

## Review

# Application of *in vivo* cryotechnique to the examination of cells and tissues in living animal organs

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**Summary.** When all biological materials in cells and tissues of living animal organs are quickly and promptly frozen, immunolocalization of their components and structural features *in situ* is necessary to understand their *in vivo* functioning states. However, these direct morphological analyses were difficult to achieve by conventional chemical fixation methods during the last century. A new cryofixation method, named the “*in vivo* cryotechnique”, in which the normal blood circulation in living animals is always retained at the moment of freezing, has become a powerful tool to visualize the real native morphology of cells and tissues with functional meaning. The “*in vivo* cryotechnique” can usually be combined with a wide range of subsequent preparation techniques, and can thereby enable us to perform various direct analyses on biological samples, reflecting the physiological functions of living animal organs.

**Key words:** *In vivo* cryotechnique, Living animal organs, Immunolocalization, Cryofixation

### Introduction

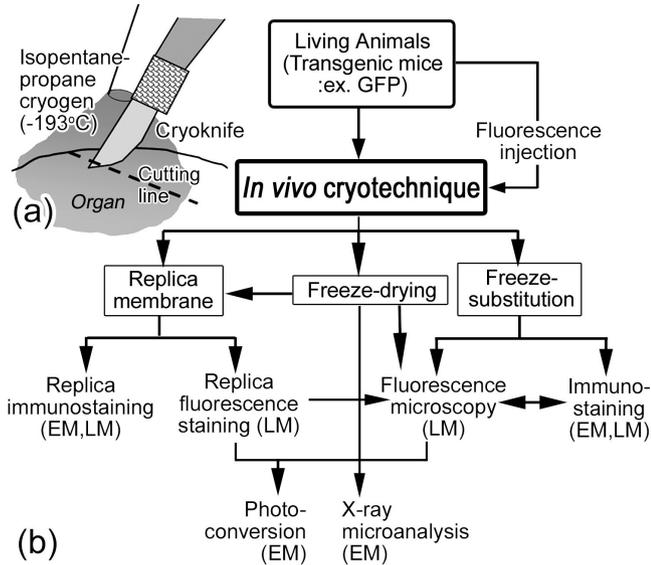
For the past decade, we have been attempting to make analyses on native immunolocalizations of biological target molecules in cells and tissues as well as simultaneous observation of their real morphology in living animals (Ohno et al., 1996a,b, 2004a,b; Terada and Ohno, 2004). To approach the demonstration of their functioning morphological states, we have already proposed that our “*in vivo* cryotechnique” is the most powerful tool for freezing the various organs of living animals *in vivo* (Fig. 1a). In this review, we briefly summarize our previous experiments which were performed with the “*in vivo* cryotechnique”, and propose further applications of this cryotechnique for different

microscopic observations, as shown in the flow-chart (Fig. 1b).

### All biological components frozen with the “*in vivo* cryotechnique”

The main purpose of “*in vivo* cryotechnique” is achieving the prompt freezing of all biological components (Ohno et al., 1996a,b) (Fig. 1). However, the times necessary for freezing at each depth from the frozen tissue surface differ, because the thermal conductance in cells and tissues is due to the continuous movement of thermal energy. Thus, only the surface tissue layer within a certain depth, such as 10 µm or 200 µm, is frozen sufficiently to prevent the formation of visible ice crystals at electron (Fig. 2b) or light (Fig. 2c) microscopic levels, respectively (Ohno et al., 1996a,b). The tissue areas at the same depth from the freezing surface can be quickly frozen at nearly the same time. Therefore, by cutting areas vertically to the freezing surface, we can achieve a similarly well-frozen morphology over a wide range. For example, we have already examined the erythrocytes flowing in the right ventricle of a living mouse heart (Fig. 3a). Previously, little was known about the state of the blood flowing inside the normally beating heart, because of the difficulty in obtaining such images with the conventional fixation method. By carefully checking the changing erythrocyte shapes and their distribution (Fig. 3b,c), a dynamic direction of flows in the blood circulation can be detected in the beating heart of a living mouse.

