2. Recent innovation of in vivo cryotechnique in neurosciences of living animals

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Abstract. The conventional chemical fixation and alcohol dehydration have been widely used as common preparation methods, but they usually yield morphological artifacts, such as tissue shrinkage and extraction of soluble components, during the preparation procedures. Both conventional quick-freezing and high-pressure freezing methods, by which animal organ tissues are resected and frozen for physical fixation, can reduce such technical artifacts produced in the chemical fixation and alcohol dehydration. However, these freezing methods inevitably expose tissue specimens to noxious stresses of anoxia and ischemia, and the results show only anoxic morphological states of living animal organs without blood circulation, because the tissue specimens have to be removed from living animal organs for the freezing. To the contrary, our in vivo cryotechnique (IVCT), by which all cells and tissues are directly cryofixed in anesthetized animal organs, can prevent such anoxic artifacts usually induced by the first resection step. The IVCT has been already used for many animal organs, including brain, kidney, liver, intestine, spleen, eyeball, and etc. In the central nervous system, it has brought about new findings on blood-brain barrier integrity, immunolocalization of...
small molecules, dynamic changes of signaling proteins, and ultrastructural features of extracellular spaces, which were difficult to demonstrate by the conventional preparation methods. The IVCT could be also combined with other bioimaging methods, including Raman microscopy, to obtain morphofunctional information about the directly cryofixed tissues. In conclusion, it allows us to perform novel investigations of living states of cells and tissues in the central nervous system, and innovate new approaches on the transition from dead tissue morphology to living animal morphology.

I. Introduction

To understand the physiological and pathological features of living animal organs, the conventional morphological study had been used as one of the indispensable approaches, since structures of cells and tissues usually reflected some functional aspects of the living animal organs. The electron microscopy has been a standard research approach for ultrastructural analyses, and is still necessary in the morphological field with many applications. On the other hand, recent progresses of computer-assisted microscopic methods were also remarkable, and new fluorescence technologies have also enabled us to get dynamic bioimaging of signal molecules and their molecular interactions in living animal cells and tissues. However, each technique has both sides of merits and demerits for the bioimaging of living animal organs, and more detailed understanding about their technical features is very important to select appropriate preparation techniques. Our in vivo cryotechnique (IVCT) has been more useful to clarify functioning morphology of living animal organs, and also get new findings on dynamically changing structures of cells and tissues in addition to immunolocalizations of intrinsic and extrinsic molecules by both light and electron microscopies (Ohno et al., 2010). The IVCT was further developed to the cryobiopsy, which would be used for clinical application in human bodies. The purpose of this review is to describe some technical aspects of the IVCT, and review the recent new findings revealed by IVCT in the field of neurosciences.

II. Problems of conventional preparation methods

In widely used morphological studies with light and electron microscopes, animal organs are usually examined after the common preparation procedures using chemical fixation, alcohol dehydration, paraffin or epoxy resin embedding, tissue sectioning, and chromatic dye or heavy metal staining steps. However, various technical artifacts are inevitably induced during these preparation steps, especially owing to ischemia/anoxia and tissue shrinkage by the chemical fixation and dehydration steps, and
In vivo cryotechnique for neurosciences

influence the final morphological findings (Hippe-Sanwald, 1993; Kellenberger, 1991). The chemical fixation is usually performed by routine perfusion of animal organs or immersion of tissues into chemical fixatives, including paraformaldehyde, glutaraldehyde or osmium tetroxide (Hopwood, 1969). One serious problem of the chemical fixation is molecular movement and structural changes always induced during this fixation step (Kellenberger, 1991). Because the molecular cross-linking effects of chemical fixatives are too slow to avoid artificial redistribution of soluble molecular components (Hippe-Sanwald, 1993). The conformation of such quickly changing molecules can be easily modified by the chemical fixatives (Kellenberger et al., 1992). Additionally, due to the necessary time intervals before completion of fixation, it is difficult to retain transient or dynamic molecular structures of cells and tissues, particularly by the immersion-fixation (Shiurba, 2001). In the subsequent alcohol dehydration step, additional morphological artifacts, including tissue shrinkage and extraction of soluble components, can be easily induced, resulting in misunderstanding of histopathological findings of animal organs (Chan and Inoue, 1994; Leu et al., 1993). Since there had been such several serious problems in preservation of morphology and molecular distribution of cells and tissues in living animal organs with the conventional preparation methods, some alternative approaches were also used to reduce such morphological artifacts during the preparation steps. One of them was the quick-freezing method of resected biological specimens.

