Histol Histopathol (2007) 22: 1281-1290

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Recent development of *in vivo* cryotechnique to cryobiopsy for living animals

N. Ohno[1], N. Terada[1], S. Saitoh[1], H. Zhou[1,2], Y. Fujii[1] and S. Ohno[1]

[1]Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo-city, Yamanashi, Japan and [2]Department of Child, Adolescent and Women's Health, School of Public Health, Peking University, Beijing, China

Summary. Various microscopic methods have been used to analyze the morphology and molecular distribution of cells and tissues. Using conventional procedures, however, ischemic or anoxic artifacts are inevitably caused by tissue-resection or perfusion-fixation. The *in vivo* cryotechnique (IVCT) was developed to overcome these problems, and was found to be useful with light microscopy for analyses of the distribution of water-soluble molecules without anoxic effects at high time resolution. But there are limitations to the application of IVCT, such as exposure of target organs of living small animals and immunoreactivity of lipid-soluble molecules owing to freeze-substitution with acetone. Recently, a new cryotechnique called "cryobiopsy" has been developed, which enables one to obtain tissue specimens of large animals including humans without ischemia or anoxia, and has almost the same technical advantages as IVCT. Both IVCT and cryobiopsy complement other live-imaging techniques, and are useful for not only the morphological observation of cells and tissues under normal conditions, but also the preservation of all components in frozen tissue specimens. Therefore, morphofunctional information *in vivo* would be obtained by freeze-substituion for light or electron microscopy, and also by other analytical methods, such as freeze-fracture replication, X-ray microanalyses, or Raman microscopy. Considering the merits of both IVCT and cryobiopsy, their application should be expanded into other microscopic fields and also from experimental animal studies to clinical medicine.

Key words: In vivo cryotechnique, Cryobiopsy, Morphofunctional study, Pathophysiological application

Offprint requests to: Shinichi Ohno, M.D., Ph.D., Professor and Chairman, Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-city, Yamanashi 409-3898, Japan. e-mail: sohno@yamanashi.ac.jp

Introduction

Various conventional analyses of morphology and molecular distribution with light or electron microscopy have been essential in the biological and medical fields for the past several decades. In those analyses, a morphological approach with fixed tissue specimens has been used, because a large variety of fixation or hydration steps have been steadily improved (Hopwood, 1969; Plattner and Bachmann, 1982). On the other hand, the conventional chemical fixation or quick-freezing of resected tissues yields technical problems during the tissue preparation steps, such as molecular movement, structural alterations and anoxic modification of cells (Kellenberger et al., 1992; Hippe-Sanwald, 1993; Chan and Inoue, 1994; Shiurba, 2001; Zea-Aragon et al., 2004). One effective way to overcome such problems is the live-imaging of structural or molecular components, mainly performed with various confocal or multi-photon laser scanning microscopes (Selvin, 2000; Stephens and Allan, 2003). This has been widely used, because of major advancements in fluorescence-imaging technology, but live-imaging still has limitations and drawbacks (Fricker and Meyer, 2001; Tauer, 2002; Molitoris and Sandoval, 2005).

Another approach, the in vivo cryotechnique (IVCT), has been used to demonstrate the functional morphology of cells and tissues in living animal organs for a decade (Ohno et al., 1996, 2004a). It is a method of cryofixation, in which target organs of anesthetized small animals, such as mice and rats, are frozen in vivo, indicating that they are directly embedded in ice crystals without perfusion-fixation or tissue-resection (Ohno et al., 2004b). IVCT was originally developed for ultrastructural analyses of dynamically changing glomerular capillary loops in living mouse kidneys under different hemodynamic conditions (Ohno et al., 1996, 2001), because conventional chemical fixation methods had artificial effects on the glomerular capillary loops with abundant blood supply (Furukawa et al.,

1991). Thereafter, as summarized in Table 1, IVCT was initially applied to the ultrastructural examination of organs in mice and rats, and then used for immunohistochemical analyses of soluble molecules of cells and tissues at the light microscopic level. This analytical shift from electron to light microscopy is due to the technical advantages of IVCT for clinical medicine, resulting in the recent development of "cryobiopsy" for the time-dependent examination of organs in living animals, including humans (Fujii et al., 2006). In the present article, the features of IVCT and cryobiopsy, especially at the light microscopic level, are reviewed on the basis of experimental protocols and trends.

Light microscopic applications of IVCT

As every morphological approach has its own advantages and disadvantages, an understanding of various findings is dependent on the selection of the most suitable method. Here the features of IVCT, which are summarized in Table 2, will be discussed with particular emphasis on its light microscopic or immunohistochemical applications.

(i) Immunohistochemical detection of soluble components in living animal organs

As the first application of IVCT to immunohistochemistry in 2004, serum albumin and immunoglobulin G (IgG) were detected exclusively in blood vessels of living mouse cerebellum, but quickly leaked out across the blood-brain barrier owing to anoxia with the quick-freezing of resected fresh tissues, or severely diminished with transcardial perfusion-fixation followed by alcohol-dehydration (Zea-Aragon et al., 2004). Subsequently, the extracellular or intracellular distribution of serum proteins and soluble components, such as glycogen, was shown to be well retained in kidney or liver tissues of living mice with IVCT, but significantly modified with conventional fixation or quick-freezing methods (Li et al., 2005, 2006a,b; Ohno et al., 2006a; Zhou et al., 2006). These findings indicated that IVCT would have some beneficial effects on the immunohistochemical observation of soluble molecules in cells and tissues of living animals (Ohno et al., 2006b).

The in vivo immunodistribution of such soluble components is difficult to capture in tissue sections prepared by chemical fixation, because they are easily moved or extracted during fixation and dehydration. Therefore, it was suggested that freeze-substitution would be more useful for their preservation (von Schack and Fakan, 1994; Moreira et al., 1998). The better preservation of water-soluble components seems to be due to the organic solvents used during freeze-substitution at low temperatures, because there would be much less chance of their diffusion and loss from cells

Table 1. Various applications of the "in vivo cryotechnique". Underlined references were published after 2004.