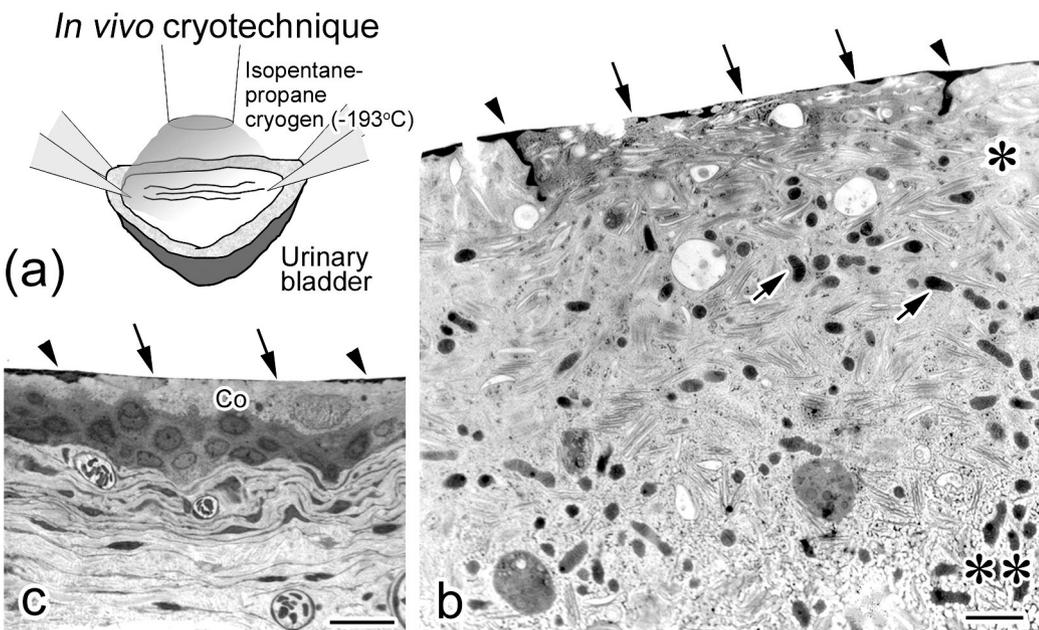
To obtain wide areas of well-frozen cells and tissues using common cryotechniques, considerable amounts of liquid cryogen are usually necessary for plunging resected fresh animal organs, which are otherwise contacted with copper blocks cooled in liquid nitrogen. With the metal contact method, some tissues are often compressed onto the metal due to the crash impact (Harreveld and Trubatch, 1975), even though a spacer and cushion are inserted between them. On the contrary, with liquid cryogen, such as an isopentane-propane mixture cooled in liquid nitrogen, the frozen tissues



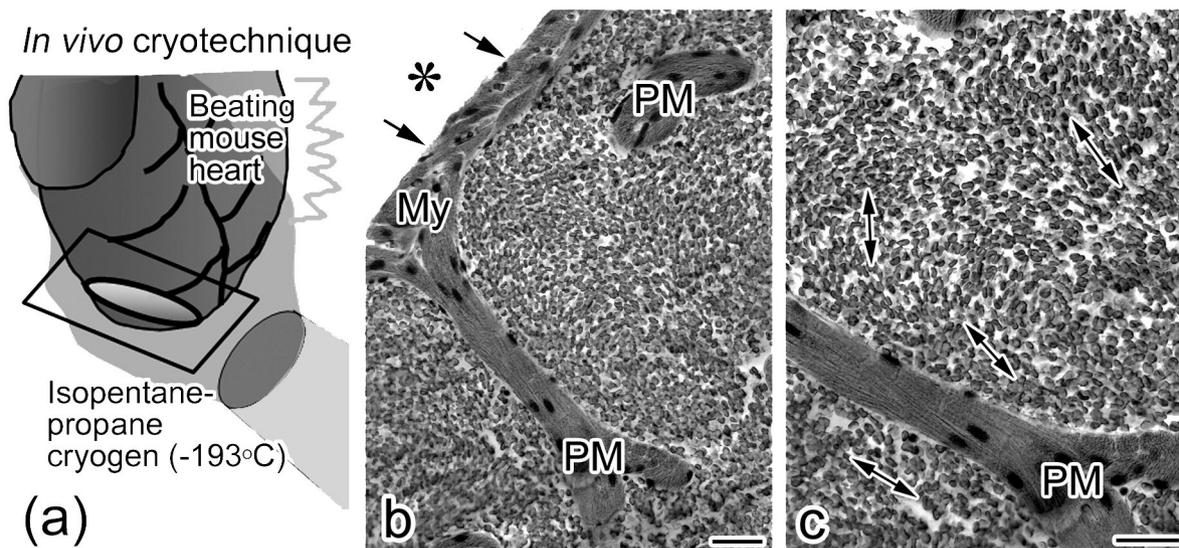
**Fig. 1. a.** Schematic representation of the "in vivo cryotechnique". A living animal organ is quickly cut with a cryoknife precooled in liquid nitrogen without disturbing the blood circulation, and immediately liquid isopentane-propane cryogen (-193°C) is poured onto it. **b.** Flow diagram of the various subsequent preparation steps following the "in vivo cryotechnique" for living animals.