The quick-freezing method is a physical fixation technique which can quickly embed biological tissues in vitrified ice (Plattner and Bachmann, 1982). The term, vitrification, means the freezing condition without any visible ice crystals at an electron microscopic level. Such vitrification is particularly important for morphological studies since ice crystals often destroy molecular structures of cells and tissues. Therefore, the best vitrification for resected fresh tissues without cryoprotectants (e.g. glycerol or sucrose solution) requires very high cooling-rate (usually more than $10^5 \degree C/sec$) or very high pressures (a few thousand times higher than atmospheric pressures) during the freezing time (Plattner and Bachmann, 1982). To meet their strict requirement, various quick-freezing methods have already been developed for the last century. In the so-called “slamming quick-freezing” method, resected tissues are slammed onto copper blocks cooled down in liquid helium (-269$\degree$C) or liquid nitrogen (-196$\degree$C). In another “plunging quick-freezing” method, tissue pieces are directly plunged into liquid cryogens, which include propane alone or isopentane-propane mixture (-193$\degree$C) cooled in liquid nitrogen. By the slamming or plunging quick-freezing methods, formation of observable ice crystals can be prevented in
tissue areas less than 10 μm deep from the contacted tissue surface. To the contrary, the high-pressure freezing is another method of freezing, by which cryofixation is always performed under very high pressures to achieve the vitrification in relatively broad tissue areas. Then, various following preparation procedures are usually available after the quick-freezing or high-pressure freezing method. One of them is the freeze-substitution (FS) fixation, in which the frozen tissues are usually incubated in cooled organic solvents containing chemical fixatives, such as osmium tetroxide, glutaraldehyde or paraformaldehyde at about -80°C (Nicolas and Bassot, 1993; Shiurba, 2001). After the FS, these tissue specimens are routinely embedded in epoxy resin, paraffin wax or other materials for the subsequent sectioning. Another preparation procedure is the deep-etching (DE) method in a high vacuum chamber (10⁻⁵Pa) after the freeze-fracturing, where replica membranes of the freeze-fractured tissues are obtained by shadowing with platinum metal and carbon. As described above, one advantage of such quick-freezing or high-pressure freezing methods is better morphological preservation with fewer technical artifacts (Plattner and Bachmann, 1982). In those cryotechniques, however, targeted cells and tissues have to be always taken out from living animal organs with dynamic functions, and so morphology and molecular distributions of these resected specimens are inevitably affected by ischemia and anoxia due to loss of blood supply (Ohno et al., 1996; Terracio and Schwabe, 1981). Thus, dynamic morphology and signal molecules of cells and tissues in living animal organs, which are always changing under different hemodynamic states or physiological/pathological conditions, are difficult to be captured by the conventional cryotechniques (Ohno et al., 2001; Ohno et al., 1996; Terada et al., 1998). Therefore, it is necessary to directly freeze living animal organs under normal blood circulation and avoid the resection step of animal tissues to overcome these ischemia and anoxia problems.

## III. Development of in vivo cryotechnique and cryobiopsy

In the in vivo cryotechnique (IVCT), living animal organs are directly cryofixed without tissue resection (Fig. 1a), as described below. After exposing target organs of animals under anesthesia, they are immediately cut with a cryoknife precooled in liquid nitrogen (-196°C), and isopentane-propylene cryogen (-193°C) followed by liquid nitrogen is simultaneously poured over the whole organs (Ohno et al., 1996). By this IVCT, good vitrification of the frozen tissues at an electron microscopic level can be achieved within the local areas of several micrometers deep from the tissue surface firstly contacted by the cryoknife (Ohno et al., 1996; Xue et al., 1998). Damaged tissue areas caused by the first
In vivo cryotechnique for neurosciences

Figure 1. A flow chart of various preparation steps for light microscopy, fluorescence microscopy, transmission electron microscopy, scanning electron microscopy and Raman microscopy after “in vivo cryotechnique” (a) or cryobiopsy (b).

cryocutting are usually limited within less than 0.5 μm from the contacted tissue edge (Ohno et al., 1996). By the IVCT, blood circulation into the living animal organs is well preserved at the exact moment of freezing, which can achieve very high time-resolution.

