds of inevitable artifacts influence their morphological findings, especially owing to the conventional fixation and dehydration steps (19,20).

The first step, chemical fixation, is usually performed with routine chemical fixatives, such as paraformaldehyde, glutaraldehyde and osmium tetroxide (21). However, some technical problems of the chemical fixation have been already pointed out during the last century. One of the problems is the rapid molecular movement and structural changes occurring during the fixation step (20). For example, small soluble molecular components in cells and tissues are inevitably redistributed before the cross-linking effect is completed as the fixation mechanism (19). In addition, the chemical fixatives can sometimes modify the molecular conformation (22). Another technical problem is that transient or dynamic structures of cells and tissues are difficult to capture by the chemical fixation, because certain time intervals are necessary to complete it, particularly by the immersion fixation (23).

There are also other technical problems during the following steps, such as alcohol dehydration, embedding in paraffin wax or epoxy resin, sectioning and staining with dye or metal, although some of them are not restricted to the conventional preparation procedure. It has been frequently pointed out that the alcohol dehydration step can cause tissue shrinkage or additional morphological artifacts, which sometimes lead to misunderstanding of functional significance (24,25). In the case of embedding in epoxy resin, the embedding step needs heating up over 50°C, which might cause additional heat damage on biological tissues. The thickness of cut sections during the sectioning step depends on the embedded specimens and following observation devices, but the sectioning usually results in two-dimentional viewing of cells and tissues. Next, various kinds of staining processes are available during the dye or metal staining, but every staining technique enhances some aspects of the specimen structures and inversely obscures the others. Even during observation in an electron microscope, ultrathin sections stained with metals have to be put into highly vacuumed chambers and undergo heating damage induced by electron beams.

Although the conventional preparation techniques have several problems with morphological preservation of cells and tissues, the morphological studies have yielded important findings during the last century, and also contributed to academic achievement in biological and medical fields. On the other hand, lots of effort has been made to reduce such morphological artifacts commonly induced during the conventional preparation steps. For that purpose, the quick-freezing (QF) method was introduced for biological specimens at the middle of 20th century, and gradually improved during the late 20th century.

QUICK-FREEZING METHOD AND ITS TECHNICAL PROBLEMS

The QF method, usually termed as "cryofixation", is one of the physical fixation methods, in which biological specimens are not chemically fixed, but quickly embedded in vitrified ice (26). The term "vitrification" means ice formation without any ice crystals seen by electron microscopy, which often destroy morphological structures of cells and tissues. In the case of fresh resected tissues without cryoprotectants (e.g. glycerol or sucrose solution), the good freezing to obtain the vitrification has to be performed either at very high cooling-rate (usually more than 10^5 ^{o}C/sec) or under very high pressures (a few thousand times higher than atmospheric pressure) (26). For the last century, various QF methods have been developed for the purpose (reviewed in 26,27). One example is called the "slamming QF" method, by which resected tissues are slammed onto copper blocks cooled down in liquid helium

(-269ºC) or liquid nitrogen (-196ºC). Another example is called the "plunging QF" method, by which they are plunged into liquid cryogens, such as propane alone or isopentane-propane mixture (-193ºC) cooled in liquid nitrogen. The slamming or plunging QF method prevents formation of detectable ice crystals in areas less than 10 µm deep from the contacted tissue surface. To the contrary, the high-pressure freezing is a different method from the other QF methods, because it is always performed under very high pressures to achieve the vitrification in relatively broad tissue areas.

There are various preparation steps after the quick-freezing and the high-pressure freezing (Fig. 1). One of them is the freeze-substitution (FS), in which the frozen specimens are usually incubated in cooled organic solvents containing fixatives, such as osmium tetroxide, glutaraldehyde and paraformaldehyde at about -80ºC (23,28). After the freeze-substitution, they are embedded in epoxy resin, paraffin wax or other materials for the following sectioning. However, the freeze-substituted specimens are considered to be affected to some extent by the organic solvents and chemical fixatives (20). Another deep-etching (DE) replication method, following the quick-freezing, has been already developed, in which replica membranes of freeze-fractured tissues are obtained by shadowing with metal platinum and carbon (29) (Fig. 1). Therefore, the QF-DE method is now considered to prevent the morphological artifacts induced by the FS method (19,30,31).

As described in the previous paragraphs, one merit of the QF method is better morphological preservation with fewer artifacts (26,27), and it has been used for revealing new ultrastructural findings for the past few decades. However, no matter what kinds of cryotechniques are used for the physical

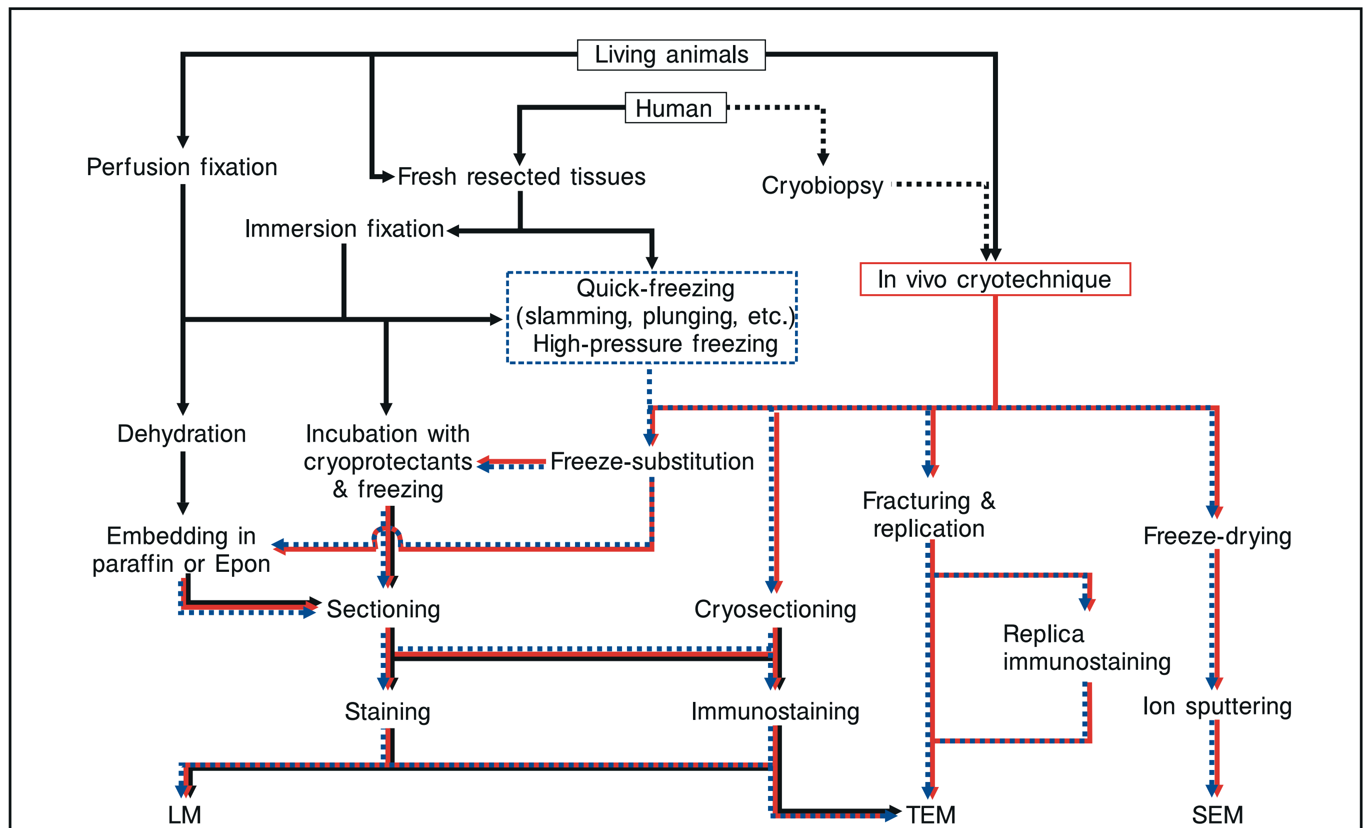

Figure 1. *A flowchart of various preparation steps for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The chemical fixation and dehydration steps (both immersion and perfusion fixation), the quick-freezing and high-pressure freezing methods (outlined with blue dotted lines), and the "*in vivo *cryotechnique" (outlined with red lines) are described with connection with their specimen preparation steps. The red arrows represent the preparation steps closely related to the "*in vivo *cryotechnique". Note that all preparation steps, following the quick-freezing and high-pressure freezing methods (blue dotted arrows), are available after the "*in vivo *cryotechnique". To apply the "*in vivo *cryotechnique" to larger animals, such as human, a new method of biopsy, termed here as "cryobiopsy", would be necessary (black dotted lines), described in* Future directions for "living morphology".

cryofixation, pieces of tissues have to be always taken out from living animal organs, and it has long been suggested that morphology of the resected tissues would be inevitably changed, because of loss of blood supply causing ischemia and anoxia (8,32). Thus, dynamic morphology of living animals, changing with various blood pressures, is hardly investigated by the conventional cryotechniques (7,8,11). Furthermore, immunohistochemical analyses of dynamically changing signal molecules would be more difficult by such conventional cryotechniques, because animal tissues have to be exposed to ischemia and anoxia during the resection process (16). To overcome these problems, it is necessary to avoid the removal of animal tissues, and directly freeze living animal organs *in vivo* under normal blood circulation. Although a few pioneering studies challenged to solve them (33,34), it was difficult to establish a new cryotechnique, which could yield satisfactory results for various organs of living animals.

DEVELOPMENT OF OUR NEW "*IN VIVO* CRYOTECHNIQUE"

The "*in vivo* cryotechnique" is a technique to directly cryofix living animal organs *in vivo* without resecting tissues. Briefly, after exposing the animal organs under anesthesia, it is performed by cutting them with a cryoknife precooled in liquid nitrogen (-196°C) and simultaneously pouring the isopentane-propane cryogen (-193°C) over them, followed by liquid nitrogen (8). By combination of the cryoknife and the cryogen, good vitrification of the frozen tissues can be obtained within the areas of several micrometers deep from the tissue surface first contacted with the cryoknife (8,14,35). Damaged areas caused by the cryoknife are usually limited within less than 0.5 µm from the contacted tissue edge (8).