ORGAN/FOCUS POINTS	MICROSCOPY	REFERENCES
Kidney		
Morphology of living mouse glomeruli under hemodynamic changes.	TEM, SEM	(Ohno et al., 1996; Yu et al., 1998; Ohno et al., 2001)
Dynamics of injected fluorescent probes in blood vessels.	LM	(Terada et al., 2005)
Immunodistribution of serum protein under hemodynamic changes.	LM	(Li et al., 2005, 2006a,b)
Serum protein in nephrons of albumin-overloaded mice.	LM	(Zhou et al., 2006)
Liver		
Morphology and serum protein under hemodynamic changes in mouse livers.	LM, TEM	(Ohno et al., 2006a)
Morphological and histochemical analyses of livers with cryobiopsy.	LM, SEM	(Fujii et al., 2006)
Different shapes of flowing erythrocytes under hemodynamic changes.	SEM	(Terada et al., 1998)
Dynamics of injected fluorescent probes.	LM	(Terada et al., 2005)
Eye		
Ultrastructure of corneal epithelium and precorneal tear film.	TEM, SEM	(Chen et al., 1995; Chen et al., 1997)
Rapid phosphorylation of rhodopsin by light exposure.	LM	(Terada et al., 2006c)
Raman microscopy in freeze-dried mouse eyeballs.	LM	(Terada et al., 2007)
Joint		
Ultrastructures of superficial layers of articular cartilage.	TEM, SEM	(Watanabe et al., 2000)
Blood vessels		
Shapes of flowing erythrocytes in aorta and inferior vena cava.	TEM	(Xue et al., 1998)
Spleen		
Erythrocyte shapes under hemodynamic changes.	SEM	(Xue et al., 2001)
Lung		
Ultrastructures of lungs during inflation.	TEM	(Takayama et al., 2000)
Intestine		
Caveolin immunolocalization in smooth muscle cells.	TEM	(Takayama et al., 1999)
Cerebellum		
Immunolocalization of serum protein after ischemia.	LM	(Zea-Aragon et al., 2004)
Antigen-retrieval effects of cryofixed tissues.	LM	(Ohno et al., 2005)
Testis		
Albumin immunodistribution changed by cadmium-treatment.	LM	(Liao et al., 2006)

and tissues (Melan and Sluder, 1992). The addition of fixatives in the organic solvents which would effectively cross-link the molecular components and further prevent their translocation is another choice for better freeze-substitution and often increases immunoreactivity, compared with conventional chemical fixation (Yamashita and Yasuda, 1992). Moreover, the immunoreactivity of some antigens in quick-frozen tissues can be significantly enhanced with freeze-substitution, probably because of the loosening of intermolecular or intramolecular cross-linkage networks which often prevent effective access of antibodies to their epitopes (Ohno et al., 2005; Terada et al., 2006c). In some cases, a common retrieval treatment may favor the immunohistochemical detection of soluble or structural molecules in compactly cross-linked tissues. Different from IVCT, it is necessary in conventional quick-freezing to resect target organ tissues prior to the cryofixation, and ischemic or anoxic stress induced by the resection easily cause the rapid translocation of soluble components in some organs of living mice (Zea-Aragon et al., 2004; Li et al., 2005, 2006a; Zhou et al., 2006). Therefore, as compared with chemical fixation, quick-freezing and freeze-substitution would have beneficial features in immunohistochemical examinations of soluble components. If ischemic or anoxic stress seriously affects their localization in cells and tissues of living animals, IVCT would be the most suitable approach for immunohistochemical analyses.

High time resolution of molecular localization in living animal organs

Some rapid morphological changes occurring within seconds are difficult to capture with conventional chemical fixation methods, because it is not clearly demonstrated how long it takes to completely cross-link the target molecules of cells and tissues during the fixation step. All the fixatives used for biology and medicine take some time to penetrate deeper areas of tissue blocks in common fixative solutions (Dykstra, 2003). Although the transvascular perfusion of chemical fixatives is used to improve their penetration of tissue blocks (Hayat, 1989), the time necessary for the chemical cross-linkage is still unclear because of a dependence on chemical reactions of the fixatives in vivo. In contrast, quick-freezing instantly immobilizes the target molecules in cells and tissues at the time of cryofixation, and molecular changes to functioning components, such as phosphorylation, would be effectively stopped in frozen tissues. This molecular finding was made in an experiment on the rapid phosphorylation of rhodopsin, in which immuno-reactivity of rhodopsin phosphorylated ^{334}Ser was detected after 30sec of exposure to light in dark-adapted mouse retinas, but not after 10sec (Terada et al., 2006c). In this report, by using IVCT, the interval between tissue-resection and cryofixation, which is necessary for quick-freezing and affects the serine-phosphorylation of rhodopsin in the mouse retina, was reduced to less than one second. Although it probably takes longer to freeze deeper tissue areas, areas near the surface would be frozen without ice crystals visible at the light microscopic level (Plattner and Bachmann, 1982). Given that the immunoreactivity of some molecular components can be better preserved with quick-freezing and freeze-substitution than conventional chemical fixation as described above, IVCT would be the most useful approach to immunohistochemical analyses of rapid molecular changes in functioning cells and tissues of living animals.

Dynamic morphology of organs under different hemodynamic conditions

In animal organs artificially perfused with fixatives, morphology is usually affected by the various pressures of perfusion (Hayat, 1989; Yu et al., 1997), and so the perfusion-pressure should be critically controlled for morphofunctional studies of living animal organs. On the other hand, in resected tissues prior to chemical fixation, the circulation of blood is inevitably stopped, resulting in most blood vessels collapsing before complete chemical fixation (Ohno et al., 2006a). In contrast, IVCT directly cryofixes functioning organs of anesthetized animals without perfusion-fixation or tissue-resection, and so dynamically changing cells and tissues can be clearly detected under different hemodynamic conditions (Ohno et al., 1996; Terada et al., 1998). This morphological advantage was shown in living mouse kidneys and livers by hematoxylin-eosin staining and also immunostaining for serum proteins at the light microscopic level (Li et al., 2005, 2006a; Ohno et al., 2006a). For the morphofunctional study of tissue organization in vivo, antigens specific for blood or lymphatic vessels would facilitate detailed

Table 2. Technical features of the "in vivo cryotechnique".

ADVANTAGES	LIMITATIONS
i) Better preservation of soluble components easily translocated during tissue-resection or perfusion-fixation.	i) Limited tissue areas for observation, because of ice crystal damage, as with the other quick-freezing methods.
ii) Higher time-resolution without any delay due to chemical fixation or tissue-resection.	ii) Surgical exposure of target organs, usually necessary for electron microscopic specimens.
iii) Direct cryofixation of target organs under blood circulation, without tissue-resection or perfusion-fixation.	iii) Lipid-soluble components may be extracted during freeze-substitution with acetone in some cases.

immunohistochemical analyses under physiological and pathological conditions (Achen et al., 2005; Emanueli and Madeddu, 2005; Gariano and Gardner, 2005). The functional morphology of blood vessels and their permeability can also be examined by using extrinsic fluorescent dyes, because soluble molecular markers are well retained with IVCT followed by freeze-substitution (Jain et al., 2002; McDonald and Choyke, 2003; Terada et al., 2005). We already reported that the intramembranous particle organization of artificially flowing human erythrocytes was significantly changed at the electron microscopic level, depending on various flowing speeds (Terada et al., 2006a), and so IVCT would also be useful for the examination of such components in flowing erythrocytes of living organs. In conclusion, the rapid preservation of morphology and molecular conformation is a technical advantage of IVCT, different from conventional chemical fixation or quick-freezing methods.