retain their original morphological states without compression by plunging them into the cryogen or pouring it over their surface layer (Jehl et al., 1981; Cole et al., 1990; Ohno et al., 1996a,b). This idea for the usage of liquid cryogen was also reconfirmed in the model system by jetting human blood samples into the liquid cryogen, referred to as the "in vitro cryotechnique for erythrocytes", to examine dynamically changing erythrocyte shapes at different blood flowing speeds (Terada et al., 1998).

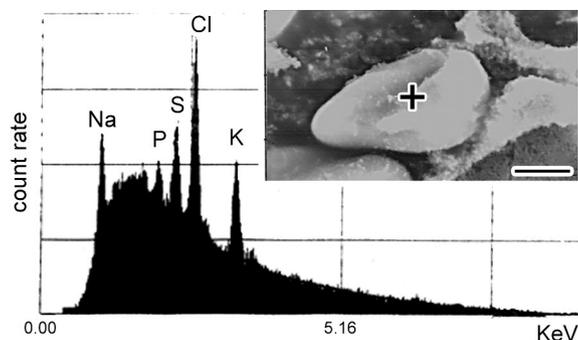
To examine deeper areas from the frozen tissue surface, we need to cryocut the organs of living animals *in vivo* under anesthesia. The original idea has been attempted; when the cryoknife itself was precooled in liquid nitrogen before cryocutting the living animal organs (Fig. 1a), the exposed tissue surface in direct contact with the cryoknife was quickly frozen in the same way as with the metal contact method (Ohno et al., 1996a,b). Surface damage to the cryocut tissue is rarely detected when the living animal organs are able to be passed quickly with the cryoknife. Then, the widely cryocut and frozen areas have another liquid cryogen of an isopentane-propane mixture (-193°C) simultaneously poured over them. In this process, the physical speeds of the moving cryoknife and freezing intensity have to be considered to decide the necessary time to complete the *in vivo* freezing of living animal organs. Practically, at an electron microscopic level, the well-frozen areas appear to occupy a very narrow band, less than 10 μm wide in



electron microscopic level, highly-developed membranous structures called discoidal and fusiform vesicles, and also cell organelles, such as mitochondria, were observed in the cytoplasm. Note that the true cell surface can be traced with extracellular electron-dense components (arrowheads in (b)). Near the frozen tissue surface (asterisk in (b)), the intracellular structures are well preserved without large ice-crystals, as compared with those in deeper cytoplasmic areas (double asterisks in (b)). Bars: (b) 1 μm, (c) 50 μm.



**Fig. 3.** **a.** Schematic representation of the method for preparing the samples of beating mouse heart using the “*in vivo* cryotechnique”. Specimens embedded in paraffin are sectioned vertically to the frozen tissue surface (parallelogram in **(a)**). **b.** The myocardium (My), papillary muscles (PM) and flowing erythrocytes of the right ventricle are well frozen, due to a thin epicardium (arrows in **(b)**) at the light microscopic level. Asterisk: outside of the heart. **c.** At a higher magnification, several flow directions of erythrocytes according to the blood circulation were detected with this cryotechnique (bidirectional arrowheads). Bars: 50  $\mu\text{m}$ .



