Review

Morphofunctional Merits of an *In Vivo* Cryotechnique for Living Animal Organs: Challenges of Clinical Applications from Basic Medical Research

Shinichi Ohno[1]

[1]*Department of Anatomy and Molecular Histology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-City, Yamanashi 409–3898, Japan*

Recent advances in molecular and genetic techniques have led to establishment of new biomedical fields; however, morphological techniques are still required for a more precise understanding of functioning cells and tissues. Conventional preparation procedures involve a series of chemical fixation, alcohol dehydration, paraffin or epoxy resin embedding, sectioning, and staining steps. In these steps, technical artifacts modify original morphologies of the cells being examined. Furthermore, difficulties are associated with capturing dynamic images *in vivo* using conventional chemical fixation. Therefore, a quick-freezing (QF) method was introduced for biological specimens in the 20th century. However, specimens have to be resected from living animal organs with blood supply, and their dynamical morphologies have not been investigated in detail using the QF method. In order to overcome these issues, the tissue resection step of organs had to be avoided and samples needed to be frozen under blood circulation. Our *in vivo* cryotechnique (IVCT) was an original technique to cryofix samples without resecting their tissues. The most significant merit of IVCT is that blood circulation into organs is preserved at the exact moment of freezing, which has been useful for arresting transient physiological processes of cells and tissues and maintaining their components *in situ*.

I. Introduction

Advances in analytical techniques with gene analysis and manipulation have recently occurred in the field of medical biology, and new biomedical evidence has been obtained for the basis of human life. Furthermore, new life sciences, such as the clinical application of iPS cells, have already been developed in the field of regenerative medicine. However, morphological methods are still required because the physiological functions of cells and tissues depend on dynamic organs in living animal bodies. Recent advances in molecular biology have occurred through the combined use of genetic manipulation and fluorescence-labeled molecules. Therefore, genetically fluorescence-labeled molecules have been examined time-dependently in the cells and tissues of living animal bodies or during dynamic intracellular transport using optical imaging methods.

In this review, we demonstrated that the development of our *in vivo* cryotechnique (IVCT) may be also useful as an optical imaging method and also for human clinical applications at a light microscopic level, because it allows

for the preparation of paraffin-embedded tissue sections, and provides functional images of living animal organs and their proteins *in vivo* [4].

II.　Basis of Cryotechniques

1. Purpose of freezing

In common perfusion-fixation and immersion-fixation methods, chemical fixatives using paraformaldehyde or glutaraldehyde are mainly used for light and electron microscopies. However, ischemia and the lack of oxygen at the time of fixation are caused by sample excision or the duration of perfusion. Therefore, a new cryotechnique that provides tissue specimens as close as possible to living states, which often uses the physical phenomenon of "freezing" to instantly enclose the organ tissues of living animal bodies in amorphous ice for electron microscopy at a very low temperature of approximately –190°C, is needed [4, 5].

2. Common quick-freezing method

In the quick-freezing (QF) method at a cooling rate of more than 10^5°C per second, fresh excised organ tissues need to be placed in any liquid cryogen as soon as possible in order to minimize the morphological changes that occur in fresh tissues following animal tissue excision. However, well-frozen tissues typically contain amorphous ice of less than 10 μm at the contact tissue surface at the electron microscopic level, and, thus, clear observations are only possible in small areas. However, good observations are possible at a depth of 200–300 μm in paraffin-embedded specimens with light microscopy because tissue damages caused by ice crystals are not visible by light microscopy with lower resolution [5].