The most significant point of the "*in vivo* cryotechnique" is preservation of blood circulation into living animal organs at the exact moment of freezing, in addition to the very high time-resolution of QF (26). Hence, it is possible to cryofix their cells and tissues with fewest artifacts induced by ischemia or anoxia (16). Furthermore, dynamic morphological changes of kidney or lung induced by different blood pressures or respiratory ventilation can be also examined in the specimens prepared by the "*in vivo* cryotechnique" (7-9). Therefore, it enables us to capture the cellular structures and molecular distributions closer to dynamic "living" states, which can't be detected by the other cryotechniques. Then, the preparation procedures following the other QF methods can be used after the "*in vivo* cryotechnique", as summarized in Figure 1. It was also shown that the cryotechniques reduce several steps of antigen retrieval treatments required for the chemically fixed and dehydrated tissues (6).

However, a few comments on the "*in vivo* cryotechnique" have to be made in this paragraph. First, well-frozen areas prepared by the "*in vivo* cryotechnique" are usually restricted to less than 10 µm depth from the surface tissue at an electron microscopic level, which is almost similar to that by the conventional QF methods (8,14,35-38). Therefore, it might be necessary to collect morphological data on ultrathin sections from the small areas of each specimen. Such troublesome efforts can be minimized by cutting the tissue sections in parallel to the cryoknife contact face, which usually increases the tissue areas with good freezing. Additionally, the tissue areas without detectable ice crystals are wider at a light microscopic level, because the spatial resolution of the light microscope is lower to detect the tiny ice crystals in cells and tissues, formed by slow freezing (6,16). To maximize the well-frozen tissue areas, it would be necessary to use several tips for the better freezing. These tips will be discussed in the paragraphs below. Second, to perform the "*in vivo* cryotechnique" and especially achieve the vitrification in wider areas, we have to expose the organs of living animals to pour the cryogen. Thus it is challenging to perform the "*in vivo* cryotechnique" if the target organs, such as lung under negative pressures, are difficult

Table 1. *Merits and demerits of "*in vivo *cryotechnique".*

Merits	Demerits
1. Preservation of blood supply into organs; hemodynamically changing morphological data and molecular immunolocalization *in vivo* can be obtained from cells and tissues in living animals.	1. Small well-preserved tissue areas, as similar to those obtained by the QF methods.
2. Highest time-resolution by immediate cryofixation, without ischemic or anoxic stresses; dynamically functioning signal, channel and receptor molecules can be precisely captured in various cells of living animal organs.	2. Surgical exposure of target organs to air atmosphere, especially in the case of electron microscopic specimens, while they are resected for the QF methods.

to expose *in vivo*. Some merits and demerits of the "*in vivo* cryotechnique" are listed in Table 1.

HOW TO PERFORM THE "*IN VIVO* CRYOTECHNQUE"

The "*in vivo* cryotechnique" was originally performed without the new "*in vivo* cryoapparatus", which will be described below (Fig. 2). For the original method, the isopentane-propane cryogen is manually poured over the exposed animal organs with or without cutting with a cryoknife. The cryogen must be poured over the target organs immediately after they are cut with the cryoknife. After pouring the cryogen for several seconds, the *in vivo* frozen tissues are cracked off from animal bodies and plunged into liquid nitrogen for preservation. When it is difficult to crack off the frozen tissues, like cerebellar tissues, the whole animal body with the target organ is first put in liquid nitrogen, and thereafter some tissue pieces can be taken out in the liquid nitrogen.

For the past several years, it was sometimes difficult to perform all procedures manually by one operator and constantly obtain well-frozen tissue specimens. To achieve them more easily, a new "*in vivo* cryoapparatus" is now commercially available all over the world (IV-12, Eiko engineering, Hitachinaka, Ibaraki, Japan) (Fig. 2a,b). The operation procedure of the "*in vivo* cryoapparatus" is described in Table 2. After performing the "*in vivo* cryotechnique" and removing the frozen tissues, various preparation steps can follow (Fig. 1).

To achieve the better freezing, there are a few tips in the performance of the "*in vivo* cryotechnique". First, the surgically exposed organ of living animals should not be dried up

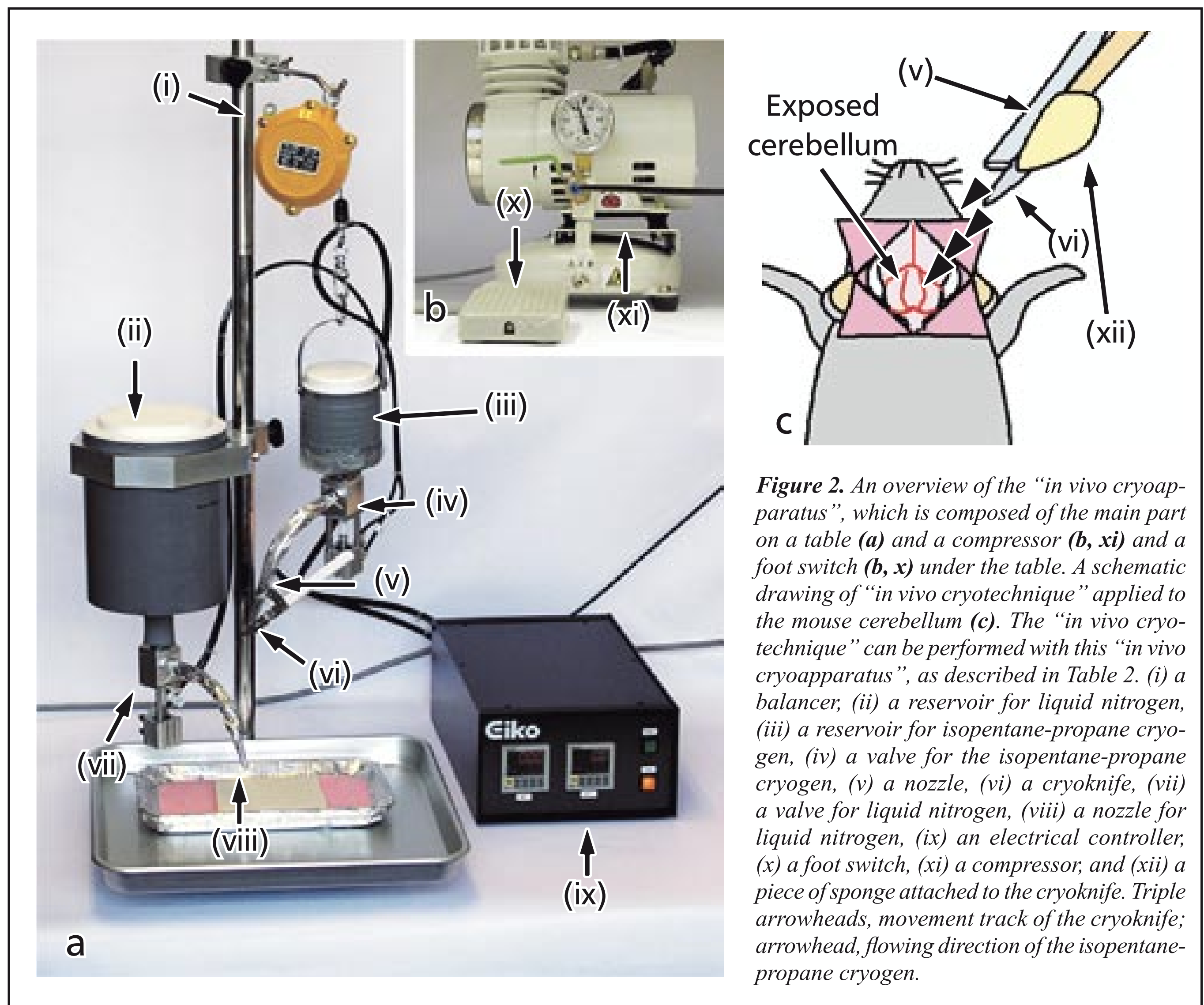

Figure 2. *An overview of the "in vivo cryoapparatus", which is composed of the main part on a table **(a)** and a compressor **(b, xi)** and a foot switch **(b, x)** under the table. A schematic drawing of "in vivo cryotechnique" applied to the mouse cerebellum **(c)**. The "in vivo cryotechnique" can be performed with this "in vivo cryoapparatus", as described in Table 2. (i) a balancer, (ii) a reservoir for liquid nitrogen, (iii) a reservoir for isopentane-propane cryogen, (iv) a valve for the isopentane-propane cryogen, (v) a nozzle, (vi) a cryoknife, (vii) a valve for liquid nitrogen, (viii) a nozzle for liquid nitrogen, (ix) an electrical controller, (x) a foot switch, (xi) a compressor, and (xii) a piece of sponge attached to the cryoknife. Triple arrowheads, movement track of the cryoknife; arrowhead, flowing direction of the isopentane-propane cryogen.*

***Table 2**. How to operate the "*in vivo *cryoapparatus".*

Step 1. Pour some amounts of liquid nitrogen into the reservoirs [Fig. 2(ii), (iii)] to be cooled down. Once the cooling of the reservoirs starts, they should be continuously cooled with liquid nitrogen. If they are warmed up, some water produced by melting of attached frost covers the valves. When the reservoirs are cooled down again, the valves are completely frozen and immobilized.

Step 2. Set timers of the controller [Fig. 2(ix)] and press the foot switch [Fig. 2(x)] to check if the liquid nitrogen in the reservoirs correctly comes through the nozzles [Fig. 2(v), (viii)]. This trial is important to prevent machinery accidents.