**Fig. 4.** Spectrum of X-ray microanalysis of a flowing human erythrocyte (cross mark in inset), as prepared with the “*in vitro* cryotechnique for erythrocytes” followed by freeze-drying. Some elements, such as sodium (Na), phosphorus (P), sulfur (S), chloride (Cl) and potassium (K), are analyzed in dynamically flowing erythrocytes at ejection pressures (100 mmHg in this case). Bars: 2  $\mu\text{m}$ .

cells and tissues (Fig. 2b). Moreover, the heat conductivity in cells and tissues gradually changes, depending on the concentrations of biological materials, including electrolytes and proteins, because some well-frozen areas are next to the damaged ones with larger ice crystals even at the same distance from the frozen tissue surface. Although careful attention has to be paid to the performance of cryotechniques, the “*in vivo* cryotechnique” is the only way to examine the native functional morphology of living animal organs and make various analyses of the dynamic biological components

in the cells and tissues with a high time-resolution.

#### Direct analyses of the frozen biological components

All biological components, including proteins, lipids, carbohydrates, electrolytes and gases, etc., of living animal organs are instantly trapped in ice *in situ* with the “*in vivo* cryotechnique”. To analyze the electrolytes in cells and tissues, they can be kept using the common freeze-drying (FD) method, resulting in dehydrated electrolyte deposits (Takayama et al., 1994). In addition, the X-ray microanalysis of elements in freeze-dried erythrocytes (inset in Fig. 4), as observed with scanning electron microscopy (SEM) after the cryofixation of the “*in vitro* cryotechnique for erythrocytes” (Terada et al., 1998), is shown in the spectrum with the different peaks of several atomic elements (Fig. 4). Thus, the flowing erythrocyte morphology and their analyses of certain electrolyte elements can be obtained simultaneously with the FD method.

However, some gases, such as oxygen or carbon dioxide, must be released from the erythrocytes during the FD preparation step. In this case, only a cryo-stage equipped inside an electron microscope can retain such gases in the erythrocytes to be analyzed. In addition, other proteins containing gas elements are also cryofixed in the cells and tissues at the same time, which may retain their molecular structures even after the freeze-substitution fixation. Therefore, as described in the next paragraph for a future study, the contents of gases can be analyzed by the examination of the protein conformation

instead of the gases themselves, such as immunostaining for their specific molecular structure.

### Merits of freeze-substitution after the “*in vivo* cryotechnique”

The freeze-substitution (FS) method is used to fix cells and tissues at low temperatures by substituting ice crystals with organic solvents, such as acetone and alcohol, in which the biological components are supposed to be immovable, as compared with conventional chemical fixation and alcohol dehydration (Leng et al., 1998). This FS method can be combined with subsequent embedding steps in epoxy resin, usually used for ultrathin sectioning of transmission electron microscopy (TEM), or transferring to organic solvents, such as *t*-butyl alcohol, for FD of SEM. We have already

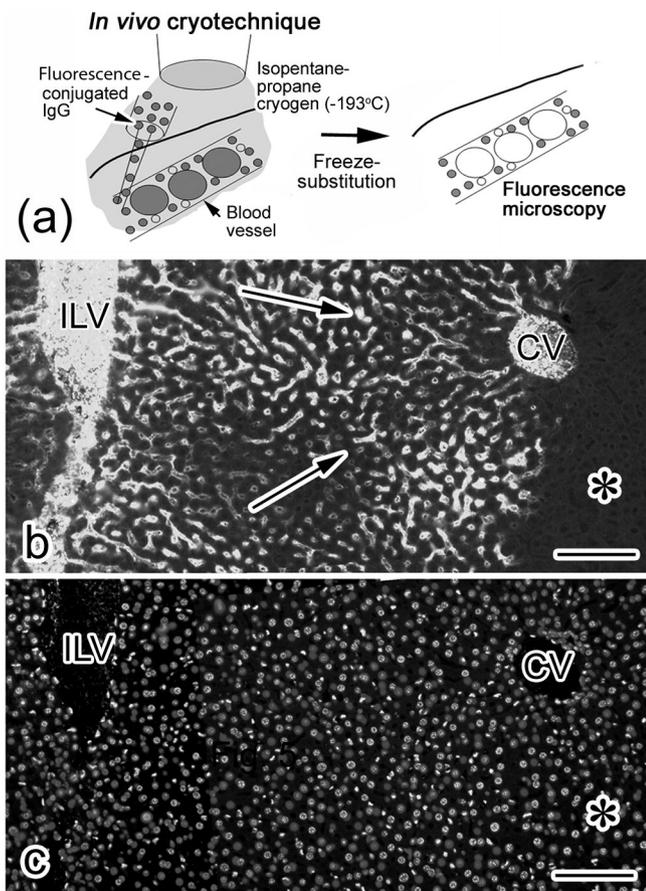
reported on the dynamic morphological changes in mouse organs, such as the lungs during respiration (Takayama et al., 2000) and the erythrocytes flowing in various vessels (Terada et al., 1998; Yu et al., 1998; Xue et al., 1998, 2001). As reported in previous papers, well-frozen areas without obvious ice crystals were obtained to examine the dynamically changing morphology of *in vivo* organs at an electron microscopic level.