3. Liquid cryogens and their physical features

Liquid nitrogen is commonly used for freezing at very low temperatures (boiling point; –196°C), but cannot be employed for morphological analyses of freezing cells and tissues as the QF of animal tissue samples. Liquid nitrogen vaporizes easily due to the narrow range of the temperature difference between melting point (–210°C) and the boiling point, and the nitrogen gas produced always reduces heat conduction within the frozen tissues, leading to the formation of larger ice crystals. Liquid helium is also used at lower temperatures (–269°C), but cannot be employed to freeze tissues for the same reason; the gap in the temperature to the melting point (–272°C) is only 3°C. In contrast, the gaps in the temperatures of each boiling point for isopentane (melting point; –160°C) and propane (melting point; –188°C) are very large, and, thus, they are often used as liquid cryogens to freeze animal organ tissues with efficiently wide gap ranges of 188°C (isopentane) and 146°C (propane). We always use mixed isopentane-propane cryogens because of the preparation merits of both cryogens [4, 5], as described below.

III.　Merits and Demerits of the Common QF Method

Soluble components in cells and tissues typically diffuse out or are washed away in conventional fixative solution when the perfusion-fixation of animal organs is performed. Furthermore, the movement of soluble components cannot be avoided during the preparation steps when parts of organ tissues are resected surgically and immersed in chemical fixative solution. In contrast, this artificial movement is preventable with the QF method, because there components are instantly enclosed in amorphous ice. Therefore, the morphological images obtained closely reflect the state of *in vivo* cells and tissues in living animal organs. Tissues frozen using the QF method are generally freeze-substituted, and processed using epoxy resin or hydrophilic resin embedding for electron microscopy and paraffin-embedding for light microscopy. Morphological analyses and immunostaining of these freeze-substituted tissues are also possible. Even though we use the conventional QF method on freshly resected organ tissues, stopping the circulation is inevitable due to their excision. Therefore, morphological changes to resected tissues occur because of the lack of oxygen. Hence, functionally dynamic molecules with real morphological features in the cells and tissues of living animals cannot be accurately examined even in samples frozen using the QF method. Therefore, cells and tissues need to be directly frozen, and, in such cases, the circulation is constantly maintained in living animal organs [5].

IV.　Procedures and Merits of IVCT

IVCT is a method by which the above-described QF method is directly performed on the organ tissues of anesthesized animals. During surgery on the abdominal cavity, it is important to prevent various organs becoming dry, because *in vivo* organ surface tissues, such as these of the liver, spleen, and intestines, are always moistened by a small amount of peritoneal fluid. Furthermore, breathing must be control in anesthesized animals using an artificial respirator after opening thoracic cavity in order to access the beating heart and respiring lungs. In our previous study, the mixed isopentane-propane liquid cryogen (approximately –193°C) was directly poured over various organs as soon as possible [5]. This step was performed more easily in the living animal body using a commercial *in vivo* cryoapparatus when target organ tissues were cryocut with a liquid nitrogen-cooled cryoknife for electron microscopic specimens [4, 5]. The critical influence of ischemia and lack of oxygen were strictly avoided by using IVCT on anesthesized animals, such that morphological images of totally functional cells and tissues were directly obtained. Although the observable tissue range was not large, we were able to collect sufficient experimental data at the light microscopic level [5].

V. Preparation Procedure of the Mixed Isopentane-Propane Cryogen

We have described the simple preparation procedure for the liquid isopentane-propane mixture. We initially prepared a home-made instrument to hang a 50 ml beaker with wires in a Styrofoam box containing liquid nitrogen. After pouring approximately 15 ml of the commercial isopentane solution into the beaker, we hung it in liquid nitrogen (−196°C) which had already been added into the Styrofoam box. We stirred the isopentane in the beaker using a magnet stirrer, inserted the nozzle tip of a gas tube connected to a commercial propane gas-tank into the isopentane, and made the liquid propane cryogen by spraying the propane gas into the cooled liquid isopentane. We should check that the magnet is well rotating inside the isopentane. The propane gas blow may sometimes be delayed, resulting in solidification of the isopentane solution; therefore, careful attention is needed when cooling isopentane in order to prevent its solidification. Propane gas generally becomes the mixed liquid isopentane-propane cryogen in this manner. The amount of the liquid isopentane-propane cryogen prepared gradually increased, reaching a volume of approximately 45 ml (isopentane : propane=1 : 2) within 4–5 min. The temperature of this mixed cryogen is known to be approximately −193°C. We covered the lid of the Styrofoam box, containing the beaker in order to prevent the atmospheric moisture becoming tiny frost and contaminating the mixed cryogen prepared. The mixed isopentane-propane cryogen is recommended for use immediately after its preparation.