Step 3. Pour the isopentane-propane cryogen into the reservoir [Fig. 2(iii)]. The isopentane-propane cryogen is prepared beforehand by bubbling propane gas in liquid isopentane precooled in liquid nitrogen and always agitated with a magnet stirrer. The ratio of isopentane to propane should be 1:2 to 1:3 to achieve the maximal cooling ability at -193°C (63).

Step 4. Expose a target organ of living animals surgically under anesthesia.

Step 5. Set timers of the controller [Fig. 2(ix)], and precool the cryoknife [Fig. 2(vi)] in liquid nitrogen pooled in another container. A piece of sponge [Fig. 2(xii)] is usually attached to the cryoknife [Fig. 2(vi)], to absorb some liquid nitrogen and keep the cryoknife cooled during transfering over the exposed organ.

Step 6. From this step, see Fig. 2c. Bring the cooled cryoknife [Fig. 2(vi)] onto the exposed target organ, and press the foot switch [Fig. 2(x)]. Immediately after the pressing, cut the organ manually with the cryoknife (triple arrowheads in Fig. 2c), then followed by pouring of the isopentane-propane cryogen through the nozzle (Fig. 2(v), arrowhead in Fig. 2c), which was already initiated by pressing the foot switch and automatically regulated by the controller. In several seconds after pouring of the isopentane-propane cryogen, liquid nitrogen is also automatically poured onto the frozen organ through another nozzle [Fig. 2(viii)].

Step 7. Put the frozen organ as a whole in liquid nitrogen, and preserve it until removal.

Step 8. Get necessary tissue parts of the frozen organ in the liquid nitrogen by a dental electric drill.

in the air atmosphere. For this purpose, physiological saline is often poured over the target organ. However, the excessive saline should be always absorbed with small pieces of filter paper just before the freezing, because the vitrification is usually obtained from the directly contact surface areas covered with much less saline. Second, it is recommended to place small and thin plates, made of plastic or rubber and wrapped with aluminum sheets, under target organs in living animal bodies, to make it easy to recover the frozen target tissues, because the frozen organs are sometimes attached to other adjacent organs in the ice block or pushed down into the deeper position without such plates.

VARIOUS APPLICATIONS OF "*IN VIVO* CRYOTECHNIQUE"

A. Flowing erythrocytes in blood vessel

It has been well known that human or rodent erythrocytes exhibit a biconcave discoid shape in blood suspension, and their shapes can be changed by different hemodynamic conditions, various drugs and experimental calcium-loading (39-42). However, there had been few studies about morphology of flowing erythrocytes in large blood vessels and capillaries of living animal organs, because of technical difficulty in their preparation. Their morphology is particularly important to be examined, considering that deformability of flowing erythrocytes would affect the efficiency of blood flow in microcirculation and then oxygen supply into the organs (40). Therefore, the "*in vivo* cryotechnique", which can directly cryofix the flowing erythrocytes and preserve their native shapes and structures, has been already applied to the morphological investigations in various mouse organs, including abdominal aorta, inferior vena cava, liver and spleen (11,13,14).

The erythrocytes flowing in the inferior vena cava exhibited the typical shapes similar to well-known biconcave discoid ones, but those in the abdominal aorta showed rather ellipsoidal or irregular shapes (Fig. 3a,b) (14). This difference of the erythrocyte shapes was caused by hemodynamic factors of blood flowing speeds and pressures, which are much higher in the abdominal aorta. To the contrary, flowing erythrocytes in mouse hepatic sinusoids exhibited various shapes in the branching blood vessels under normal circulation, as similar

to those in the splenic red pulp (Fig. 3c) (11,13). They were changed into the biconcave discoid shape after the heart arrest (Fig. 3d). Therefore, a variety of erythrocyte shapes are considered to be caused by dynamic turbulence of blood flow, which is presumably induced by complicated networks of blood vessels in some organs, such as liver and spleen. From these findings, the "*in vivo* cryotechnique" is useful for morphological analyses of flowing erythrocytes *in vivo*. Moreover, it can be applied to immunohistochemical examination of other blood cells and components flowing in blood vessels of living animal organs.

B. Abdominal organs

The "*in vivo* cryotechnique" can be also applied to examining various abdominal organs, such as kidney, intestine and liver, but a few technical points should be considered for each preparation step. First, as already mentioned above, the abdominal organs are easily moved by the external press force and pushed into deeper position by the cryoknife. Therefore, small plastic plates should be placed under the target organ in advance to remove it more easily after freezing. Second, in the case of retroperitoneal organs, such as kidney and abdominal aorta, the parietal peritoneum and surrounding connective tissues are carefully removed to achieve the better freezing. Third, as the surface parts of abdominal organs are usually covered with a little ascites and kept moist in living animals, careful attention should be paid to keep them from drying up, with physiological saline or moist filter paper, as described in

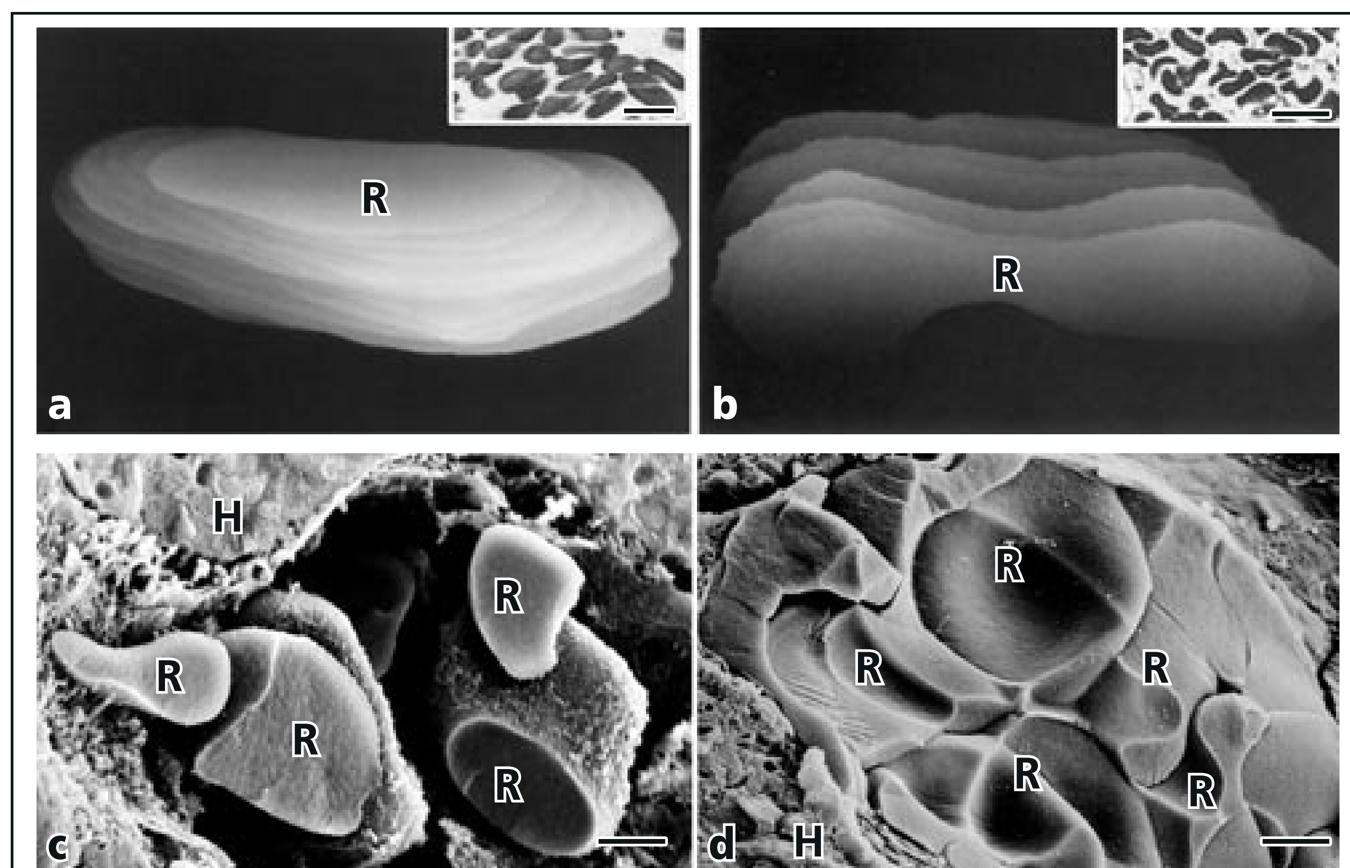

Figure 3. *Electron micrographs of flowing erythrocytes in abdominal aorta (AAo)* ***(a)****, inferior vena cava (IVC)* ***(b)*** *and hepatic sinusoid* ***(c,d)*** *under normal blood circulation* ***(a-c)*** *or the heart arrest condition* ***(d)****. Two reconstructed images of flowing erythrocytes in AAo* ***(a)*** *and IVC* ***(b)*** *are obtained on serial ultrathin sections by TEM. Although erythrocytes flowing in IVC* ***(b, inset)*** *exhibit shapes resembling traditional biconcave discoid shapes, which are more evident in the reconstructed image* ***(b)****, those flowing in AAo* ***(a, inset)*** *have ellipsoidal shapes* ***(a)****. Erythrocytes flowing in hepatic sinusoids exhibit various shapes, as observed by SEM* ***(c)****. The free spaces among erythrocytes, which are considered to be filled with plasma in the living mouse, are well preserved under normal blood circulation* ***(c)****, although such free spaces become collapsed under the heart arrest condition, resulting in typical biconcave discoid shapes of congested erythrocytes* ***(d)****. H, hepatocyte; R, erythrocyte. Bars: 10 µm in insets, and 1µm in c and d.*

the previous section. However, the excessive fluid covering the surface tissues of target organs should be wiped off just before the freezing.