As the molecular structures of the components to be examined are not markedly changed during the preparation process of FS, several specimen treatments can be applied before and after the “*in vivo* cryotechnique”. An example of such a treatment before the “*in vivo* cryotechnique” is to label living animal organs under anesthesia with some fluorescent probes. The fluorescent probes retain their excitation and emission wavelengths even after the FS, which enables us to visualize their localization in the living animal organs at the moment when the “*in vivo* cryotechnique” is performed (Fig. 5) (Terada et al., 2005). A fluorescence-conjugated immunoglobulin (IgG) was directly injected into the blood vessels in a living mouse liver (Fig. 5a), showing its time-dependent distribution, which reflected the blood flow in the living state (Fig. 5b).

As for the other treatments following the FS procedure, the common immunohistochemical step can be used to demonstrate the distribution of components in the cells and tissues as per usual. In a simple way with the “*in vivo* cryotechnique”, we have already obtained light microscopic images of leaking serum proteins, albumin and IgG, through the changeable blood-brain barriers under the ischemic condition (Zea-Aragon et al., 2004a). This procedure was also applied to examination on the reabsorption of the serum proteins which passed through glomerular capillary loops in acute hypertensive mouse kidneys (Li et al., 2005).

Moreover, it is well known that most functioning molecules change their molecular conformation during various signaling cascade processes in dynamically active cells, because of their interactions with modified biological molecules, including phosphorylation (Fig. 6a). If the molecular structure of the functioning molecule is retained *in situ* during the FS step, it is possible to analyze any component with the immunostaining technique for the original distribution of the biological materials, when the “*in vivo* cryotechnique” is performed (Fig. 6a). Using immunohistochemistry, we have already reported such an idea for the phosphorylated form of a signal molecule, phosphorylated cAMP-responsive element binding protein (pCREB), in the nuclei of granule cells of living mouse cerebellar tissues (Fig. 6b) (Ohno et al., 2005). Therefore, the “*in vivo* cryotechnique” enables us to examine such dynamic changes of molecular structures with a higher time-resolution, with various kinds of monoclonal and polyclonal antibodies against specific molecular structures of functioning proteins.