Therefore, it has become possible to cryofix cells and tissues with fewest molecular artifacts induced by ischemia or anoxia, as reported before (Zea-Aragon et al., 2004). We can capture the cellular structures and molecular distributions closer to their dynamic living states. Multiple preparation procedures, which are following the common cryotechniques, are also available after the IVCT, as summarized in Figure 1. In addition, it is also shown that several steps of antigen retrieval treatments required for the chemically fixed and dehydrated tissues can be omitted by using the IVCT (Ohno et al., 2005). However, there are a few limitations of the IVCT. First, well-frozen areas prepared by the IVCT are usually restricted to less than 10 μm depth from the tissue surface at an electron microscopic level, which are almost similar to those by the conventional quick-freezing methods (Chen et al., 1995; Ohno et al., 1996; Van Harreveld et al., 1974; von Schack et al., 1993; Xue et al., 1998). Therefore, tissue areas available for better observation is usually limited within several micrometers’ depth, even by using new technologies for observation of wide tissue areas, such as focused ion beam-scanning electron microscopy. It may be necessary to collect lots of data on thin banding tissue areas from multiple specimens. For standard transmission electron microscopic observation, the well-frozen tissue areas can be maximized by obtaining ultrathin tissue sections in parallel to the tissue surface directly contacted by a cryoknife. At a light microscopic level,
the observable regions are about a few hundred micrometers deep from the contact tissue surface, because the spatial resolution of the light microscope is too low to detect tiny ice crystals formed in cells and tissues by slower freezing speeds (Ohno et al., 2005; Zea-Aragon et al., 2004). Second, it should be necessary to expose the target organs of living animals to pour the liquid cryogen over them and obtain wide tissue areas without observable ice crystals. The good cryofixation are always challenging, if such target organs of anesthetized animals are difficult to expose within their bodies.

The IVCT was initially used for electron microscopic analyses of organs in living mice and rats, but the number of bioimaging technologies dramatically increased for the last few decades. Such technological shifting from electron to light microscopic analyses revealed multiple technical advantages of IVCT probably applied for clinical medicine, resulting in the recent development of cryobiopsy for the time-dependent examinations of living animal organs (Fujii et al., 2006), including humans in the near future (Fig. 1b). One technical limitation of above-mentioned IVCT is a difficulty to obtain a series of tissue specimens from an identical animal, since the liquid cryogen should be directly poured only once over the animal organs. By contrast, in cryobiopsy, targeted organ tissues of anesthetized animals are quickly pinched off with the cryobiopsy forceps precooled in liquid nitrogen (-196°C) and immediately plunged into the isopentane-propane cryogen (-193°C). In the following preparation steps of these cryobiopsied tissues, the similar procedures for the commonly frozen tissues can be used (Fig. 1d~f), and some morphological findings were already reported by observation with light microscopy or scanning electron microscopy (Fujii et al., 2006; Ohno et al., 2008). The new cryobiopsy technique would be useful for repeated examination of functioning organs of living animals (Saitoh et al., 2010), including humans for clinical medicine.

As described above, the IVCT has lots of characteristics which would be very useful in research of neuroscience fields. In the following sections, our recent innovation of IVCT will be discussed with particular emphasis on four aspects of the IVCT; (i) maintaining precise in vivo distributions of soluble molecules, (ii) visualizing rapid changes of signaling molecules, (iii) preserving cerebellar morphology without ischemia/anoxia, and (iv) combining with new bioimaging techniques.