Morphological analyses of kidney

It is generally accepted that the glomerular basement membrane constitutes a major part of filtration barriers between blood capillaries and urinary spaces. Although morphology of the basement membrane would be affected by hemodynamic factors, probably causing modification of normal barrier functions (43-48), the precise ultrastructures of glomerular capillary loops couldn't be analyzed by the conventional preparation methods, because of some technical problems. For example, the morphology of glomerular capillary loops can be easily modified by artificial pressures during the perfusion fixation and also components of fixatives (49,50). In addition, the glomerular ultrastructures can be also changed by renal ischemia during the resection step (44), which is inevitable in the conventional QF methods. To overcome these problems, we have applied the "*in vivo* cryotechnique" to the morphological analyses of glomerular capillary loops of living mouse kidneys under different hemodynamic conditions.

A mouse kidney was exposed under pentobarbital anesthesia, and then the "*in vivo* cryotechnique" was routinely performed, as already described above. Additionally, to investigate morphological changes of glomerular capillary loops by either increase or decrease of renal blood flow, it was also performed after the ligation of abdominal aorta at the point just distal to branching renal arteries (a hyperflow state), or after the heart arrest by intraperitoneal injection of excessive amounts of pentobarbital (a heart arrest state). After resecting the frozen kidney tissues, some of them were freeze-substituted in acetone containing 2% osmium tetroxide, and routinely processed for scanning electron microscopy (SEM) (7,15). Other specimens were embedded in epoxy resin after the freeze-substitution, sectioned under an ultramicrotome, stained with uranyl acetate and lead citrate, and finally observed with a transmission electron microscope (TEM) (8). For comparison, mouse kidney tissues were prepared by the conventional chemical fixation with glutaraldehyde and osmium tetroxide, followed by the routine dehydration with a graded series of alcohol (8,15). The conventional QF method was also performed, in which fresh kidney tissues were resected and quickly frozen by plunging into the isopentane-propane cryogen (8).

The slit spaces between foot processes were widely open in the specimens prepared by the conventional chemical fixation (Fig. 4a). In addition, such foot processes exhibited their irregular surface contours, indicating that they were shrunken during the chemical fixation and dehydration steps, as reported before (Fig. 4a) (8). To the contrary, in the specimens prepared by the conventional QF method (Fig 4b), the width of the slit spaces became narrow, because the shrinkage artifacts could be hardly produced with the cryofixation. Moreover, in the specimens prepared by the "*in vivo* cryotechnique" (Fig. 4c), the slit spaces between foot processes were more widely open under normal blood circulation, and their height was a little shorter than that as revealed by the conventional QF method. In the specimens of mouse kidneys under the hyperflow state (Fig. 4d), they were much more widely open, and their height was dramatically shorter, which might reflect extension of foot processes around blood capillaries due to the increase of renal blood flow (8). By SEM (Fig. 5), both elongation of foot processes and wideness of their slit spaces could be more obviously confirmed in the view of three-dimension (Fig. 5e-f), as compared with those under normal blood circulation (Fig. 5a, b) (7). On the other hand, deep interdigitation of compact foot processes was seen in the specimens of mouse kidney under the heart arrest, and the slit spaces between foot processes became narrower (Fig. 5c,d).

The widely open slit spaces between foot processes in the specimens prepared by the conventional chemical fixation were considered to be artifacts induced by perfusion pressures of chemical fixatives and shrinkage of foot processes during the alcohol dehydration (8). The new findings obtained by the "*in vivo* cryotechnique" indicate that the renal glomerular capillary loops can be dynamically changed *in vivo*, depending on the renal blood flow condition. The "*in vivo* cryotechnique" would be useful for physiological and pathological analyses of dynamic morphology in kidney and other organs, which has been difficult to be examined with the conventional chemical fixation or QF methods.

Dynamic ultrastructures of smooth muscle cells

Intestinal organs of normal mice or dystrophin-deficient mdx mice under pentobarbital anesthesia were surgically exposed in their abdomen and put on a thin plastic plate. The "*in vivo* cryotechnique" was similarly performed, as described above. Some of the frozen specimens were routinely freeze-substituted in acetone containing osmium tetroxide, embedded in epoxy resin, sectioned, stained with uranyl acetate and lead citrate, and observed with TEM. Others were freeze-fractured, deeply etched under high vacuum conditions and replicated with platinum and carbon in the freeze-fracture apparatus (Eiko FD-3AS, Eiko Engineering, Ibaraki, Japan), as reported before (10). Some tissue components attached to the prepared replica membranes were partially dissolved by incubation in both 5% sodium dodecyl sulfate (SDS) and 0.5% collagenase.

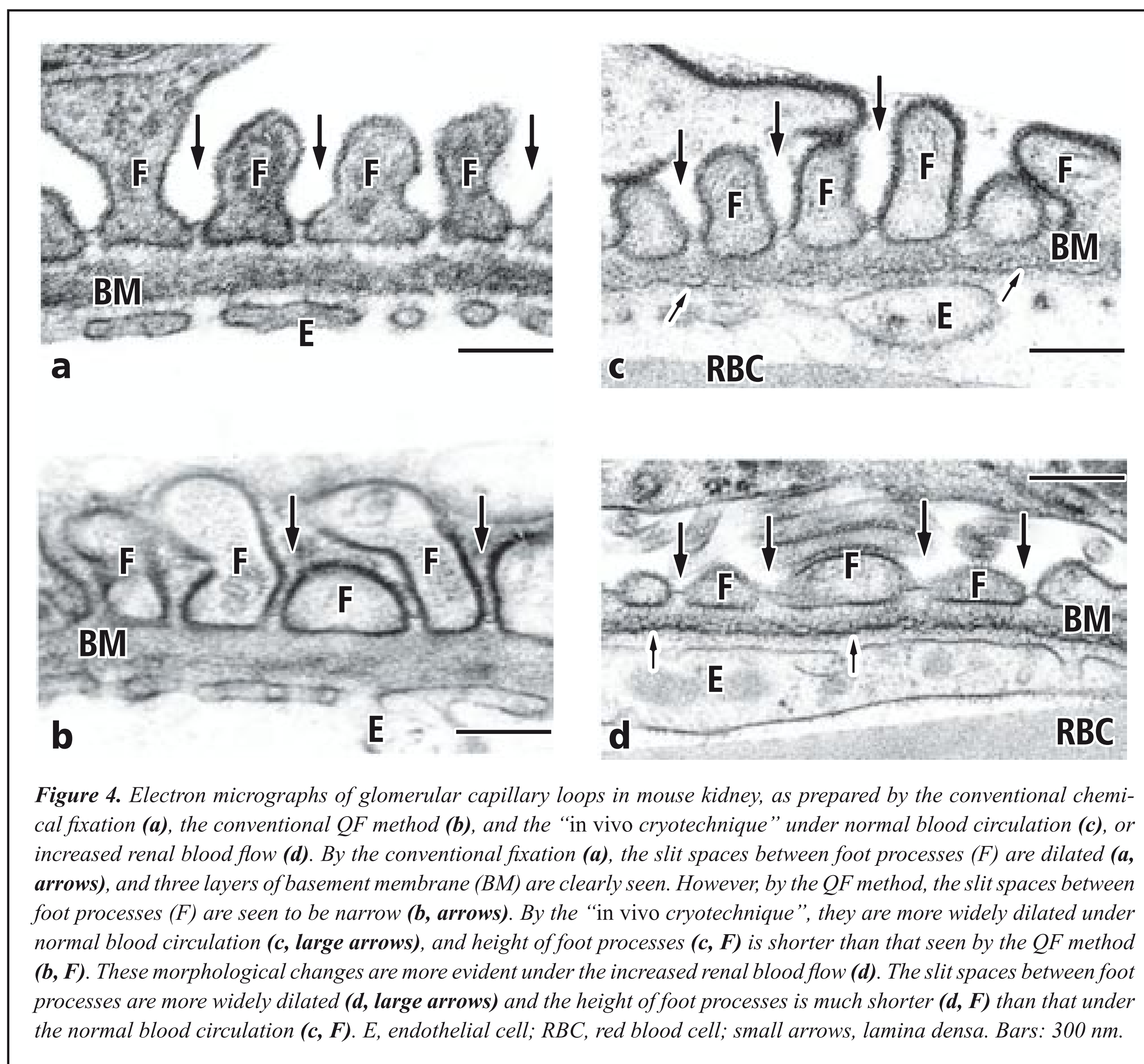

Figure 4. *Electron micrographs of glomerular capillary loops in mouse kidney, as prepared by the conventional chemical fixation **(a)**, the conventional QF method **(b)**, and the "*in vivo *cryotechnique" under normal blood circulation **(c)**, or increased renal blood flow **(d)**. By the conventional fixation **(a)**, the slit spaces between foot processes (F) are dilated **(a, arrows)**, and three layers of basement membrane (BM) are clearly seen. However, by the QF method, the slit spaces between foot processes (F) are seen to be narrow **(b, arrows)**. By the "*in vivo *cryotechnique", they are more widely dilated under normal blood circulation **(c, large arrows)**, and height of foot processes **(c, F)** is shorter than that seen by the QF method **(b, F)**. These morphological changes are more evident under the increased renal blood flow **(d)**. The slit spaces between foot processes are more widely dilated **(d, large arrows)** and the height of foot processes is much shorter **(d, F)** than that under the normal blood circulation **(c, F)**. E, endothelial cell; RBC, red blood cell; small arrows, lamina densa. Bars: 300 nm.*

The replica membranes with remaining proteins were first immunostained with primary rabbit anti-caveolin antibody and then with secondary goat anti-rabbit IgG antibody conjugated with 10 nm gold particles (10). The immunostained replicas were mounted on Formvar-filmed copper grids and observed with TEM.