VI. Comment on Optical Imaging Methods

Optical imaging methods, which have developed rapidly, are now frequently used to analyze various kinds of functional molecules involved in intracellular signaling in animal organ tissues and cultured cells. However, difficulties are associated with analyzing functional morphology in optical images of the cells and tissues of living animal organs, using conventional preparation methods for light microscopy. IVCT was initially assumed to be capable of capturing all cells and tissues of the living mouse kidneys in amorphous ice at an electron microscopic level [4] and we already mentioned that it was also very useful to solve the conventional preparation problems at a light microscopic level [5]. Therefore, it is now possible to prepare living animal organs in order to obtain living morphological images for the analysis of functional molecules using light microscopy, thereby supporting digitized imaging data.

VII. Biomedical application of IVCT

1. Immunohistochemical analysis of serum proteins in beating heart tissues

IVCT, which immediately cryofixes target organs *in situ*, has been used to clarify the morphological features of beating heart tissues in living mice [9]. We initially attached an electrocardiogram (ECG) and respirator to anesthetized mice, and their beating hearts were quickly frozen under the diastolic condition by IVCT. We also injected bovine serum albumin (BSA) into mice via the tail vein, and the hearts were then frozen as described above 4, 8, and 48 hr later as well as after 5 and 30 min. After routine freeze-substituting fixation, samples were embedded in paraffin wax. We immunostained deparaffined tissue sections with antibodies, such as albumin, IgG1, connexin 43, and BSA. The exact stop time of the beating mouse heart was determined by ECG, and open blood vessels with flowing erythrocytes were clearly observed with less artificial tissue shrinkage than those prepared using conventional preparation methods. With normal hemodynamics in living mouse heart tissues, albumin and IgG1 were immunolocalized in the matrix substrate between cardiac muscle cells, not only in blood vessels. However, albumin alone was also detected in T-tubules and at intercalated discs immunolabeled by connexin 43. On the other hand, the injected BSA was temporarily immunolocalized at the intercalated discs only 30 min later. These findings clearly demonstrated dynamic immunohistochemical changes in soluble serum proteins in beating heart tissues together with the circulation. Therefore, IVCT is regarded as being more useful for morphofunctional examinations of dynamically changing heart tissues than conventional preparation methods [9].

2. Visualization of adenosine triphosphate in skeletal muscle tissues

Adenosine triphosphate (ATP) is a well-known energy source for muscle contractions. A luciferin-luciferase reaction was performed in tissue sections of mouse skeletal muscle prepared by IVCT in order to visualize the localization of soluble ATP [12]. We exposed the gluteal muscle tissues of anesthetized mice, which were frozen *in vivo*. We then performed common freeze-substitution fixation in acetone containing 0.2% glutaraldehyde and initiated the luciferin-luciferase reaction for ATP visualization in cryostat tissue sections [12]. The amount of ATP was significantly higher in skeletal muscle fibers than in the matrix substrate, and a relative difference in the quantity of ATP that depended on the number of relative photons was also detected between each muscle fiber when we quantitatively imaged the intensity of luminescence, as revealed with a light microscope. Thus, it has become possible to perform microscopic evaluations of the relative amounts of ATP molecules in mouse skeletal muscles using IVCT-prepared specimens [12], which mostly reflect their living states.