(i). Maintaining precise distributions of soluble molecules

Blood-brain barrier (BBB) is a selective molecular barrier separating the central nervous system from blood circulation. The BBB integrity also plays important roles in pathophysiology of brain diseases, such as neurodegenerative disorders (Zlokovic, 2008). The in vivo injection of dye probes was initially used to examine the BBB integrity (Broman et al., 1966).
The unexpected problem of this injection approach was that these dyes easily bind to serum proteins and their color got mostly lost in the biological tissues. Because of such difficulties in dye usage, alternative common tracers for injection, such as horseradish peroxidase (HRP) (Broadwell and Sofroniew, 1993; Reese and Karnovsky, 1967), radio-labeled tracers (Zucker et al., 1983) or fluorescent dyes (Miller et al., 1996), were later developed in the tracer experiments for the past decades. However, the intravascular injection of extrinsic molecules can’t be used for subsequent immunohistochemistry with perfusion-fixation, since artificial perfusion of the animal brains with fixatives easily washes out these molecules from cells and tissues. Trypan blue was also transcardially perfused and used as a tracer for alteration of BBB integrity (Reynolds and Morton, 1998), but it had the similar diffusion problem to examine the BBB integrity during the preparation steps. In this context, alternative immunohistochemical detection of serum proteins, such as intrinsic albumin, was used to reveal extravasation of the serum proteins into extracellular matrices of animal brains (Hamilton and Gould, 1987; Nishino et al., 1995).

In our previous study (Zea-Aragon et al., 2004), the IVCT was used to examine rapid leakage of soluble serum proteins through the BBB from the view-point of hemodynamic effects on living mouse cerebellum. Since their dynamic leakage across BBB could be time-dependently seen after ischemia of the mouse cerebellum, the IVCT in combination with FS, which minimizes artificial diffusion and translocation of soluble molecules at about -80°C, clarified the precise distributions of serum IgG and albumin in the cerebellar tissues. Indeed, both distributions of the serum IgG and albumin were restricted inside cerebellar blood vessels under the normal hemodynamic condition. To the contrary, in cerebellar tissues resected from brains and quickly frozen in the isopentane–propane cryogen at about -193°C within a minute, they were already diffusely immunolocalized in large matricial areas around blood capillaries. Such immunolocalizations of the serum IgG and albumin following the tissue resection are attributable to easy leakage of the serum components through BBB in short time of anoxia. In addition, another time-dependent detection of injected extrinsic probes have been successfully performed in other organs prepared with IVCT (Fig. 1c) (Ohno et al., 2008; Saitoh et al., 2012; Terada et al., 2005). Since no soluble serum protein could be identified outside blood capillaries under normal hemodynamic conditions of the anesthetized mice, the IVCT in combination with FS is a powerful tool among several immunohistochemical approaches to examine the extravascular leakage of intrinsic and extrinsic molecules across BBB under pathological conditions of living animal brains.
Another immunohistochemical study using IVCT followed with FS was performed to examine distributions of a neurotransmitter, glutamate (Terada et al., 2009), which had been a previously controversial issue in inner segments of photoreceptors. In the study of such small signal molecules, retinal tissues of anesthetized mice were directly frozen with IVCT, processed for FS fixation in acetone containing glutaraldehyde fixatives, and embedded in paraffin wax. Deparaffinized sections were immunostained with anti-glutamate antibody. By perfusion-fixation and alcohol dehydration, stable immunoreactivity of glutamate in retina was usually difficult to obtain, probably because of technical problems, such as its diffusion artifact and/or antigen-masking of shrunken tissues. By contrast, the soluble glutamate was clearly immunolocalized in the inner segments, outer and inner plexiform layers, and ganglion cell layers of living mouse retina prepared with IVCT. The best immunoreactivity of glutamate was detected in the specimens followed by FS with low concentrations of glutaraldehyde, whereas no immunoreactivity was obtained without the chemical fixatives. With various immunohistochemical approaches, a controversial issue had arisen until then as to whether the glutamate was immunolocalized in the inner segment or not. In enucleated eyes of goldfish (Marc et al., 1990), cat (Pourcho and Owczarzak, 1991), chicken (Kalloniatis and Fletcher, 1993; Sun and Crossland, 2000), rat (Kalloniatis et al., 1996), and monkey (Kalloniatis et al., 1996), which were conventionally immersion-fixed, resulting in glutamate immunoreactivity only in the inner segment. However, the glutatione immunoreactivity was not detected in the inner segment by perfusion-fixation (Sasoh et al., 2006; Sasoh et al., 1998). Therefore, the immunolocalization of glutamate only in the inner segment might be attributed to postmortem changes during the conventional fixation induced by ischemia/anoxia. To the contrary, the clear immunoreactivity of glutamate was revealed not only in the inner segment of photoreceptors, but also other retinal layers by IVCT followed with FS, which enables immunohistochemical analyses without ischemia/anoxia, suggesting that the glutamate immunoreactivity in the inner segment is not a technical artifact of preparation procedures. The inner segment has a special mitochondrial metabolic cycle involving glutamate (Tsacopoulos et al., 1998). Therefore, such mitochondria abundant in the inner segment are strong candidates to localize glutamate. Several enzymes for glutamate metabolism, including glutamate dehydrogenase, phosphate-activated glutaminase, aspartate aminotransferase, and ornithine aminotransferase, were reported to have high activity in the inner segment (Endo et al., 1999; Ross and Godfrey, 1987). The best glutamate immunoreactivity was obtained in the IVCT-prepared specimens with low concentrations of glutaraldehyde. The alteration of
glutamate immunoreactivity under different concentrations of glutaraldehyde may be due to alteration of its cross-linking mechanism of tissue structures, which efficiently retain glutamate in the retinal tissues, but it sometimes hinders the specific antibody binding sites at its higher concentrations. These findings collectively demonstrate that the IVCT is useful for examination of the immunohistochemical distribution of small molecules like glutamate in living animal retinal tissues.