Morphological comparison with the other fixation methods

First, as IVCT is a method of cryofixation, acceptable tissue areas without visible ice crystals are usually limited to within a few hundred micrometers or several micrometers from the frozen tissue surface at the light or electron microscopic level, respectively (Ohno et al., 1996; Zea-Aragon et al., 2004). The limitation of such areas is also shown in other quick-freezing methods (Plattner and Bachmann, 1982). However, with immersion-fixation, the fixatives penetrate the tissue into deeper areas, which are inevitably damaged by post-mortum changes (Hayat, 1989). Considering the cryofixation methods, the presence of visible ice crystals should always be checked in serial sections before immunostaining or other histochemical analyses. Hematoxylin-eosin staining is sufficient for such an examination.

Second, it is usually better to expose target organs of anesthetized animals before cryofixation of IVCT, because tissue areas without visible ice crystals are necessary for direct contact with the isopentane-propane (IP) cryogen and also the cryoknife (Ohno et al., 1996). Moreover, such careful exposure of the target organs in situ was not necessary for conventional quick-freezing, because all tissues are removed from the living animals and immediately cut into small pieces for cryofixation. Therefore, it was a challenge to apply IVCT to target organs which were difficult to expose in living animals, such as functioning lungs under the negative pressures of physiological respiration. Moreover, when they are prepared under anesthesia for IVCT, excessive body fluid or unnecessary blood on the tissue surface also lowers the structural quality of images with the cryofixation. In such a case, they should be carefully wiped off with soft paper sheets just before the freezing (Ohno et al., 2004b).

Third, when frozen specimens are prepared for immunohistochemical analyses, the antibodies used should be optimized to obtain the best results. In other words, the specific immunoreactivity can be increased by the cryofixation method in many cases as described above, but unexpectedly decreased in some cases in comparison with chemical fixation. Most antibodies are usually used in tissue sections prepared by conventional chemical fixation. On the other hand, cryofixation and freeze-substitution would retain lots of other soluble molecules to mask binding sites of the antibodies with specific molecular epitopes. For example, well-preserved soluble serum molecules in the glomerular capillary loops of living mouse kidneys were compactly cryofixed by IVCT, which disturbed the immuno-detection of structural components of glomerular basement membranes, such as type IV collagen. In such a case, much smaller probes for histochemical staining, such as PAS, were helpful to identify the glomerular basement membrane (Li et al., 2006b). Moreover, hydrophobic molecules in lipid membranes may not be well preserved during freeze-substitution with acetone (Giddings, 2003; Yang et al., 2006). As described above, the tissue preparation steps after cryofixation significantly affect the availability of antibodies on paraffin sections similarly to conventional chemical fixation. Some antibodies commonly used on cryosections don't work so well on alcohol-dehydrated paraffin sections (Shiurba et al., 1998). Such problems in the detection of cross-linked or paraffin-embedded molecules are often solved by using various antigen-retrieval techniques or methods such as metal replication or freeze-drying for electron microscopy.

Time-dependent morphology in comparison with live-imaging

Although IVCT is useful to quickly capture tissue and cellular morphology under normal blood circulation, it is a kind of cryofixation to immobilize every component in vitreous ice crystals. So, it is impossible to examine how the intracellular or extracellular molecules behave, following the time course of target organs in living animals. However, cryofixed specimens are repeatedly used to examine different functional or structural molecules by using specific probes or antibodies on serial or same sections, which can be compared with the histological architectures. On the other hand, time-lapse observation of living animal organs realized by a new live-imaging technology has also become a powerful tool in the fields of biological and medical science. Considering such technological progress, our goal with IVCT is not a simple observation of morphology closer to the living state under normal or pathological conditions. Besides freeze-substituion for standard light or electron microscopy, IVCT can also be combined with other methods, such as freeze-fracture replication or X-ray microanalyses for electron microscopy (Takayama et al., 1999; Terada et al., 2006b). A new application of IVCT to Raman microscopy was already tried to demonstrate the molecular organization of the mouse retina (Terada et

al., 2007). In this experiment, IVCT followed by freeze-drying was able to successfully exhibit specific Raman spectra dependent on the retinal tissue's architecture, which were confirmed by subsequent embedding of epoxy resins for light microscopy. As the Raman spectra appeared to correspond to abundant molecules localized in each tissue structure of living animal organs, rapid conformational or molecular changes, such as translocation or phosphorylation, would be precisely analyzed at high time-resolution by the combination of IVCT and Raman microscopy. Therefore, the most important point about IVCT is that all the soluble or structural components with functional information are instantly immobilized in the iced tissue blocks at the time of freezing (Fig. 1). The morphofunctional information obtained always depends on the procedure, employed after IVCT, indicating that it would be a complementary cryotechnique rather than a completely independent approach.

New development and improvement of cryobiopsy for living animals

As described above, IVCT has several advantages over the conventional methods, promoting its application to light microscopy. It was also speculated to be applicable in clinical medicine, in which most pathological and immunohistochemical data are obtained by the conventional immersion-fixation of biopsied human tissues. However, it was another challenge to apply IVCT to larger animals including humans, because the liquid IP cryogen had to be directly poured over the target organs (Fig. 1a). Although there have been a few trials of quick-freezing in vivo (Chang et al., 1980; von Zglinicki et al., 1986), some technical problems remained. To solve these problems, it has been necessary to freeze only a small part of target organs in vivo. For that purpose, we have recently developed a new cryotechnique, named "cryobiopsy" (Fig. 1b), and invented a home-made device, cryoforceps (Fig. 2a) (Fujii et al., 2006). By using this new tool, functional morphology with open sinusoids and flowing erythrocytes of living mouse livers, which were similarly preserved with IVCT, was clearly detected as with a conventional biopsy without killing the host mice during cryobiopsy (Fig. 1c). Such morphological features of living mouse livers cannot be obtained by conventional immersion-fixation or quick-freezing methods, which inevitably need tissue-resection. Although there were some tissue areas with morphological deformation, especially around the cryocut tissue edge with the cryoforceps, an almost native morphology was well preserved in the areas adjacent to such compressed surface tissues (Fujii et al., 2006). As cryobiopsy enables us to obtain tissue specimens of larger animals including humans with minimal effects of ischemia or anoxia, and also has the morphological significance of living animal organs similar to IVCT, it will surely contribute to new pathological analyses in the field of clinical medicine.