In the specimens of normal living mice, smooth muscle cells in outer muscle layers of intestine were located close to each other, and their spontaneous contraction could be often observed in some areas of the cytoplasm (Fig. 6a). To the contrary, intercellular spaces between smooth muscle cells were enlarged, and extracellular matrices were partially disrupted under the muscle contracting condition in the specimens obtained from the dystrophin-deficient mdx mice (Fig. 6b). Additionally, irregularly shaped caveolae and fusion of several caveolae could be more often seen in the mdx mice than those in the normal mice (Fig. 6b, inset). By the replica immunoelectron microscopy, many immunogold particles for caveolin were mainly localized around caveolae in smooth muscle cells of normal mice (Fig. 6c), but those of the mdx mice were found to be also localized on flat cell membranes without caveolar pits (Fig. 6d) (10). In addition, the caveolar pits of the dystrophin-deficient mdx mice were larger in neck

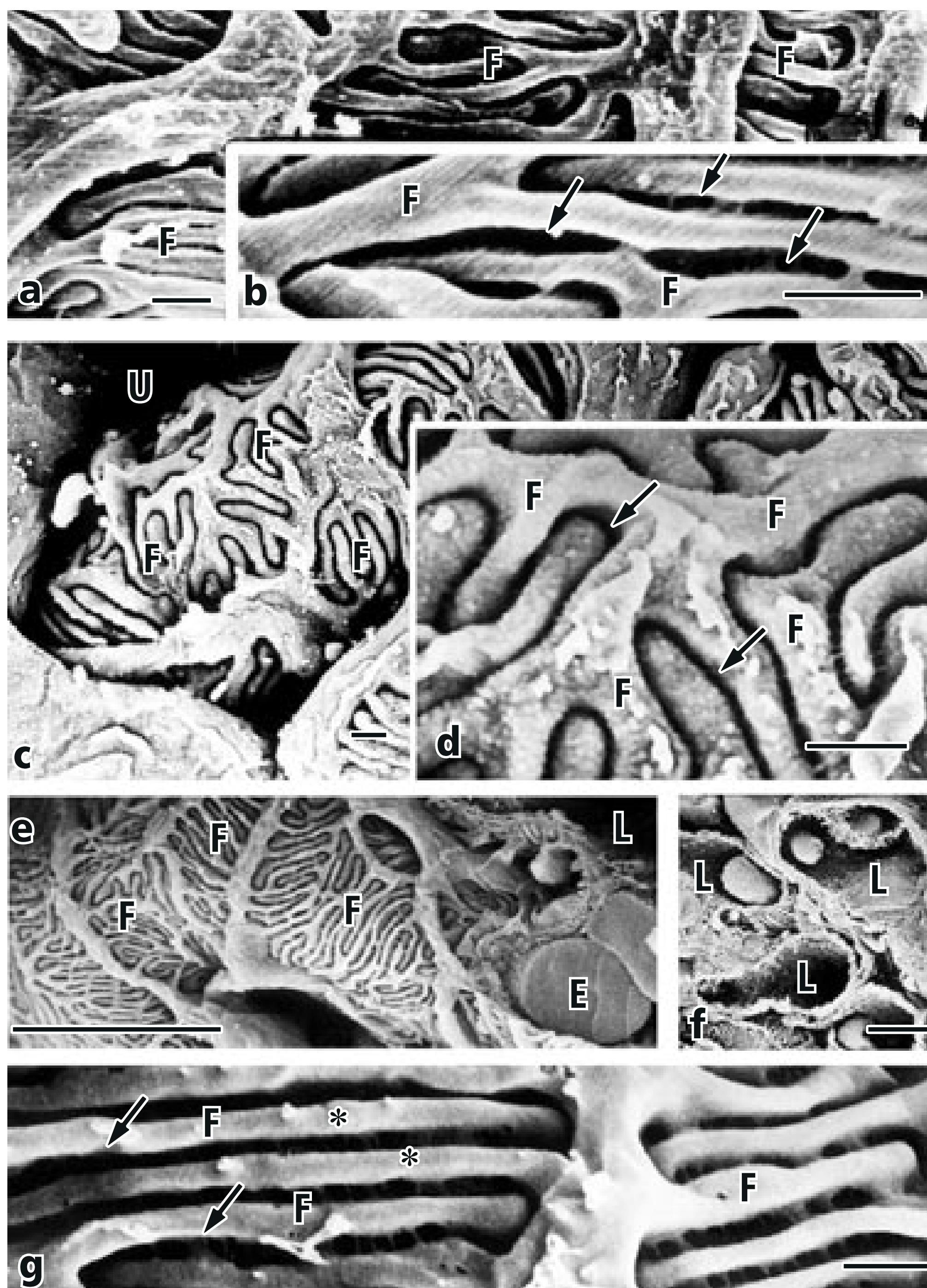

*Figure 5. Scanning electron micrographs of living mouse kidney obtained by the "in vivo cryotechnique" under normal blood circulation **(a,b)**, heart arrest **(c,d)** and increased renal blood flow conditions **(e,f,g)**. As seen with foot processes under the normal blood circulation **(b, arrows)**, slit spaces between foot processes (F) are widely open under the increased renal blood flow **(g, arrows)**, although the slit spaces become narrow under the heart arrest condition **(d, arrows)**. Additionally, the foot processes become thinner under the increased renal blood flow **(g, asterisks)**. E, erythrocyte; L, luminal space; U, urinary space. Bars: 5 μm in (e) and (f), and 500 nm in the others.*

size than those of the normal mice (Fig. 6c,d).

Thus, the "*in vivo* cryotechnique" has revealed morphological and immunohistochemical features of spontaneously contracting smooth muscle cells in the normal living mouse intestine and clarified their clear difference of extracellular matrix and caveolae from those in the dystrophin-deficient mdx mice. From these findings, the "*in vivo* cryotechnique" combined with the replica immunoelectron microscopy would be highly useful for examining functional ultrastructures of living animal cells and in situ immunodistribution of signal molecules.

Light microscopic analyses of liver specimens

Mouse liver tissues were prepared by the "*in vivo* cryotechnique", as compared with those frozen by the plunging QF method with isopentane-propane cryogen. The both frozen specimens were freeze-substituted in acetone containing 2% PFA and embedded in the paraffin wax. Then thin sections were routinely stained with hematoxylin-eosin (HE) and observed with a light microscope. Other sections were immunostained with goat anti-mouse albumin or IgG antibody and visualized by the routine immunoperoxidase method.

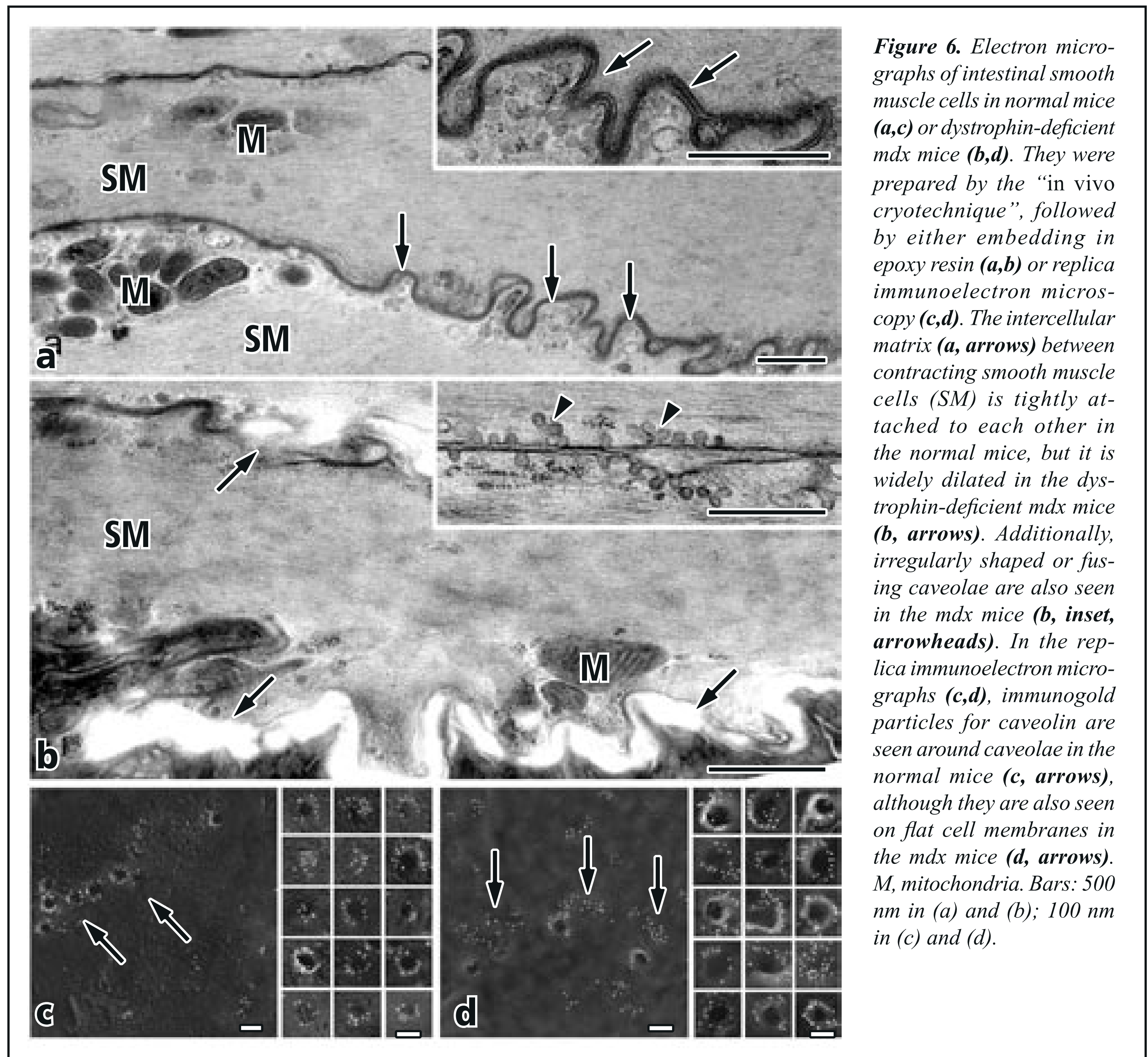

Figure 6. *Electron micrographs of intestinal smooth muscle cells in normal mice* ***(a,c)*** *or dystrophin-deficient mdx mice* ***(b,d)****. They were prepared by the "*in vivo *cryotechnique", followed by either embedding in epoxy resin* ***(a,b)*** *or replica immunoelectron microscopy* ***(c,d)****. The intercellular matrix* ***(a, arrows)*** *between contracting smooth muscle cells (SM) is tightly attached to each other in the normal mice, but it is widely dilated in the dystrophin-deficient mdx mice* ***(b, arrows)****. Additionally, irregularly shaped or fusing caveolae are also seen in the mdx mice* ***(b, inset, arrowheads)****. In the replica immunoelectron micrographs* ***(c,d)****, immunogold particles for caveolin are seen around caveolae in the normal mice* ***(c, arrows)****, although they are also seen on flat cell membranes in the mdx mice* ***(d, arrows)****. M, mitochondria. Bars: 500 nm in (a) and (b); 100 nm in (c) and (d).*