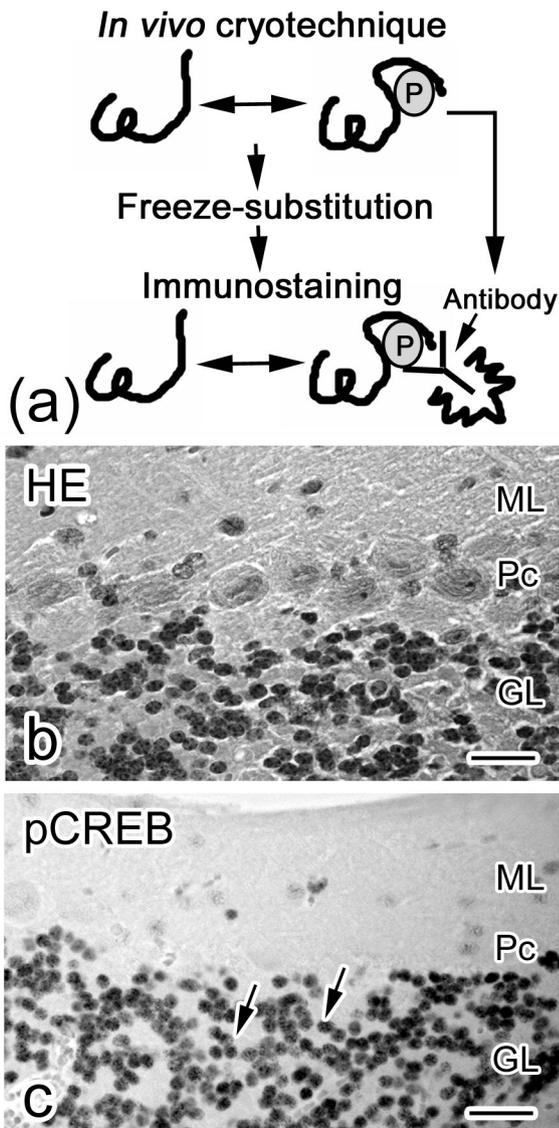
It is also possible to perform the FS or FD method to



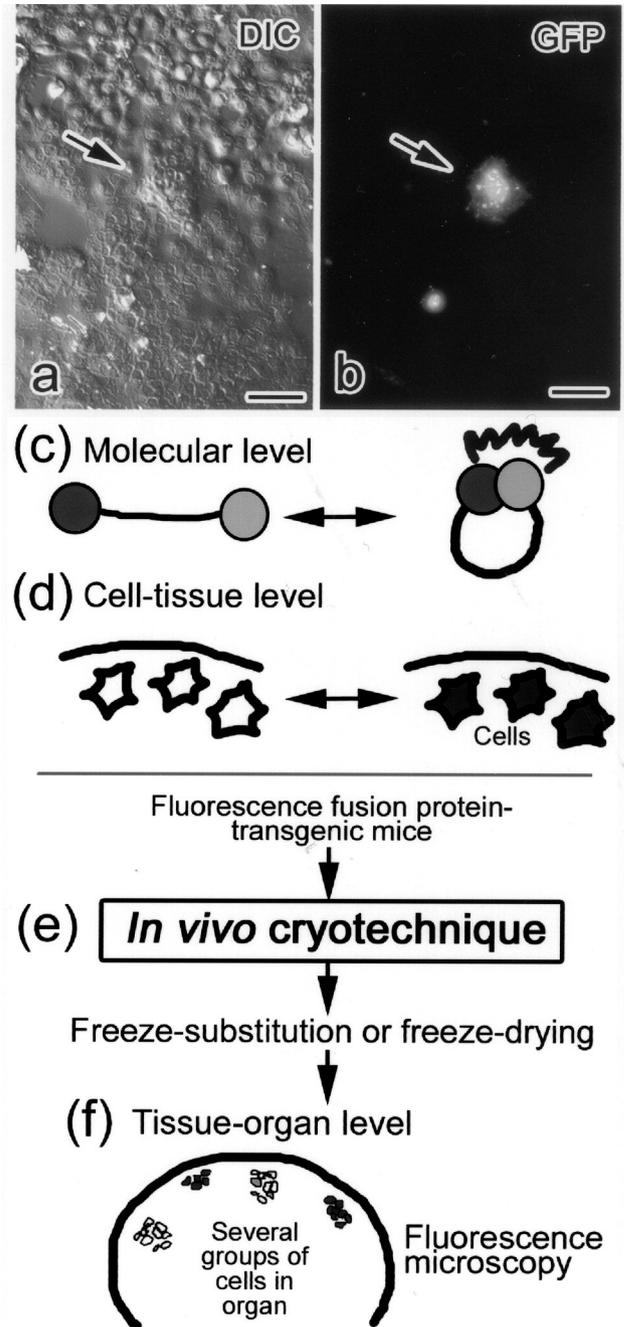
**Fig. 5** a. Schematic representation of the “*in vivo* cryotechnique” for the living mouse liver a few seconds after the injection of fluorescence-conjugated IgG, followed by freeze-substitution. b. Under fluorescence microscopy, fluorescent signals were clearly observed in the sinusoidal cavities, central veins (CV) and interlobular vessels (ILV). Note that the entirety of the hepatic lobules (asterisks in (b, c)) is not fluorescence-labeled, because of the functional blood flow (arrows in (b)) as a portal lobule from ILV to CV. Bar: 200  $\mu$ m.