Summarized methodological details of IVCT and cryobiopsy

To date, there have been two slightly modified methods for IVCT; the original IVCT with or without an "in vivo cryoapparatus" and cryobiopsy (Ohno et al., 2004b; Fujii et al., 2006). The research goal is to achieve complete cryofixation without tissue-resection or organ-perfusion as quickly as possible to prevent the formation of visible ice crystals (Terada et al., 2006b). To that end, a liquid IP cryogen with high cooling ability plays a crucial role in the successful cryofixation of target organs in living animals. The liquid IP cryogen (i) has high cooling ability (Jehl et al., 1981), (ii) facilitates IP preparation and (iii) is inexpensive, being made up of commercially available materials (Ohno et al., 1996). The cooling speed of the IP cryogen was previously reported to be higher than that of isopentane or propane alone, when the sample was small enough to freeze (Jehl et al., 1981). Additionally, it is easy to handle as a liquid, because its freezing point is below the boiling point of liquid nitrogen (77K), and the frozen tissues can be kept in the same liquid cryogen for a while (Jehl et al., 1981). For the past two decades, the liquid IP cryogen has been

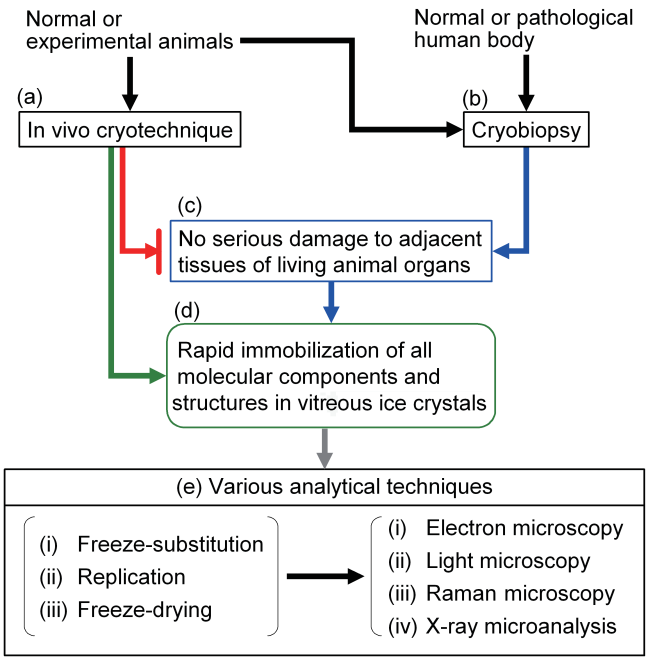

Fig. 1. A schema showing features of the in vivo cryotechnique and "cryobiopsy". The cryotechnique is used to freeze target organs of experimental animals in vivo and immobilize all components and structures in situ with vitreous ice crystals (green arrow). Therefore, various analytical techniques can be used to obtain morphofunctional information (e). However, serious damage to adjacent tissues is often caused by pouring the liquid isopentane-propane cryogen (red arrow). In contrast, pinching off limited parts of target organs enables both the preservation of all components and structures, and also prevention of serious damage (blue arrows). Human application of the cryobiopsy system would be a future challenge.

used for the conventional quick-freezing of resected tissues. By plunging them into the IP cryogen, few visible ice crystals were produced within the areas several micrometers away from the tissue surface at an electron microscopic level (Furukawa et al., 1991; Ohno et al., 1992). In the case of light microscopic observation, the acceptable tissue areas without visible ice crystals appear to be deeper, a few hundred micrometers from the tissue surface, because of the low spatial resolution (Ohno et al., 2004b).

Anyone can make cheaper home-made devices and get commercially available agents for IVCT. To make the liquid IP cryogen, a coolite box (Fig. 3a), a 50ml glass beaker with hanging wires containing a stirring magnet bar (Fig. 3b), liquid nitrogen (-196ºC), liquid isopentane and propane gas (Fig. 3d) are needed. After checking if the stirring magnet bar rotates in the beaker hung inside the coolite box on a magnet stirrer machine (Fig. 3b), the beaker is taken out and an appropriate amount of liquid nitrogen is poured into the coolite box.