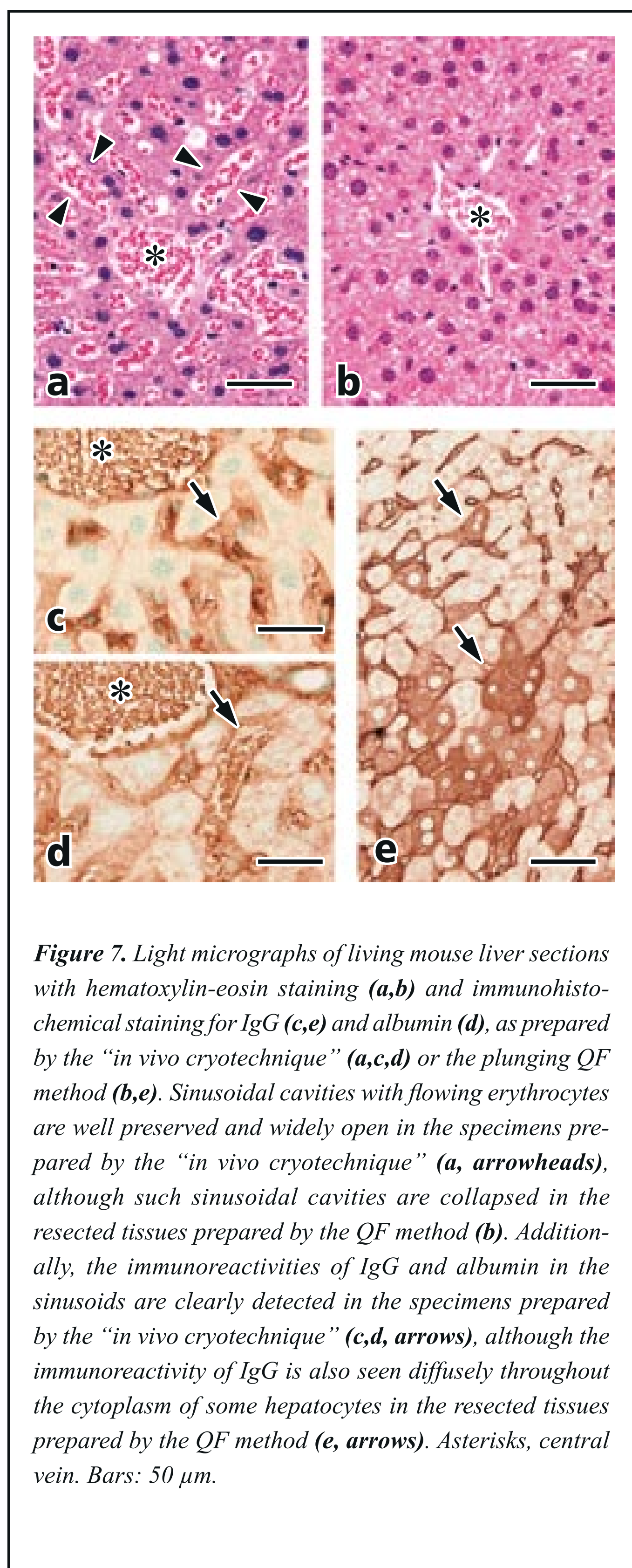

Figure 7. *Light micrographs of living mouse liver sections with hematoxylin-eosin staining* ***(a,b)*** *and immunohistochemical staining for IgG* ***(c,e)*** *and albumin* ***(d)****, as prepared by the "in vivo cryotechnique"* ***(a,c,d)*** *or the plunging QF method* ***(b,e)****. Sinusoidal cavities with flowing erythrocytes are well preserved and widely open in the specimens prepared by the "in vivo cryotechnique"* ***(a, arrowheads)****, although such sinusoidal cavities are collapsed in the resected tissues prepared by the QF method* ***(b)****. Additionally, the immunoreactivities of IgG and albumin in the sinusoids are clearly detected in the specimens prepared by the "in vivo cryotechnique"* ***(c,d, arrows)****, although the immunoreactivity of IgG is also seen diffusely throughout the cytoplasm of some hepatocytes in the resected tissues prepared by the QF method* ***(e, arrows)****. Asterisks, central vein. Bars: 50 µm.*

As compared with the resected liver tissues, sinusoidal cavities between hepatocytes in living mouse liver prepared by the "*in vivo* cryotechnique" were maintained to be widely open, accompanied by many flowing erythrocytes (Fig. 7a). The serum albumin and IgG were clearly immunolocalized in the sinusoidal cavities of living mouse liver (Fig. 7c,d), and cytoplasmic immunoreactivity of synthesized albumin could be also detected in almost all hepatocytes. However, in the resected liver tissues prepared by the plunging QF method, both albumin and IgG immunoreactivities could be detected in the cytoplasm of some hepatocytes, in addition to their immunolocalization in the collapsed sinusoids (Fig. 7b, e). These findings indicate that anoxia and ischemic stresses with the tissue resection might induce rapid changes of cell membrane permeability of hepatocytes and distribution of blood components in the liver tissues. Therefore, the "*in vivo* cryotechnique" would be also useful for immunohistochemical analyses on such rapid translocation of serum proteins in other organs.

Visualization of injected fluorescent dye

A living mouse kidney was surgically exposed under pentobarbital anesthesia, and directly injected inside its renal capsule with FITC-conjugated goat IgG (green). They were quickly frozen in a few seconds immediately after the injection by the "*in vivo* cryotechnique". The frozen tissues were routinely freeze-substituted in acetone containing 2% PFA, incubated with 30% sucrose, and sectioned in a cryostat machine after freezing. In the cryostat sections, the green fluorescent dye could be observed in glomelular capillary loops in addition to blood vessels around proximal or distal tubules (Fig. 8). Therefore, the "*in vivo* cryotechnique" in combination with direct injection of fluorescent dyes would be useful for dynamic analyses of time-dependent changes of blood flow in living animal organs.

C. Central nervous system (cerebellum)

The central nervous regions, including cerebellum and cerebrum, are covered with the cranial bone, which must be carefully opened in the case of living mice under pentobarbital anesthesia. Some devices, such as dental electric drills, are useful for opening the cranial bone. When highly concentrated attention has been paid to minimizing their bleeding and preventing them from drying, the "*in vivo* cryotechnique" can be successfully applied to their morphological and immunohistochemical analyses.

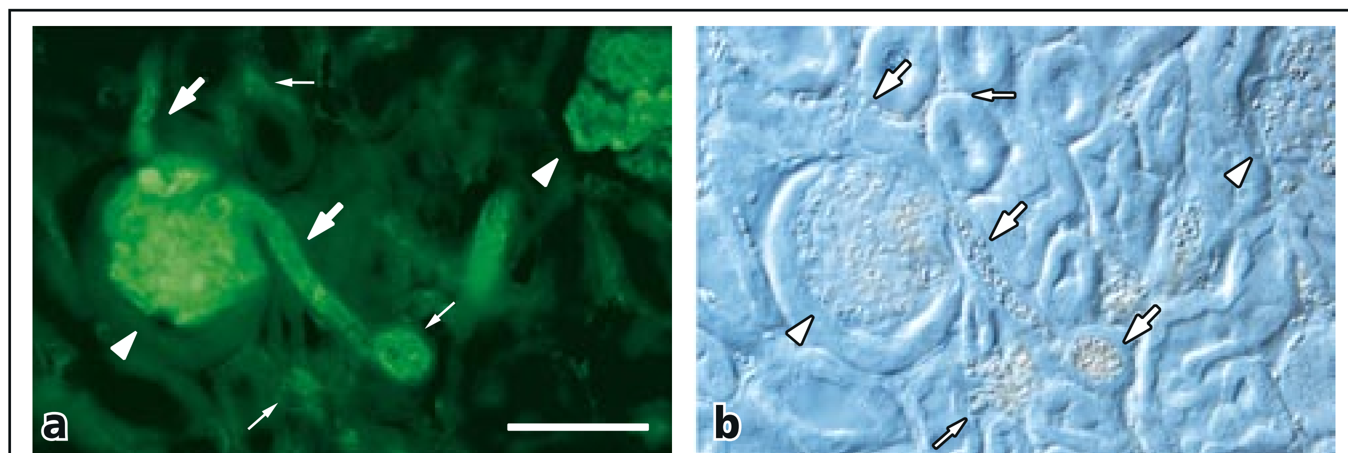

Figure 8. *Light micrographs of fluorescence **(a)** or DIC image **(b)** of a living mouse kidney after direct injection of a green fluorescent dye, as prepared by the "in vivo cryotechnique". The fluorescent dye circulating in an afferent or efferent arteriole (large arrows), glomerular capillary loops (arrowheads), and blood vessels among renal tubules (small arrows) can be clearly observed under normal blood circulation. Bar: 100 µm.*