(ii). Visualizing rapid changes of signaling molecules

The common rhodopsin (Rho) phosphorylation has provided an example of the ubiquitous regulatory pattern of specific kinases downregulating the activity of G protein-coupled receptors (Maeda et al., 2003). The Rho is localized in a photoreceptor quickly changing its molecular structure by light stimulation (Menon et al., 2001). In such a case, the phosphorylation of Rho and subsequent binding of arrestin to the Rho quickly inactivate an active form of Rho (Fain et al., 2001). During these time-dependent processes of activation and inactivation, the Rho phosphorylation is considered to play an important role for visual functions of living animals, ranging from the ability of rod photoreceptor cells to generate reproducible electrical responses to the dark adaptation after light exposure (Arshavsky, 2002). The Rho is usually phosphorylated at three epitopes, such as 334Ser, 338Ser, and 343Ser, near its carboxyl terminus (Ohguro et al., 1996). The biochemical analysis with mass spectrometry using resected mouse retinas already revealed that the phosphorylation reaction quickly started in response to various light conditions (Kennedy et al., 2001). Recently, specific and well-characterized antibodies against phosphorylated Rho (P-Rho) were produced and used to examine the duration of P-Rho immunoreaction after the dark adaptation of retinas (Ohguro et al., 2003). However, immunohistochemical visualization of the rapid phosphorylation in response to light stimuli was difficult with the conventional preparation methods, since rapid conformational changes of the molecular structure could not be captured during the chemical fixation (Adamus et al., 1988; Hicks and Barnstable, 1987). To overcome this problem, IVCT followed by FS was used for immunohistochemical examination of time-dependent molecular changes of Rho and P-Rho under dark-adaptation and light-stimulation conditions in living mouse retinas (Terada et al., 2006). In the previous study, deparaffinized sections of mouse retinal tissues were routinely immunostained with anti-phosphorylated 334Ser-Rho (P-Rho334) antibody to detect their time-dependent molecular changes. The common immunoreactivity of P-Rho334 was specifically recognized in the outer segments of living mouse retinas which were exposed
to daylight. Following 12 hr of dark-adaptation, P-Rho334 immunoreactivity was completely eliminated. In addition, after 12 hr or 36 hr of dark adaptation, exposure to the safety red light for 120 sec in a dark room did not increase the P-Rho334 immunoreactivity. By contrast, other various time exposures to strong visible light for 30, 60, and 180 sec dramatically increased P-Rho334 immunoreactivity to the level similar to that under the daylight condition, although 10 sec exposure time did not increase its immunoreactivity. Such a highly temporal resolution of IVCT was useful to capture photo-signal transduction in the living animal retina, which can quickly proceed (Menon et al., 2001). The IVCT can physically cryofix the cells and tissues within microseconds, when vitreous ice is produced. In the serial Rho-phosphorylation steps, only 343Ser was the most rapidly phosphorylated, while the other 338Ser and 344Ser were more slowly phosphorylated (Kennedy et al., 2001). In conclusion, the higher sensitivity of immunostaining with IVCT facilitated immunohistochemical demonstration of rapid phosphorylation of P-Rho334, which was induced within 10-30 sec after the strong light exposure in the living mouse retina.