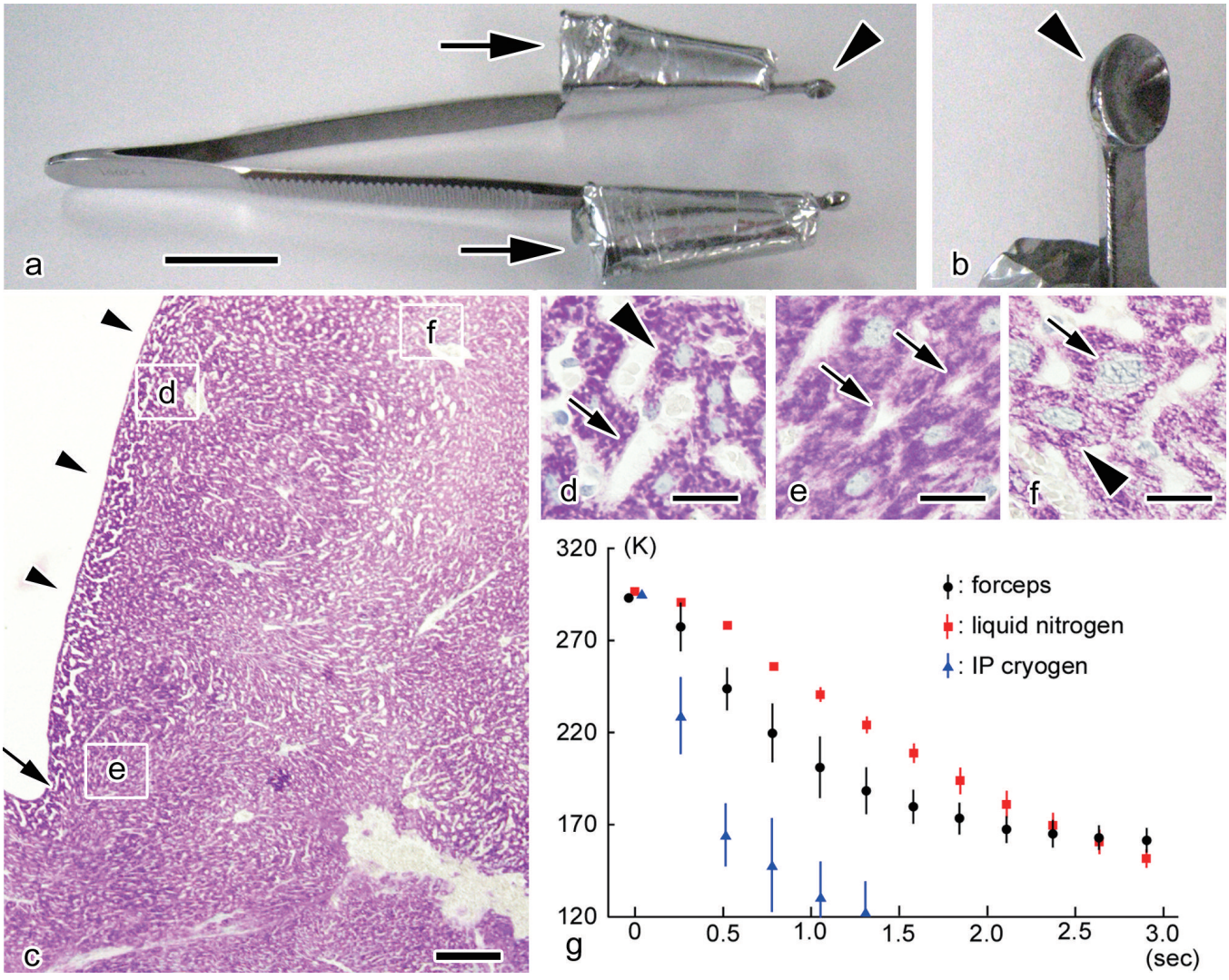

Fig. 2. a, b. Pictures showing the original cryoforceps. The tips of the cryoforceps are round cups, whose edges are sharpened (**a**, **b**, arrowheads). There are sponges just above the tips to absorb liquid nitrogen, which flows down and cools the tips to a liquid nitrogen temperature (**a**, arrows). Bar: 2cm. **c-f.** Light micrographs showing a living mouse liver specimen obtained by cryobiopsy and stained with periodic acid-Schiff (PAS). Highly magnified images (**d~f**) are obtained from the corresponding areas shown in the figure (**c**). Near the surface of specimens (**c**, arrowheads), which were direct contact with the cryoforceps, few visible ice crystals can be seen, and open hepatic sinusoids (**d**, arrow) and PAS-positive glycogen (**d**, arrowhead) are well preserved. However, near the tissue edge compressed by the brink of the cups (**c**, arrow), tissue-compression is obvious (**e**, arrows). In deeper areas, ice crystal is clearly visible especially in nuclei (**f**, arrow), and PAS-staining is also weakened (**f**, arrowhead). Bars: 200μm in **c**; 20 μm in **d~f**. **g.** The cooling speed of the cryobiopsy system (black). To check the cooling speed of the tissue areas in contact with the cryoforceps, the probe of a thermocoupler was bound between the tips of forceps cooled in liquid nitrogen. The cooling is faster than that of liquid nitrogen (red), but not as fast as that of the IP cryogen (blue). Means (circles, rectangles, triangles) and standard deviations (bars) are shown under the different conditions.

Another 15ml liquid isopentane is first poured into the beaker at room temperature, which is then hung in the coolite box containing the liquid nitrogen. The stirring magnet bar will automatically mix the isopentane-propane cryogen, which is cooled down by the liquid nitrogen (-196ºC). At first, extensive bubbling of the liquid nitrogen occurs, but this gradually decreases, when the peripheral part of the isopentane is almost

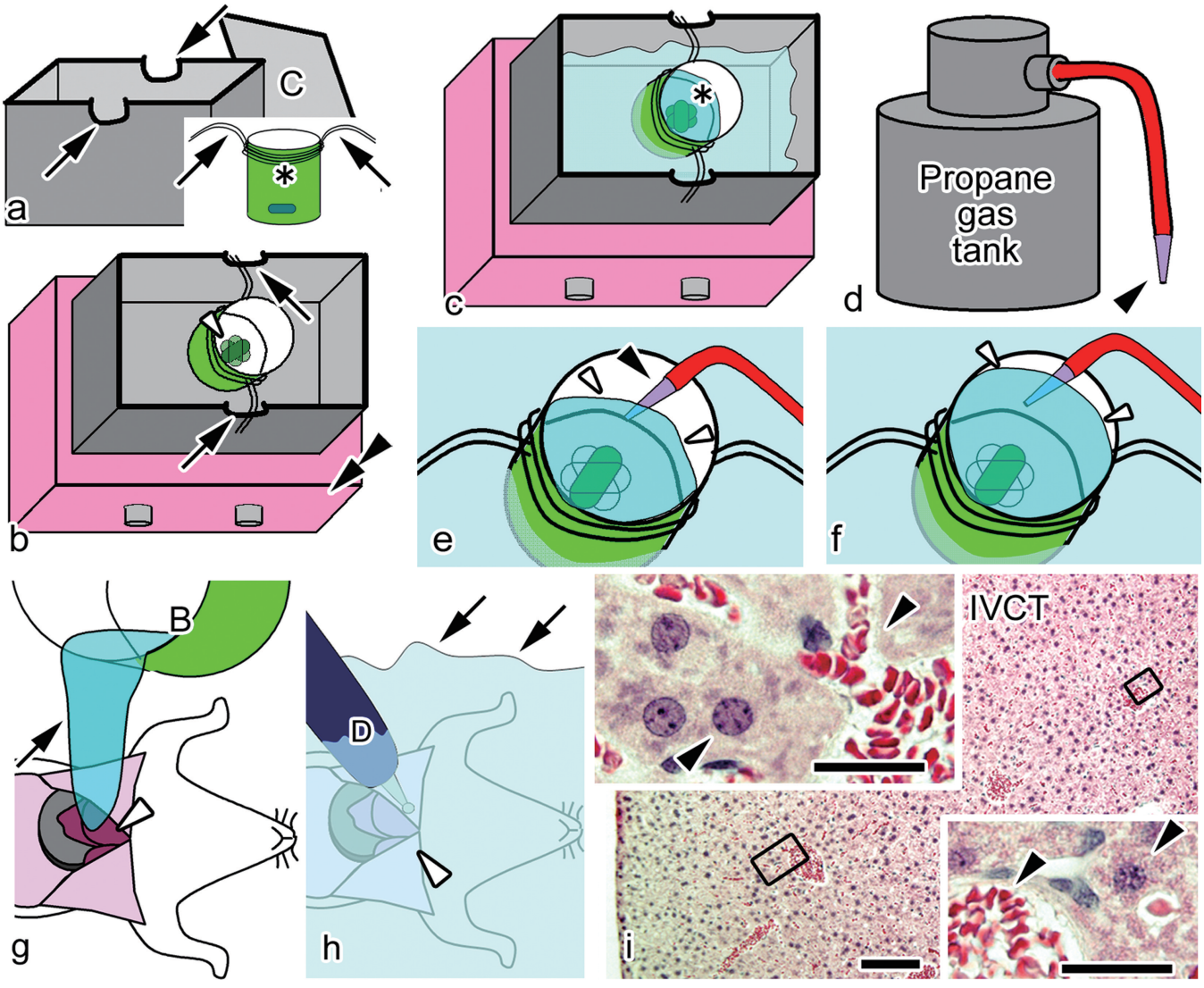

Fig. 3 a-f. Schematic drawings showing how to prepare the isopentane-propane (IP) cryogen. **a.** A coolite box with slits (a, arrows) to hold the wire tips around a beaker, and its lid (C). The beaker with a magnet stirring bar inside (**a**, inset, asterisk) and wire loops wrapped around to hang the 50ml beaker on the coolite box (**a**, inset, arrows). **b.** The beaker is hung over the box on a magnet stirring apparatus (b, double arrowhead), with the two tips of wires (**b**, arrows). So, the magnet bar can smoothly rotate in the beaker (**b**, white arrowhead). **c.** Liquid nitrogen (-196ºC) is poured into the coolite box, in which the beaker with about 15ml of liquid isopentane (**c**, asterisk) is hanging, and its bottom side is dipped in the liquid nitrogen, resulting in the nitrogen gas bubbling. The magnet bar is continuously stirring the liquid isopentane (c, asterisk). **d.** The propane gas is blown out with a narrow tip to be quickly liquidized (**d**, arrow). **e.** After the gas bubbling almost stops, the liquid level of isopentane can be identified (**e**, white arrowheads). Then the propane gas is jetted into the cooled isopentane (e, arrowhead), and the liquid cryogen level gradually elevates. **f.** The liquid level of mixed IP cryogen is finally elevated up to about 45ml (**f**, white arrowheads). Its exposure to the atmosphere should be avoided to prevent contamination of frost. **g**, **h.** Performance of the in vivo cryotechnique (IVCT) without an in vivo cryoapparatus. The liquid IP cryogen (**g**, arrow) is directly poured over the living mouse liver (**g**, white arrowhead) from the beaker (**g**, B). Immediately after the pouring is finished, the whole mouse body with the target liver is directly transferred into liquid nitrogen, and thereafter frozen liver tissues (**h**, white arrowhead) are removed with a dental electric drill (**h**, D) in liquid nitrogen (**h**, arrows). **i.** HE-staining of liver sections prepared with IVCT. The frozen specimens were freeze-substituted in acetone containing 2% paraformaldehyde and embedded in paraffin wax as described previously (Ohno et al., 2005). There are few visible ice crystals near the frozen tissue surface (**i**, left upper inset, arrowheads), whereas some ice crystal damage of flowing erythrocytes and nuclei of hepatocytes is induced in the deeper tissue areas (**i**, right lower inset, arrowheads). Each inset is shown with a rectangle in the low magnification images. Bars: 100μm; 20μm in insets.