3. Morphology of living mouse lungs and pulmonary hemodynamics

Microscopic imaging of blood vessels and serum proteins is essential for analyzing hemodynamics in living animal lungs under normal respiration or respiratory diseases. IVCT was used in combination with freeze-substitution fix-

ation to demonstrate the dynamically changing morphology of living mouse lungs and immunohistochemical analyses of serum proteins of their living states [7]. We attached a respirator to anesthetized mice in order to maintain functional lungs, and then opened the thoracic cavity to expose the lungs. We directly froze the living mouse lungs using IVCT at expiration or inspiration. We also froze the lungs 1, 2, or 10 sec after glutathione-coated Q-dots (quantum dots), which emit red fluorescence light with a wavelength of 650 nm, were injected into the right ventricle.

By using hematoxylin-eosin staining, morphological features, such as the shape of the alveolar septum and size of the alveolar lumen, were captured, reflecting their respiratory conditions *in vivo*. Moreover, blood capillaries in the alveolar septum were filled with flowing erythrocytes of various shapes. Q-dots were detected in arterioles and blood capillaries 1 sec after their injection, but not observed in venules. IVCT enabled us to examine pulmonary tissue images together with hemodynamic visualization *in vivo* at the time of functional expiration or inspiration [7].

4. Experimental pulmonary hypertension with soluble albumin exudates

IVCT has also been applied to the mouse lungs of an acute pulmonary hypertension model [7]. With a clipping of the ascending aorta in an anesthetized mouse, we time-dependently prepared the lungs using IVCT. Hemagglutination was observed in many blood capillaries after 7 min, and a large amount of an albumin exudate was mostly detected in the alveolar cavity. Thus, IVCT combined with immunohistochemical approaches for serum proteins enabled us to capture native images of dynamically changing lung structures and the microvascular permeability of living mouse lungs [7].

5. Imaging of thrombosis and microcirculation in melanoma lung metastasis

The microscopic bioimaging of blood flow and cancer cells in experimental animal lungs is essential for analyzing the mechanisms underlying lung cancer metastasis. Metastasis has been assumed to induce hypercoagulable states in cancer patients in the clinical field. However, it is challenging to examine the rapid phenomenon of thrombus formation in conventional tissue sections due to the technical difficulties associated with microscopic specimens. We considered it important to determine the dynamic histological features reflecting the living states. Therefore, IVCT was used to bioimage the hypercoagulable states and thrombosis, induced in living mouse lungs by the early metastasis of mouse B16-BL6 melanoma [8]. Glutathione-coated Q-dots were injected in order to examine blood flow at different time intervals following a melanoma cell injection via the right ventricle. The injected melanoma cells attached to the endothelia of blood capillaries within 1 min in the initial stage, and a large number of platelets aggregated in the neighborhood areas within 5 min. We also

confirmed the local existence of flowing Q-dots around some tumor clots, demonstrating persistent blood circulation [8]. We used the Q-dot injection and immunostaining of the soluble serum proteins, albumin and fibrinogen, together with IVCT-prepared tissue sections of living mice, and obtained tumor tissue images *in vivo* for the light microscope, which may correspond to Q-dot live imaging.

VIII. Confocal Raman Microscopy for IVCT-Prepared Specimens

1. Molecular organization of the retina in freeze-dried eyeball slices

The wavelength pattern of Raman-scattered light is known to reflect the molecular composition of the tissue structure. Therefore, we directly froze an anesthetized mouse eyeball, which was prepared by the freeze-drying method, using IVCT, and then examined retinal tissues in unstained specimens using confocal Raman microscopy [10]. The Raman wavelength showed a characteristic pattern in response to each retinal layer localized with specific molecules. The choroid membrane and pigment epithelium cell layer both showed the characteristic wavelength pattern of melanin granules. We also observed other specific wavelength patterns that corresponded to hemoglobin of erythrocytes flowing in sclera blood vessels and the photorecepter protein, rhodopsin, in the visual cell layer. Therefore, functional molecules in retinal tissues were identified without any staining in the IVCT-prepared specimens of living mice [10].