The IVCT was also used to examine functional activities of receptors in the central nervous system (CNS) by focusing on angiotensin II (AT) receptors in living mouse cerebellum (Huang et al., 2013). The AT receptors, type 1 (AT1R) and type 2 (AT2R), are seven-transmembrane G-protein-coupled receptors, which mediate the physiological functions of AT (Basso and Terragno, 2001; de Gasparo et al., 2000). Although AT receptors are also expressed in CNS in addition to well-established adrenal glands and play functional roles in hemodynamic control, differentiation, neuronal plasticity and cell survival (Allen et al., 1998; Arce et al., 2001; Arce et al., 2011; Changarlis et al., 1978; Cote et al., 1999; Fogarty and Matute, 2001; Johren et al., 1998; Phillips et al., 1993; Reagan et al., 1994; Saavedra, 1992), their distributions and activation states have not been well understood in the animal cerebellum. To address this question, specific antibodies against amino-terminal domains of AT receptors, which undergo conformational changes upon the binding of ligands, were used in the study, and immunohistochemical analyses of AT receptors were performed in the living mouse cerebellum with our IVCT followed by FS (Fig. 1d). Immunoreactivities of both AT1R and AT2R were detected in the cerebellum, and largely overlapped with glial fibrillary acidic protein (GFAP), a marker of Bergmann glia under normal blood circulation and 1 min hypoxia conditions (Fig. 2a~c). Surprisingly, the AT1R immunoreactivity was remarkably reduced in the cerebellar cortex following 5 min and 10 min of hypoxia (Fig. 2d~g), or direct administration of an AT1R antagonist, losartan. Such hypoxia-induced decrease of immunoreactivity was similarly observed by immunostaining of AT2R in the
Figure 2. (a) Schematic summary of AT1R and AT2R immunolocalizations in the molecular, Purkinje cell and granular layers of mouse cerebellar cortex, as detected using IVCT. The immunolocalizations of AT1R (green) are closely related to those of GFAP-positive processes of Bergmann glia (red) in the molecular layer. (b~g) Light microscopic images of HE staining (b, d, f) and immunostaining for AT1R under 1 (b, c), 5 (d, e) and 10 (f, g) min hypoxia in the mouse cerebellum. After 1 min of hypoxia, AT1R immunoreaction products are still detected as dot-patterns, while after 5 and 10 min of hypoxia, the immunoreaction intensities of AT1R are remarkably reduced in the cerebellar cortex. Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer. Bars 20 μm.
same cerebellar specimens. These findings suggested that functional activities of both AT receptors were time-dependently modulated under hypoxia in the cerebellar tissues. Furthermore, it is also proposed that the IVCT is useful to visualize rapid alteration of signaling receptor molecules without anoxia in CNS of living animals.