cooled down to the liquid nitrogen temperature and gets solidified (Fig. 3c). The tip of a gum tube from the propane gas tank (Fig. 3d) is immediately placed in the cooled down isopentane, which the propane gas is vigorously blown into (Fig. 3e). When the liquid isopentane is cooled down nearly to the liquid nitrogen temperature, all of the propane gas can be completely liquidized. The total cryogen level gradually increases in the beaker to reach the volume of about 50 ml (Fig. 3f), and the propane gas flow should be stopped when the ratio of isopentane to propane reaches 1:2~1:3 to obtain the maximal cooling speed (Fig. 3f) (Jehl et al., 1981). The time necessary for preparation of the liquid IP cryogen is usually less than 5 min. The top of the coolite box should always be covered to minimize exposure of the prepared cryogen to humid air, because frost easily gets into the cryogen, resulting in a decrease in its cooling speed.

IVCT was originally performed with a combination of the liquid IP cryogen and a cryoknife, such as a disposable surgical blade precooled in liquid nitrogen (-196ºC) (Ohno et al., 1996). Therefore, the cut tissue surface of target organs in living animals was immediately cryofixed by the metal of the cryoknife, and also deeper areas below the tissue surface were simultaneously cooled by the poured IP cryogen. By sharply cutting the target organs with the cryoknife, we can increase the size of surface areas in contact with the liquid IP cryogen and expose deeper areas to make them directly contact the cryogen. A series of such procedures have become more simply handled by using a commercially available "in vivo cryoapparatus" (IV-11, Eiko Engineering Co., Ibaraki, Japan) (Ohno et al., 2004b). By using this cryoapparatus, both outflows of IP cryogen and liquid nitrogen are automatically controlled for the cryofixation of target organs in anesthetized animals. A more detailed description about the in vivo cryoapparatus was already reported elsewhere (Ohno et al., 2004a). The combination of both a cryoknife and IP cryogen is recommended for electron microscopy at a higher spatial resolution, but also for light microscopy, especially when deeper areas of target organs need to be examined.

For light microscopy alone, the easiest way to perform IVCT is just to pour the liquid IP cryogen over target organs of anesthetized animals, especially around the local tissue area (Fig. 3g). In such a case, the cryofixed tissues should then be plunged into the liquid nitrogen (-196ºC) as soon as the pouring of IP cryogen is finished. In our experience, the pouring of the IP cryogen should last for more than several seconds. Afterwards, the frozen tissue samples are stored in liquid nitrogen until the next step. An example of liver specimens prepared by simple IVCT is shown in Figure 3i, indicating that areas 300~400 μm deep from the surface can be well preserved without any visible ice crystals (Fig. 3i, left upper inset), because of the lower spatial resolution of light microscopy. In the deeper tissue areas, however, structures damaged by ice crystals in nuclei of hepatocytes and irregularly shaped flowing erythrocytes in blood vessels are detected (Fig. 3i, right lower inset). The depth of acceptable tissue areas with IVCT is almost similar to that prepared with the conventional quick-freezing of freshly resected tissues (Ohno et al., 2004b), although a previous report suggested that smaller pieces of tissue blocks would be totally cooled down by the cryotechnique (Plattner and Bachmann, 1982). Apparently well-frozen areas can be widely obtained in the direction of tissue-sectioning in the case of IVCT.

To perform another cryobiopsy, a new device, such as cryoforceps, is first cooled down in liquid nitrogen and then used to pinch off a piece of the target organ in an anesthetized animal. The liquid IP cryogen should also be prepared beforehand, and the target organ exposed under anesthesia as for IVCT. After the tip of the cryoforceps has cooled enough in liquid nitrogen (-196ºC) just before their use, a part of it is quickly pinched off with the cryoforceps, and then plunged into the IP cryogen in a 50ml beaker. By using this cryobiopsy, the tissue within 350~400μm of the surface is well preserved without any visible ice crystals, as already shown in Figure 2 (Fujii et al., 2006). The frozen specimens prepared by freeze-substitution and then sputtered with platinum-palladium could be also examined under a scanning electron microscope. However, because the tip of the home-made cryoforceps had dull edges, there was some compressed tissue in the cryobiopsied samples. It is essential to find the areas without ice crystal damage and mechanical tissue-compression (Fig. 2c). Actually, the compressed tissues act as a kind of cushion, resulting in the absorption of excessive mechanical force, which usually happens during conventional quick-freezing with attachment to a copper metal block in liquid nitrogen or helium (Bennett, 1998). The cooling speed of the metal cryoforceps in liquid nitrogen (-196ºC) was measured using a thermocoupler, which was found to be higher than that of liquid nitrogen and lower than that of the IP cryogen (Fig. 2g). Actually, in the procedure of the cryobiopsy, the surface areas of pinched tissues are quickly frozen by the metal cryoforceps at a liquid nitrogen temperature, and further cooled down by the liquid IP cryogen. Therefore, it is assumed that the cooling would be faster than that of simple pinching off with the cryoforceps.

Future development of cryobiopsy for clinico-pathological applications

Many technical advances of IVCT and its development toward the cryobiopsy for living animals have been achieved as already described above, but there still remain some problems to be solved. First, the practical application of cryobiopsy to clinical medicine would be the greatest challenge at the present time. Although the cryobiopsy system works in experimental animals (Fujii et al., 2006), we need to invent a new device for its application to the human biopsy system, such as gastric endoscopes attached with cryoforceps. As

the preparation of the IP cryogen is thought to be difficult in clinical situations, the new device would better equipped with other cooling systems. The pathophysiological merits, which could be obtained with the present cryobiopsy in pathological animals, would have significant meaning for human diseases, and so it is now under progress by using experimental animal models.