Morphofunction by *in vivo* cryotechnique

detect a fluorescent probe signal, such as green fluorescent protein (GFP), from jellyfish (Fig. 7b), which is usually fused with many host proteins to make fluorescent chimeras from cells to animal bodies *in situ* (Chalfie et al., 1994). Therefore, the dynamic movement of synthesized chimera proteins with GFP in target cells can be directly observed in the tissue specimens, as prepared with the “*in vivo* cryotechnique” (Fig. 7c-e). This experiment indicates another merit of directly



**Fig. 6.** a. Schematic representation of immunohistochemistry for examining the molecular changes, such as phosphorylation, using the “*in vivo* cryotechnique” followed by freeze-substitution. b, c. In the living mouse cerebellum on thin sections as prepared with hematoxylin and eosine (HE)-staining, the nuclei of granule cells (arrows in (c)) were clearly immunostained with a specific antibody against phosphorylated cAMP-responsive element binding protein (pCREB). ML: molecular layer, Pc: Purkinje cell layer, GL: granular layer. Bars: 20  $\mu$ m.



**Fig. 7.** a, b. In cultured COS cells transfected with GFP-fused caveolin gene (a; DIC image), fluorescence wavelengths are retained after quick-freezing and freeze-drying (arrows in (b)). c. This idea can also be applied to visualizing the intramolecular changes with different fluorescence colors, which is known as the fluorescence resonance energy transfer (FRET) technology. d. At another cell-tissue level, such fluorescence color changes are observed in the target cells. e, f. A combination of the “*in vivo* cryotechnique” and the freeze-substitution or freeze-drying method (e), some groups of labeled cells showing different fluorescence colors can be directly analyzed in living animal organs (f). Bars: 20  $\mu$ m.

labeling proteins with fluorescent probes, in the case of combination with the “*in vivo* cryotechnique”. It may also enable us to visualize the intermolecular relationship of each protein and its intramolecular changes by using various fluorescent probe colors, including fluorescence resonance energy transfer (FRET) technology (Fig. 7c). As described for GFP, other fluorescent biological proteins probably retain their own fluorescent colors after both FS and FD procedures, similar to the fluorescence activity of FRET itself (Fig. 7c,d). Therefore, it is important that rapid molecular events such as signal activation can be visualized in the specimens prepared with the “*in vivo* cryotechnique” (Fig. 7e). Other transgenic mice inserted with several kinds of genes would be more useful for morphological analyses of the intracellular distribution of labeled components with the “*in vivo* cryotechnique”, because of the production of different fluorescent signals (Fig. 7f).