(iii). Preserving cerebellar morphology without ischemia/anoxia

For the past several decades, about 10–20% of CNS tissue areas were considered to be made up of extracellular spaces among neuropils and glial processes. However, direct visualization of such extracellular spaces was difficult, because they are easily diminished by the conventional chemical fixation and alcohol dehydration or anoxia with tissue resection (Van Harreveld et al., 1965; Van Harreveld and Khattab, 1968). Such dramatic loss of extracellular spaces was usually attributed to translocation of extracellular water-soluble molecules and electrolytes into intracellular compartments (Van Harreveld, 1962). In our previous study, ultrastructural analyses of living mouse cerebellar cortex were done using IVCT (Ohno et al., 2007). With the IVCT, large extracellular spaces were definitely preserved between many cytoplasmic profiles and synaptic areas in molecular and Purkinje cell layers of the living mouse cerebellar cortex. Although the conventional quick-freezing method for resected cerebelar tissues or 8 min anoxia before IVCT performance largely diminished the amount of extracellular spaces, which were strictly preserved by the IVCT without anoxia, another 30 sec anoxia just before the IVCT did not decrease the amount of extracellular spaces. In addition, abundant fluid content existing in the extracellular spaces was also diminished after prolonged exposure of the cerebellum in the air.

This ultrastructural study also revealed that such extracellular spaces were well maintained around some synaptic clefts (Fig. 3a), although others were totally or partially enclosed with surrounding glial processes (Fig. 3b–c). Interestingly, the calculated sizes of extracellular spaces around the synaptic clefts were not significantly changed between open and enclosed synaptic clefts. In addition, rapid increases in sizes of Bergmann glial cytoplasm and parallel fibers after anoxia and tissue resection indicated translocation of various extracellular components induced by the conventional technical problems (Ohno et al., 2007), as reported before (Van Harreveld, 1961). Such ultrastructural changes easily caused by anoxia could be minimized by using IVCT, as described in the previous paragraph. Deep local areas far from the cerebellar tissue surface, such as Purkinje cell layers in the cerebellar cortex, were appropriately prepared by cutting target organs with a cryoknife, as previously reported (Ohno et al., 1996), and so the IVCT could well preserve lots of extracellular spaces in both deep molecular and
Figure 3. Electron micrographs of molecular layers of living mouse cerebellar tissues, prepared by IVCT and FS. Synapses are represented by presynaptic (Pre) and postsynaptic (Post) terminals. Glial processes (arrows in b and c) can freely extend or retract through the abundant perisynaptic extracellular spaces (asterisks) among processes of other cells. Ax: axon, De: dendrite, Gl: glia. Arrowheads: synapse with open extracellular spaces.

Purkinje cell layers of the living mouse cerebellar cortex. Since some ultrastructural differences among tissue areas of the cerebellar molecular layers were already suggested using the conventional chemical fixation followed by alcohol dehydration (Gundappa-Sulur et al., 1999; Napper and Harvey, 1988; Sultan, 2000), more careful re-evaluation of the CNS morphology will be necessary without any anoxic effects by our IVCT.

Some synapses in molecular layers of the living mouse cerebellum appeared to be exposed to open perisynaptic extracellular spaces with various sizes, as discussed in the previous paragraph. Bergmann glial processes are considered to ensheath most dendritic spines of Purkinje cells bearing synapses (Spacek, 1985). Such cytoplasmic sheaths of Bergmann glia were reported to cover more than half of the circumferential length around the synaptic clefts (Spacek, 1985). But the proportion of the enclosed synapses revealed might be overestimated by the conventional preparation methods, since cytoplasmic profiles artificially got swollen during tissue resection steps, then resulting in loss of extracellular spaces (Ohno et al., 2007), and totally shrunken during alcohol dehydration. Therefore, more numbers of synapses in the molecular layers would be, at least partially, exposed to the open perisynaptic extracellular spaces. Although various preparation steps in the conventional fixation methods probably caused artificial changes of
ultrastructures of cells and tissues (Eisenberg and Mobley, 1975; Van Harreveld and Khattab, 1968), our previous findings obtained with IVCT strongly supported the concept of widely open synapses (Ohno et al., 2007), as shown in another study of murine CNS with the novel chemical fixation methods (Cragg, 1980). In addition, no significant difference was seen in the mean size of perisynaptic extracellular spaces between opened and enclosed synapses. Therefore, these findings additionally suggest that the glial processes ensheathing the synapses dynamically extend or retract throughout the open perisynaptic extracellular spaces with few structural changes of the neighboring neuropils (Ohno et al., 2007), and that the perisynaptic extracellular spaces would give spatial support for the dynamic changes of glial processes (Fig. 3). It was already reported that astroglial cells affecting synaptic neurotransmission were involved in structural development, maintenance, and remodeling of synapses in the CNS (Auld and Robitaille, 2003). Therefore, perisynaptic glial processes play significant roles in these glial functions around synapses, which are considered to dynamically change, depending on the synaptic activities (Iino et al., 2001; Oliet and Piet, 2004). A variety of sizes of perisynaptic extracellular spaces and distribution of dynamically changing glial processes indicate a possible diversity of their contributions to the synaptic signal transduction.