2. Erythrocyte oxygen saturation with confocal Raman cryomicroscopy

The relative peak ratios of 1378 cm^{-1} and 1355 cm^{-1} generally change, depending on oxygen saturation, when the two resonance Raman wavelength patterns of erythrocyte hemoglobin are examined. Therefore, we detected various amounts of hemoglobin oxygenicity in IVCT-prepared flowing erythrocytes in blood vessels at a low temperature (approximately $-150°C$) [11]. We showed that it was possible to calculate oxygen saturation levels from the two resonance Raman shifts at approximately 1355 cm^{-1} and 1378 cm^{-1}. We clearly visualized the different levels of hemoglobin oxygenicity in flowing erythrocytes in anesthetized mouse livers. The findings of morphofunctional analyses of the different hemoglobin oxygenicities raise the question of oxygen saturation in localized tissue areas *in vivo* at the time of freezing [11].

IX. Development of Cryobiopsy for Future Clinical Applications

A limitation of IVCT for living animal organs is that pieces of tissue samples cannot be obtained from an individual animal organ in a time-dependent manner. Therefore, we developed a novel "cryobiopsy" system [2, 6] that

allows for easy tissue sampling in a similar manner to clinical on-site human biopsies. We made a homemade forceps with sharp edges, which were cooled in liquid nitrogen (−196°C), and then used them to pinch off small pieces of tissue from anesthetized mouse livers [2]. Using the cryobiopsy system, we previously demonstrated that glycogen particles in hepatocytes were efficiently consumed under local anoxic conditions, and this was clearly observed as a zonal difference in the hepatic lobule [6]. We also maintained the normal hemodynamics of the living mouse liver using this cryobiopsy system, and time-dependently examined the *in vivo* liver tissues.

X. Pathological Application of IVCT and Cryobiopsy

A tumor tissue mass of cultured human lung cancer cells was produced in a transplant nude mouse in order to determine the future clinical applications of IVCT and cryobiopsy [1, 3]. Frozen specimens were freeze-substituted and routinely embedded in paraffin wax. They were cut into 4–5-μm-thick sections and routinely stained using hematoxylin and eosin. The artificial congestion of erythrocytes was frequently observed in the blood vessels of conventionally prepared specimens, and blood capillaries had completely collapsed. Furthermore, the shrinkage and transformation of tumor cells were also clearly detected in dehydrated tissues. In contrast, using cryobiopsy and IVCT, flowing erythrocytes and tumor cells with dyskaryosis were clearly observed in the blood vessels of tumor tissues [3]. Functional blood vessels, containing flowing erythrocytes had developed in some tissue areas in a manner that depended on local parts of the proliferating tumor mass. On the other hand, the expression of VEGF, which is known to participate in vascularization, was strongly detected in tissue areas with undeveloped blood vessels [3]. Therefore, IVCT and cryobiopsy are available for the freezing of transplanted tumor mass tissues in living animal models and applicable to human pathological analyses of cryobiopsied specimens.

XI. Development of a New Cryobiopsy Device for Humans

The morphological shrinkage and transformation of cells and tissues with ischemia and lack of oxygen are inevitable in the conventional preparation method. Therefore, specimen preparation for human pathological applications is needed using the new cryobiopsy system. As described above, technical artifacts are avoidable in IVCT-prepared and cryobiopsied specimens, and it is possible to directly fix various organs of the living animal body. Furthermore, recent advances have been made in optical technologies, and a confocal laser scanning endoscope has already been developed for clinical applications. Confocal laser imaging of human organ surface tissues at a depth of

approximately 250 μm is now possible in the field of clinical medicine. However, a large morphological gap exists between conventional histology and the latest "live tissue imaging". Therefore, we recently developed a new cryobiopsy device (prototypic type III) for the future application of the cryobiopsy system to human organs, and now hold its patent in Japan and abroad. The most important feature of this cryobiopsy device is the application of the human biopsy forceps typically used in common gastrofiberscopes. Furthermore, it consists of a sheath that cools the inner tube to approximately −190°C, and passes the biopsy forceps under the temperature of liquid nitrogen. As for the cooling system, liquid nitrogen from a liquid nitrogen storage tank is pressurized using an electric heater for its perfusion, and cools the inner tube to approximately −190°C. The cryobiopsy device is also equipped with a collection tank to recycle vaporized nitrogen gas in a closed system. When the new cryobiopsy device is applied for practical use in the clinical field, the functional pathology of living human organs will be compatible with live images analyzed by a confocal laser scanning endoscope.