Morphological analyses of molecular layer

The mouse cerebella frozen by the "*in vivo* cryotechnique" were freeze-substituted in acetone containing 2% osmium tetroxide and embedded in epoxy resin. Other cerebella, which had been routinely perfusion-fixed with glutaraldehyde, were postfixed with osmium tetroxide, dehydrated in a graded series of alcohol, and embedded in the epoxy resin. Both embedded cerebellar tissues were cut on an ultramicrotome, routinely stained with uranyl acetate and lead citrate and observed with TEM. Extracellular spaces among neuronal and glial cells in molecular layers were well maintained to be widely open in the specimens prepared by the "*in vivo* cryotechnique" (Fig. 9a). Some synaptic densities, where the intercellular spaces became narrow, could be observed in the widely open extracellular spaces (Fig. 9a, arrowhead). To the contrary, the extracellular spaces were not well preserved and lost in the specimens prepared by the conventional fixation and dehydration method (Fig. 9b). It was reported that the ischemia of cerebral tissues

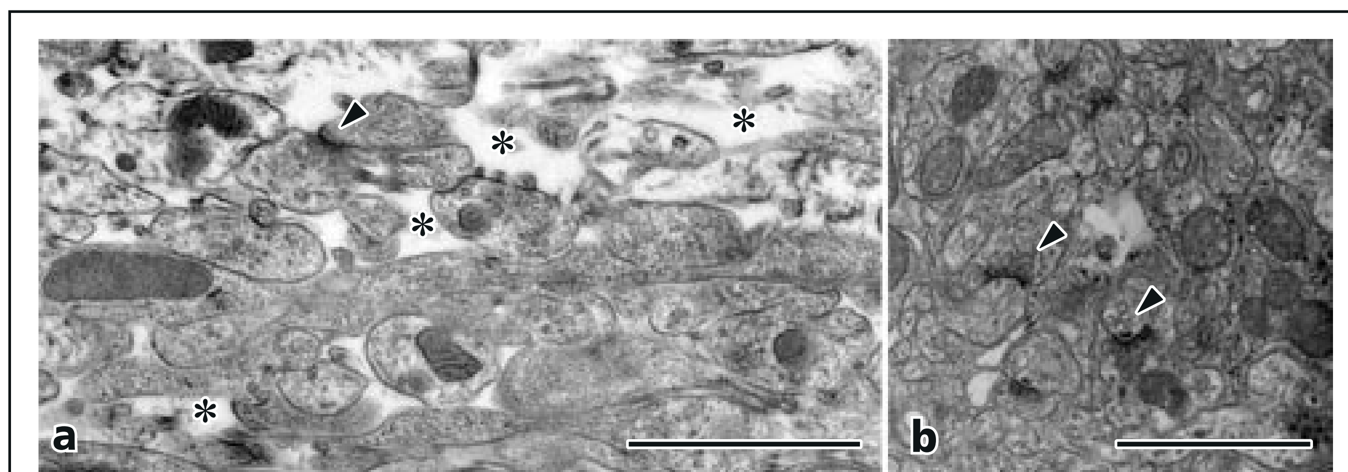

Figure 9. *Electron micrographs of molecular layers in mouse cerebellum, as prepared by either the "*in vivo *cryotechnique" **(a)** or the conventional chemical fixation and dehydration method **(b)**. In the specimens prepared by the "*in vivo *cryotechnique", lots of extracellular spaces **(a, asterisks)** are well preserved, and synaptic clefts become narrow near synaptic densities **(a, arrowheads)**, although such extracellular spaces are eliminated by the conventional method **(b)**. Arrowheads, synaptic density. Bars: 2 µm.*

might cause some structural changes, resulting in a decrease of electrical conductivity (51-53). However, the real ultrastructural changes could never been clarified by the conventional QF methods. In our study, the "*in vivo* cryotechnique" can avoid such artificial modifications due to ischemia. Actually, extracellular spaces filled with intercellular fluid are widely open under normal blood circulation, which are easily collapsed by the resection of cerebellar tissues and stop of blood circulation. As such extracellular spaces are suggested to be important for physiological functions of remodeling neural or glial cells, the "*in vivo* cryotechnique" would be useful as a morphological approach for functional analyses of dynamically changing cells and tissues in the central nervous system.

Immunolocalization of functional proteins

Mouse cerebellar tissues under normal blood circulation were cryofixed by the "*in vivo* cryotechnique", freeze-substituted in acetone containing 2% PFA and routinely embedded in the paraffin wax. The conventional QF method was also performed by resecting cerebellar tissues and then plunging them into the isopentane-propane cryogen. The frozen specimens were similarly embedded in the paraffin wax. The conventional chemical fixation was performed by perfusion with buffered 2% PFA, followed by alcohol dehydration, and the cerebellar tissues were embedded in the paraffin wax. All specimens were sectioned at 4~5µm thickness, mounted on glass slides and immunostained with four kinds of antibodies against calcium-binding calbindin, serum albumin, mouse IgG and phosphorylated cyclic-AMP responsive element binding protein (pCREB) by either immunoperoxidase or immunofluorescence method (6,16).

The immunoreactivity of calbindin in the cytoplasm of Purkinje cells could be more clearly detected in the specimens prepared by the "*in vivo* cryotechnique" than in those by the conventional perfusion-fixation method (Fig. 10a,b). Additionally, the immunoreactivities of soluble serum proteins, albumin and IgG, could be detected exclusively within blood vessels in the specimens prepared by the "*in vivo* cryotechnique" (Fig. 10d), although such immunoreactivities were completely eliminated in the specimens by the conventional perfusion-fixation method (Fig. 10e). They were detected outside of blood vessels in the resected specimens prepared by the plunging QF method (Fig. 10c,f-h) (16). Furthermore, the immunoreactivity of intranuclear pCREB in granule cells was clearly detected in the specimens prepared by the "*in vivo* cryotechnique" even without the microwave treatment for antigen-retrieval (Fig. 10k,l). However, the immunoreactivity of pCREB was not clearly detected in the specimens prepared by the conventional fixation method (Fig. 10i), and its enhancement was heterogeneously obtained with the microwave treatment (Fig. 10j) (6).

The leakage of serum proteins might be caused by the disruption of blood-brain barriers, immediately after the ischemic stress and anoxia (16). The "*in vivo* cryotechnique" would be useful for immunohistochemical analyses of intranuclear signal molecules, soluble serum proteins and calcium-binding proteins, which can be easily washed out during the preparation steps or translocated by various biological stresses. Moreover, the time-resolution of cryotechniques for physical fixation, depending on their cooling rates, is very high (26), and they were used for analyses of dynamic phenomena, such as neurotransmitter release or exocytotic processes (54,55). As the "*in vivo* cryotechnique" can directly cryofix the living animal tissues without ischemia or anoxia, it would be more useful for examining dynamically changing components *in vivo*, such as signal molecules, at an ultimate time-resolution.

D. Alveolar structures of living mice during respiration

Although some ultrastructural features of animal lungs were described in the previous reports (56-58), they could provide not enough information about physiological functions of the lungs. This is because the conventional electron microscopy couldn't reveal pulmonary ultrastructures of living animals during functional respiration. Therefore, the "*in vivo* cryotechnique" was applied to morphological analyses of mouse lungs inflated by mechanical ventilation (9). Some BALB/c mice were intubated under pentobarbital anesthesia, and then mechanical ventilation was started with air. Thereafter, their lungs were surgically exposed and cryofixed at the end of inspiration by the "*in vivo* cryotechnique". The frozen tissues were freeze-substituted in acetone containing 2% osmium tetroxide, embedded in epoxy resin and routinely processed for light or electron microscopy.

In thick sections stained with toluidine blue as observed with a light microscope (Fig. 11a), dynamic structures of pleura and subpleural regions of the mouse lung, including inflated alveoli, interstitial or alveolar epithelial cells and flowing erythrocytes in blood vessels, were well preserved without any remarkable ice crystal damage. Moreover, close association between pulmonary alveoli and blood capillaries with flowing erythrocytes could be detected in ultrathin sections, as observed with TEM (Fig. 11b). The flowing erythrocytes exhibited various shapes different from the typical biconcave discoid (Fig. 11c, inset), as seen in other organs (11,13). Some of them were partially attached to endothelial cells (Fig. 11c),