(iv). Further combining with new bioimaging techniques

Various microscopic technologies, such as ultraviolet, infrared, Raman, or fluorescence microscopy, have been recently used to evaluate the intracellular localization of biological molecules and/or exogenous probes (Choi et al., 2005). These optical methods can provide detailed information about the molecular structure of cells and tissues, and also have attracted considerable attention on their molecular evaluation. In addition, biological components of cells and tissues are dynamically changing their molecular structures, which Raman microscopy can be used to distinguish (Wood et al., 2001). As the first step to examine rapid molecular changes in lung tissues of living mice, electron microscopy was combined with the IVCT followed by freeze-drying method to retain localizations and structures of various biomolecules (Yang et al., 2006). In another study with the freeze-drying method, the Raman spectrum mapping of retinal structures was obtained from the living mouse eyeballs (Fig. 1f) (Terada et al., 2007). Briefly, eyeballs of anesthetized mice were directly cryofixed using IVCT, and then thick cryosections (100 μm thickness) were obtained under a cryostat apparatus. The eyeball tissue slices were sandwiched between glass slides and freeze-dried in a high vacuum chamber, and various tissue layers were analyzed
with confocal laser Raman microscopy (Fig. 1h). In the specimen images obtained by bright-field microscopy, Raman spectra were electronically mapped and could be largely classified into four typical patterns. Then, the tissue layer organization was confirmed by re-embedding the observed eyeball tissue slice into epoxy resin and preparing thick sections with an inverted gelatin capsule method. Each of four Raman spectral patterns mostly represented different histological layers of the eyeball. The Raman spectrum with two obvious peaks corresponded to melanin in the choroid and pigment cell layers. Another Raman spectrum obtained from blood vessels in sclera corresponded to hemoglobin, and the third Raman spectrum of photoreceptor cell layers was similar to that obtained from the purified rhodopsin. Importantly, there were little Raman spectral changes by observation of the freeze-dried tissues, probably because structural alteration of biological molecules was almost little during the freeze-drying step after IVCT.

Furthermore, the IVCT was recently combined with confocal laser Raman cryomicroscopy at a low temperature (-150°C) (Fig. 1g, h), and oxygen saturation of flowing erythrocytes was directly measured in the frozen tissues of living mouse organs (Terada et al., 2008). This new Raman cryomicroscopy technique in combination with IVCT will be useful to facilitate direct analyses of the in vivo oxygen saturation, reflecting their living states in various organ tissues of a whole animal body. These findings collectively suggest that different tissue components can be distinguished in unstained sections with Raman microscopy, and therefore such combination of IVCT with Raman microscopy would be very useful for examining rapidly changing distributions and structures of biological molecules and chemical components in living animal organs.

IV. Concluding remarks

Various components and structures of organelles, cells and tissues, and organs of living animals are dynamically changing to work for their functions at second to millisecond orders, and their dynamic alterations are always taking place in abundant extracellular or intracellular fluid in vivo. The commonly used cryofixation is based on the concept to change water content into vitreous ice to keep all components and structures in vivo for subsequent morphological, immunohistochemical or other analytical techniques. The IVCT presented in this review is the first step to capture various functional organs of living animals without anoxia under normal blood circulation at the exact moment of freezing, and can be followed by various light and electron microscopic procedures (Fig. 1). It was successfully used for various morphofunctional applications in CNS and brought about new biological
findings, which had been difficult to detect by the other conventional preparation methods. The IVCT is a powerful tool to reveal the functional morphology of cells and tissues closer to their living states (Ohno et al., 2010), and will open the door for a new field of living animal morphology during the twenty-first century.

References