XII. Conclusion

We clarified the immunohistochemical localization of soluble components and morphological characteristics of living mouse organs [5]. The local existence of functional molecules in living animal organs and their dynamic morphological changes may be consistent with those revealed by live imaging methods. The combination of IVCT or cryobiopsy with genetically manipulated probes will also be beneficial for examining the organ tissues of living animal bodies. The new cryobiopsy device currently being developed will enable *in vivo* pathological applications in the near future.

XIII. References

1. Bai, Y., Ohno, N., Terada, N., Saitoh, S., Nakazawa, T., Nakamura, N., Katoh, R. and Ohno, S. (2011) Differential distribution of blood-derived proteins in xenografted human adenocarcinoma tissues by in vivo cryotechnique and cryobiopsy. *Med. Mol. Morphol.* 44; 93–102.
2. 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.* 55; 113–122.
3. Ohno, N., Terada, N., Bai, Y., Saitoh, S., Nakazawa, T., Nakamura, N., Naito, I., Fujii, Y., Katoh, R. and Ohno, S. (2008) Application of cryobiopsy to morphological and immunohistochemical analyses of xenografted human lung cancer tissues and functional blood vessels. *Cancer* 113; 1068–1079.
4. 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.
5. Ohno, S., Terada, N., Ohno, N., Saitoh, S., Saitoh, Y. and Fujii, Y. (2010) Significance of 'in vivo cryotechnique' for morphofunctional analyses of living animal organs. *J. Electron Microsc.* 59; 395–408.

6. Saitoh, Y., Terada, N., Saitoh, S., Ohno, N., Fujii, Y. and Ohno, S. (2010) Histochemical approach of cryobiopsy for glycogen distribution in living mouse livers under fasting and local circulation loss conditions. *Histochem. Cell Biol.* 133; 229–239.

7. Saitoh, Y., Terada, N., Saitoh, S., Ohno, N., Jin, T. and Ohno, S. (2012) Histochemical analyses and quantum dot imaging of microvascular blood flow with pulmonary edema in living mouse lungs by "in vivo cryotechnique". *Histochem. Cell Biol.* 137; 137–151.

8. Saitoh, Y., Terada, N., Ohno, N., Hamano, A., Okumura, N., Jin, T., Saiki, I. and Ohno, S. (2014) Imaging of thrombosis and microcirculation in mouse lungs of initial melanoma metastasis with in vivo cryotechnique. *Microvasc. Res.* 91; 73–83.

9. Shi, L., Terada, N., Saitoh, Y., Saitoh, S. and Ohno, S. (2011) Immunohistochemical distribution of serum proteins in living mouse heart with in vivo cryotechnique. *Acta Histochem. Cytochem.* 44; 61–72.

10. Terada, N., Ohno, N., Saitoh, S., Fujii, Y. and Ohno, S. (2007) Raman microscopy of freeze-dried mouse eyeball-slice in conjunction with the "in vivo cryotechnique". *Microsc. Res. Tech.* 70; 634–639.

11. Terada, N., Ohno, N., Saitoh, S. and Ohno, S. (2008) Application of "in vivo cryotechnique" to detect erythrocyte oxygen saturation in frozen mouse tissues with confocal Raman cryomicroscopy. *J. Struc. Biol.* 163; 147–154.

12. Terada, N., Saitoh, Y., Saitoh, S., Ohno, N., Fujishita, K., Koizumi, S. and Ohno, S. (2012) Visualization of ATP with luciferin-luciferase reaction in mouse skeletal muscles using an "in vivo cryotechnique". *Microsc. Microanal.* 18; 1030–1036.