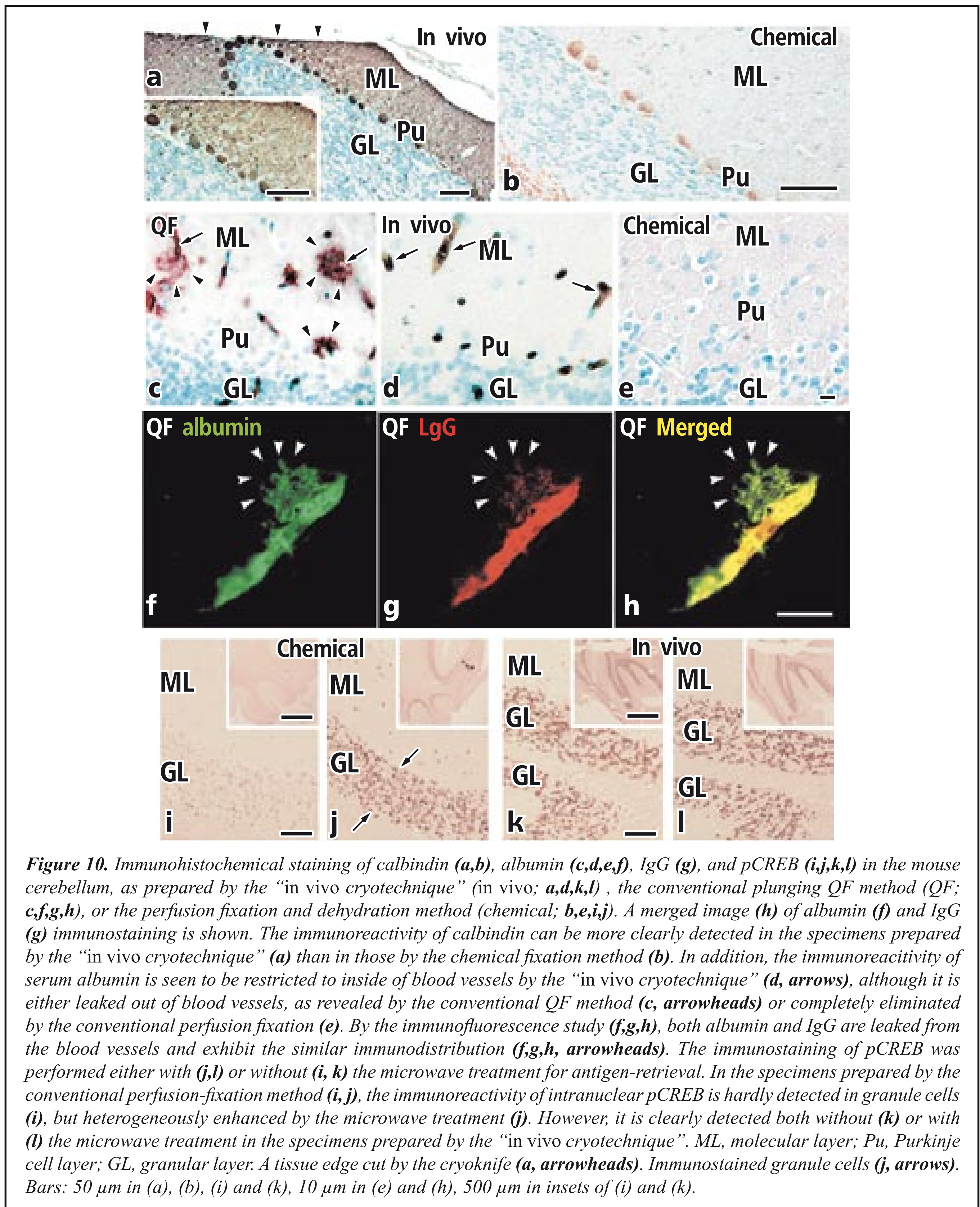

Figure 10. *Immunohistochemical staining of calbindin* ***(a,b)****, albumin* ***(c,d,e,f)****, IgG* ***(g)****, and pCREB* ***(i,j,k,l)*** *in the mouse cerebellum, as prepared by the "*in vivo *cryotechnique" (*in vivo*;* ***a,d,k,l****) , the conventional plunging QF method (QF;* ***c,f,g,h****), or the perfusion fixation and dehydration method (chemical;* ***b,e,i,j****). A merged image* ***(h)*** *of albumin* ***(f)*** *and IgG* ***(g)*** *immunostaining is shown. The immunoreactivity of calbindin can be more clearly detected in the specimens prepared by the "*in vivo *cryotechnique"* ***(a)*** *than in those by the chemical fixation method* ***(b)****. In addition, the immunoreacitivity of serum albumin is seen to be restricted to inside of blood vessels by the "*in vivo *cryotechnique"* ***(d, arrows)****, although it is either leaked out of blood vessels, as revealed by the conventional QF method* ***(c, arrowheads)*** *or completely eliminated by the conventional perfusion fixation* ***(e)****. By the immunofluorescence study* ***(f,g,h)****, both albumin and IgG are leaked from the blood vessels and exhibit the similar immunodistribution* ***(f,g,h, arrowheads)****. The immunostaining of pCREB was performed either with* ***(j,l)*** *or without* ***(i, k)*** *the microwave treatment for antigen-retrieval. In the specimens prepared by the conventional perfusion-fixation method* ***(i, j)****, the immunoreactivity of intranuclear pCREB is hardly detected in granule cells* ***(i)****, but heterogeneously enhanced by the microwave treatment* ***(j)****. However, it is clearly detected both without* ***(k)*** *or with* ***(l)*** *the microwave treatment in the specimens prepared by the "*in vivo *cryotechnique". ML, molecular layer; Pu, Purkinje cell layer; GL, granular layer. A tissue edge cut by the cryoknife* ***(a, arrowheads)****. Immunostained granule cells* ***(j, arrows)****. Bars: 50 µm in (a), (b), (i) and (k), 10 µm in (e) and (h), 500 µm in insets of (i) and (k).*

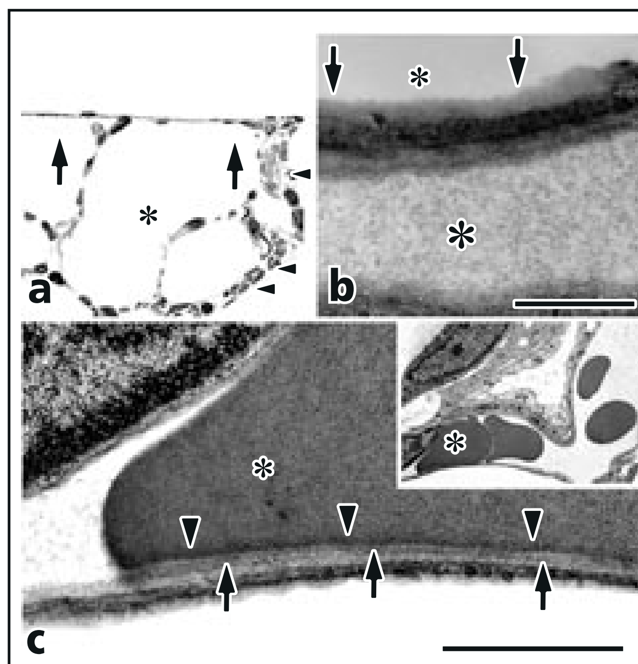

Figure 11. *Light* ***(a)*** *and electron* ***(b,c)*** *micrographs of pleura and pulmonary structures of inflating lung of living mice, as prepared by the "*in vivo *cryotechnique". In the light micrograph* ***(a)****, ballooning pulmonary alveoli* ***(a, asterisk)****, structures of pleura* ***(a, arrows)*** *and flowing erythrocytes in septal blood capillaries* ***(a, arrowheads)*** *are well preserved in a functioning lung of living mice. In the electron micrograph of blood-air barriers* ***(b)****, pulmonary alveoli* ***(b, small asterisk)*** *and blood capillaries* ***(b, large asterisk)*** *are closely associated, and the surfactant layer is well preserved on the alveolar epithelium* ***(b, arrows)****. In the electron micrograph of septal blood capillaries* ***(c)****, flowing erythrocytes exhibit various shapes* ***(c, asterisks)*** *instead of typical biconcave discoid shapes, and some erythrocytes* ***(c, arrowheads)*** *are attached to endothelial cells* ***(c, arrows)****. Bars: 1µm.*

indicating that such erythrocyte-endothelium attachment might be effective for physiological functions of gas exchange (9). The "*in vivo* cryotechnique" would be more useful for morphofunctional analyses of dynamically changing cells and tissues, which were hardly examined by the conventional preparation methods.

E. Ultrastructures of tear film and epithelium of rat cornea

The "*in vivo* cryotechnique" was used to investigate the native ultrastructures of corneal superficial layers and precorneal tear films of living rats (35,59). In these ultrastructural studies, the isopentane-propane cryogen was directly poured over the corneal surface of living rats under pentobarbital anesthesia. The freeze-substitution was routinely performed as described before, followed by embeddeing in the epoxy resin. For comparison, the conventional chemical fixation was also performed with the common glutaraldehyde and osmium tetroxide. The double-fixed specimens were dehydrated with a graded series of alcohol and embedded in the epoxy resin. They were sectioned, routinely stained and observed with TEM. In the living rat cornea as prepared by the "*in vivo* cryotechnique", closed intercellular spaces between squamous epithelial cells and three-layered tear films covering them could be clearly detected, which had been hardly preserved by the conventional chemical fixation (35,59).

F. Upper surface layer of rat articular cartilage

The "*in vivo* cryotechnique" was also applied to examination of the upper surface layer on rat articular cartilage (12,60). Although this fragile layer was easily lost during the conventional preparation steps (12), it was well preserved in the specimens prepared by the plunging QF method or the "*in vivo* cryotechnique" (12). Small lipid-like globular structures, which were considered to be effective for joint lubrication, could be well maintained by the cryotechniques (60). From these findings, the "*in vivo* cryotechnique" were useful for maintaining amorphous liquid layers on the surface tissue of articular cartilage, which were almost lost by the conventional chemical fixation.

FUTURE DIRECTIONS FOR "LIVING MORPHOLOGY"

The "*in vivo* cryotechnique" has been applied to examining several organs of living mice and rats, and also revealed new findings which were difficult to demonstrate by the conventional fixation methods. However, a few comments are still remaining to be made. The first comment is its application to pathological analyses of human tissues. Most data in the field of human pathology have been accumulated by morphological comparison between normal and abnormal tissues, which are prepared by the conventional chemical fixation. Such obtained data are sometimes reported to be modified by technical artifacts during the conventional preparation steps (61,62). For this reason, the conventional QF methods or the "*in vivo*

cryotechnique" would be useful for solving some technical problems in such pathological cases. As already discussed above, however, the conventional QF methods also yield some morphological and immunohistochemical artifacts induced by the tissue resection, resulting in ischemia and anoxia. The "*in vivo* cryotechnique" would be more suitable for these cases than the QF methods. For its human application, we need to develop a new device or improve the "*in vivo* cryoapparatus" for the human biopsy system, named as "cryobiopsy" (Fig. 1).

Another comment will be a technical advancement, which is necessary not only for its human application as described in the previous paragraph, but also for obtaining more data with the cryotechniques. Especially in the case of electron microscopy, the "*in vivo* cryotechnique" can quickly freeze only small limited areas. Moreover, its application is restricted to living animal organs exposed to air with minimal bleeding or less mechanical stresses. For its further effective application, such limitations should be overcome. It might be partially realized by making use of cryogens with much lower temperature, such as liquid helium.

CONCLUSION

By the "*in vivo* cryotechnique", it is now possible to make morphological and immunohistochemical studies of living animal organs and tissues without any major stresses of anoxia and ischemia. We have already obtained various new findings, which had never been demonstrated with the other conventional methods. As the "*in vivo* cryotechnique" can reveal the dynamic morphology closer to "living" states with normal blood circulation, it will develop a new morphological field of "living morphology" during this 21th century, and allow us to understand physiology and pathology of cells and tissues in living animal organs.

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