For the past decade, IVCT has been used in only ten organs of anesthetized animals under some physiological and pathological conditions, in spite of its unique merits (Table 1). Some rapid changes of cellular structure, molecular distribution and signal transduction molecules, were more precisely analyzed under various functional conditions by IVCT. Therefore, blood-brain or blood-testis barrier functions and their regulatory mechanisms would be further examined by IVCT, because of its superiority for the observation of soluble serum molecules. Another future topic would be to clarify the relationship between angiogenesis and its signaling molecules, because of the better preservation of blood vessel morphology in vivo. Lastly, an additional improvement of IVCT, especially in its cooling speed, would facilitate broader usage in all biological or clinical fields. In other words, with such an improvement, IVCT would become easier to perform, because the timing and duration of the pouring of both IP cryogen and liquid nitrogen should be controlled to achieve the best cooling speed in the IVCT system.

In conclusion, IVCT has become widely used for various organs of living animals from electron microscopy to light microscopy. Considering its advantages over conventional methods, the application of IVCT should be expanded from standard microscopic analyses to other new analytical fields, and also from animal experiments to clinical medicine.

Acknowledgements. This study was partly supported by a grant-in-aid for scientific research from Japan Society for the Promotion of Science to N.O. (#18890075).

References

Achen M.G., McColl B.K. and Stacker S.A. (2005). Focus on lymphangiogenesis in tumor metastasis. Cancer Cell 7, 121-127.

Bennett P.M. (1998). Structural changes in samples cryofixed by contact with a cold metal block. J. Microsc. 192, 259-268.

Chan F.L. and Inoue S. (1994). Lamina lucida of basement membrane: an artefact. Microsc. Res. Tech. 28, 48-59.

Chang S.H., Mergner W.J., Pendergrass R.E., Bulger R.E., Berezesky I.K. and Trump B.F. (1980). A rapid method of cryofixation of tissues in situ for ultracryomicrotomy. J. Histochem. Cytochem. 28, 47-51.

Chen H.B., Yamabayashi S., Ou B., Ohno S. and Tsukahara S. (1995). Ultrastructural studies on the corneal superficial epithelium of rats by in vivo cryofixation with freeze substitution. Ophthalmic Res. 27, 286-295.

Chen H.B., Yamabayashi S., Ou B., Tanaka Y., Ohno S. and Tsukahara S. (1997). Structure and composition of rat precorneal tear film. A study by an in vivo cryofixation. Invest. Ophthalmol. Vis. Sci. 38, 381-387.

Dykstra M.J. (2003). Specimen preparation for electron microscopy. In: Biological electron microscopy: theory, techniques, and troubleshooting. 2nd ed. Dykstra M.J. and Reuss L.E. (eds). Kluwer Academic/Plenum Publisher. New York. pp 1-73.

Emanueli C. and Madeddu P. (2005). Changing the logic of therapeutic angiogenesis for ischemic disease. Trends Mol. Med. 11, 207-216.

Fricker M.D. and Meyer A.J. (2001). Confocal imaging of metabolism in vivo: pitfalls and possibilities. J. Exp. Bot. 52, 631-640.

Fujii Y., Ohno N., Li Z., Terada N., Baba T. and Ohno S. (2006). Morphological and histochemical analyses of living mouse livers by new 'cryobiopsy' technique. J. Electron Microsc. (Tokyo) 55, 113-122.

Furukawa T., Ohno S., Oguchi H., Hora K., Tokunaga S. and Furuta S. (1991). Morphometric study of glomerular slit diaphragms fixed by rapid-freezing and freeze-substitution. Kidney Int. 40, 621-624.

Gariano R.F. and Gardner T.W. (2005). Retinal angiogenesis in development and disease. Nature 438, 960-966.

Giddings T.H. (2003). Freeze-substitution protocols for improved visualization of membranes in high-pressure frozen samples. J. Microsc. 212, 53-61.

Hayat M.A. (1989). Chemical fixation. In: Principles and techniques of electron microscopy. 3rd ed. Hayat M.A. (ed). Macmillan press. Houndmills. pp 1-78.

Hippe-Sanwald S. (1993). Impact of freeze substitution on biological electron microscopy. Microsc. Res. Tech. 24, 400-422.

Hopwood D. (1969). Fixatives and fixation: a review. Histochem. J. 1, 323-360.

Jain R.K., Munn L.L. and Fukumura D. (2002). Dissecting tumour pathophysiology using intravital microscopy. Nat. Rev. Cancer 2, 266-276.

Jehl B., Bauer R., Dorge A. and Rick R. (1981). The use of propane/isopentane mixtures for rapid freezing of biological specimens. J. Microsc. 123, 307-309.

Kellenberger E., Johansen R., Maeder M., Bohrmann B., Stauffer E. and Villiger W. (1992). Artefacts and morphological changes during chemical fixation. J. Microsc. 168, 181-201.

Li Z., Ohno N., Terada N. and Ohno S. (2006a). Immunolocalization of serum proteins in living mouse glomeruli under various hemodynamic conditions by "in vivo cryotechnique". Histochem. Cell Biol. 126, 399-406.

Li Z., Ohno N., Terada N., Zhou D., Yoshimura A. and Ohno S. (2006b). Application of periodic acid-Schiff fluorescence emission for immunohistochemistry of living mouse renal glomeruli by an "in vivo cryotechnique". Arch. Histol. Cytol. 69, 147-161.

Li Z., Terada N., Ohno N. and Ohno S. (2005). Immunohistochemical analyses on albumin and immunoglobulin in acute hypertensive mouse kidneys by "in vivo cryotechnique". Histol. Histopathol. 20, 807-816.

Liao X., Terada N., Ohno N., Li Z., Fujii Y., Baba T. and Ohno S. (2006). Immunohistochemical study of serum albumin in normal and cadmium-treated mouse testis organs by "in vivo cryotechnique". Histol. Histopathol. 21, 35-40.

McDonald D.M. and Choyke P.L. (2003). Imaging of angiogenesis: from microscope to clinic. Nat. Med. 9, 713-725.

Melan M.A. and Sluder G. (1992). Redistribution and differential extraction of soluble proteins in permeabilized cultured cells. Implications for immunofluorescence microscopy. J. Cell Sci. 101, 731-743.

Molitoris B.A. and Sandoval R.M. (2005). Intravital multiphoton microscopy of dynamic renal processes. Am. J. Physiol. Renal Physiol. 288, 1084-1089.

Moreira J.E., Dodane V. and Reese T.S. (1998). Immuno-electron microscopy of soluble and membrane proteins with a sensitive postembedding method. J. Histochem. Cytochem. 46, 847-854.

Ohno S., Hora K., Furukawa T. and Oguchi H. (1992). Ultrastructural study of the glomerular slit diaphragm in fresh unfixed kidneys by a quick-freezing method. Virchows Arch. B. Cell Pathol. Incl. Mol. Pathol. 61, 351-358.

Ohno S., Terada N., Fujii Y., Ueda H. and Takayama I. (1996). Dynamic structure of glomerular capillary loop as revealed by an in vivo cryotechnique. Virchows Arch. 427, 519-527.

Ohno S., Kato Y., Xiang T., Terada N., Takayama I., Fujii Y. and Baba T. (2001). Ultrastructural study of mouse renal glomeruli under various hemodynamic conditions by an "in vivo cryotechnique". Ital. J. Anat. Embryol. 106, 431-438.

Ohno N., Terada N., Fujii Y., Baba T. and Ohno S. (2004a). "In vivo cryotechnique" for paradigm shift to "living morphology" of animal organs. Biomed. Rev. 15, 1-19.

Ohno N., Terada N. and Ohno S. (2004b). Advanced application of the in vivo cryotechnique to immunohistochemistry for animal organs. Acta Histochem. Cytochem. 37, 357-364.

Ohno N., Terada N., Murata S., Katoh R. and Ohno S. (2005). Application of cryotechniques with freeze-substitution for the immunohistochemical demonstration of intranuclear pCREB and chromosome territory. J. Histochem. Cytochem. 53, 55-62.

Ohno N., Terada N. and Ohno S. (2006a). Histochemical analyses of living mouse liver under different hemodynamic conditions by "in vivo cryotechnique". Histochem. Cell Biol. 126, 389-398.

Ohno S., Terada N., Ohno N., Fuji Y. and Baba T. (2006b). "In vivo cryotechnique" for examination of living animal organs, further developing to "cryobiopsy" for humans. Recent Res. Devel. Mol. Cell. Biol. 6, 65-90.

Plattner H. and Bachmann L. (1982). Cryofixation: a tool in biological ultrastructural research. Int. Rev. Cytol. 79, 237-304.

Selvin P.R. (2000). The renaissance of fluorescence resonance energy transfer. Nat. Struct. Biol. 7, 730-734.

Shiurba R. (2001). Freeze-substitution: origins and applications. Int. Rev. Cytol. 206, 45-96.

Shiurba R.A., Spooner E.T., Ishiguro K., Takahashi M., Yoshida R., Wheelock T.R., Imahori K., Cataldo A.M. and Nixon R.A. (1998). Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections. Brain Res. Brain Res. Protoc. 2, 109-119.

Stephens D.J. and Allan V.J. (2003). Light microscopy techniques for live cell imaging. Science 300, 82-86.

Takayama I., Terada N., Baba T., Ueda H., Kato Y., Fujii Y. and Ohno S. (1999). "In vivo cryotechnique" in combination with replica immunoelectron microscopy for caveolin in smooth muscle cells. Histochem. Cell Biol. 112, 443-445.

Takayama I., Terada N., Baba T., Ueda H., Fujii Y., Kato Y. and Ohno S. (2000). Dynamic ultrastructure of mouse pulmonary alveoli revealed by an in vivo cryotechnique in combination with freeze-substitution. J. Anat. 197, 199-205.

Tauer U. (2002). Advantages and risks of multiphoton microscopy in physiology. Exp. Physiol. 87, 709-714.

Terada N., Kato Y., Fujii Y., Ueda H., Baba T. and Ohno S. (1998). Scanning electron microscopic study of flowing erythrocytes in hepatic sinusoids as revealed by 'in vivo cryotechnique'. J. Electron Microsc. (Tokyo). 47, 67-72.

Terada N., Ohno N., Li Z., Fujii Y., Baba T. and Ohno S. (2005). Detection of injected fluorescence-conjugated IgG in living mouse organs using "in vivo cryotechnique" with freeze-substitution. Microsc. Res. Tech. 66, 173-178.

Terada N., Ohno N., Fujii Y., Baba T. and Ohno S. (2006a). Dynamic study of intramembranous particles in human fresh erythrocytes using an "in vitro cryotechnique". Microsc. Res. Tech. 69, 291-295.

Terada N., Ohno N., Li Z., Fujii Y., Baba T. and Ohno S. (2006b). Application of in vivo cryotechnique to the examination of cells and tissues in living animal organs. Histol. Histopathol. 21, 265-272.

Terada N., Ohno N., Ohguro H., Li Z. and Ohno S. (2006c). Immunohistochemical detection of phosphorylated rhodopsin in light-exposed retina of living mouse with in vivo cryotechnique. J. Histochem. Cytochem. 54, 479-486.

Terada N., Ohno N., Saitoh S., Fujii Y., Ohguro H. and Ohno S. (2007). Raman microscopy of freeze-dried mouse eyeball-slice in conjunction with the "in vivo cryotechnique". Microsc. Res. Tech. (In press).

von Schack M.L. and Fakan S. (1994). Retention of glycogen in cryosubstituted mouse liver. Histochemistry 102, 451-455.

von Zglinicki T., Rimmler M. and Purz H.J. (1986). Fast cryofixation technique for X-ray microanalysis. J. Microsc. 141, 79-90.

Watanabe M., Leng C.G., Toriumi H., Hamada Y., Akamatsu N. and Ohno S. (2000). Ultrastructural study of upper surface layer in rat articular cartilage by "in vivo cryotechnique" combined with various treatments. Med. Electron Microsc. 33, 16-24.

Xue M., Kato Y., Terada N., Fujii Y., Baba T. and Ohno S. (1998). Morphological study by an 'in vivo cryotechnique' of the shape of erythrocytes circulating in large blood vessels. J. Anat. 193, 73-79.

Xue M., Baba T., Terada N., Kato Y., Fujii Y. and Ohno S. (2001). Morphological study of erythrocyte shapes in red pulp of mouse spleens revealed by an in vivo cryotechnique. Histol. Histopathol. 16, 123-129.

Yamashita S. and Yasuda K. (1992). Freeze-substitution fixation for immunohistochemistry at the light microscopic level: effects of solvent and chemical fixatives. Acta Histochem. Cytochem. 25, 641-650.

Yang C., Terada N., Ohno N., Fujii Y. and Ohno S. (2006). Morphological analysis of lamellar structures in mouse type II pneumocytes by quick-freezing and freeze-drying with osmium tetroxide vapor-fixation. Med. Mol. Morphol. 39, 88-96.

Yu Y., Leng C.G., Kato Y. and Ohno S. (1997). Ultrastructural study of glomerular capillary loops at different perfusion pressures as revealed by quick-freezing, freeze-substitution and conventional fixation methods. Nephron 76, 452-459.

Yu Y., Leng C.G., Terada N. and Ohno S. (1998). Scanning electron microscopic study of the renal glomerulus by an in vivo cryotechnique combined with freeze-substitution. J. Anat. 192, 595-603.

Zea-Aragon Z., Terada N., Ohno N., Fujii Y., Baba T. and Ohno S. (2004). Effects of anoxia on serum immunoglobulin and albumin leakage through blood-brain barrier in mouse cerebellum as revealed by cryotechniques. J. Neurosci. Methods 138, 89-95.

Zhou D., Ohno N., Terada N., Li Z., Morita H., Inui K., Yoshimura A. and Ohno S. (2006). Immunohistochemical analyses on serum proteins in nephrons of protein-overload mice by "in vivo cryotechnique". Histol. Histopathol. 22, 137-145.

Accepted May 2, 2007