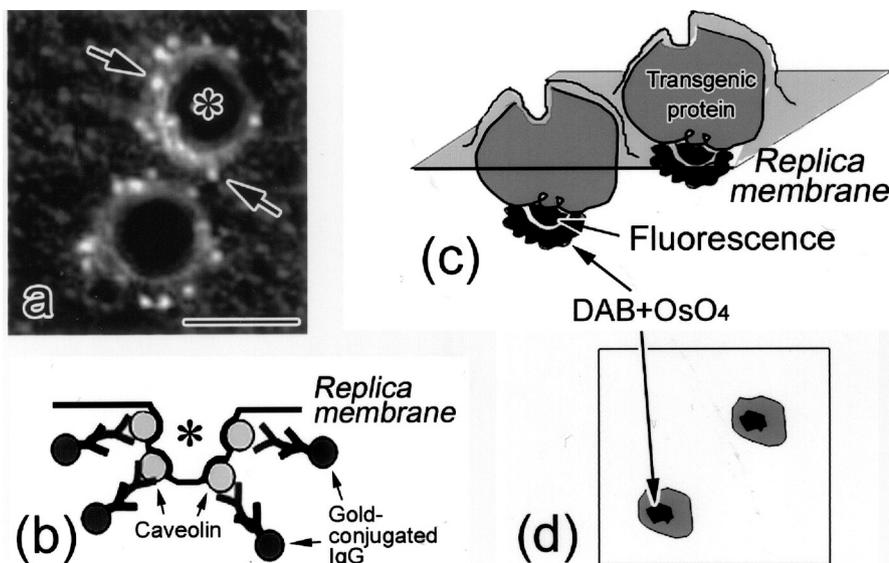
Concerning the recent advancement of fluorescence technology, a specific gene sequence (Cys-Cys-Xaa-Xaa-Cys-Cys; Xaa is a non-cystein amino acid), which is very much lower (585 Da) in molecular weight than the GFP, was demonstrated to form a complex molecule with certain cell-permeable fluorescent probes, termed fluorescein arsenical helix binder (FAsH) (Griffin et al., 2000; Adams et al., 2002). If the target protein inserted with the exact sequence retains its original characteristic of interacting with the permeable fluorescent probe after FS, the “*in vivo* cryotechnique” would be very useful for future studies of living transgenic animals. To improve the labeling technique at the electron microscopic level, certain fluorescent probes are able to form a diaminobenzidine (DAB) complex via a photo-conversion mechanism (Bentivoglio and Su, 1990), enabling us to observe its precise localization in cells and tissues (Gaietta et al., 2002). This labeling technique

will be another way to detect specific molecules using both light and electron microscopy.

### Immunohistochemistry of the *in vivo* frozen components

Even using the FS method, a slight amount of extraction or movement of the lipid-soluble components from their original site in cells and tissues is probably inevitable, due to the transferring of the frozen tissues into organic solvents, although they are instantaneously immobilized into ice crystals by freezing. It is critical to preserve all components in the cells and tissues at the frozen tissue surface *in situ*, when cryofixation is performed, to analyze their original localizations. One simple way to attach the frozen tissue sections to highly dense  $\text{NH}_3^+$ -coated glass slides was applicable for a certain lipid component, phosphatidylcholine, in the rat mandibular joint cavity (Zea-Aragon et al., 2005). In the previous study, whole resected temporomandibular joints with their disks attached were immediately plunged into an isopentane-propane cryogen ( $-193^\circ\text{C}$ ) and routinely embedded in OCT compound. Several cryostat sections were cut and mounted on the  $\text{NH}_3^+$ -coated glass slides, and then fixed with paraformaldehyde fixative. They were immunostained with a specific anti-mouse phosphatidylcholine antibody (Zea-Aragon et al., 2005). Positive immunostaining was achieved on the upper surface layers of the articular cartilage and also in the joint cavity, which easily gets lost during conventional preparation steps (Zea-Aragon et al., 2005).

With replica immunoelectron microscopy (Takayama et al., 1999; Zea-Aragon et al., 2004b), replica membranes in combination with the cryotechniques retain the original immunolocalization of the biological components at a molecular level. We have



**Fig. 8.** a. Replica immunoelectron micrograph of the caveolin around the caveolae (asterisk) in smooth muscle cells of the living mouse duodenum, visualized using the secondary immunogold method (arrows). b. Schematic representation of the antigen-antibody binding steps with the replica immunostaining method. c. More precise molecular analysis may be possible by using photo-conversion of the fluorescent signal attached to specific transgenic proteins on replica membranes, to make electron-dense DAB-reactive osmium deposits. d. Upper-side overview of the replica membrane, as shown in (c). Bar: 100 nm.

already reported the three-dimensional ultrastructures of various cells and tissues on the replica membranes, prepared after applying the quick-freezing and deep-etching method (Ohno et al., 1996a,b; Terada et al., 1997; Kubo et al., 1998; Matsuda et al., 1998; Yoshida et al., 2004). While replicating the freeze-fractured cells and tissues with platinum metal and carbon in the freeze-fracture apparatus, the prepared replica membranes are supposed to hold the etched molecules probably by tightly sealing them *in situ*. The concept behind this mechanism is well recognized by the observation of the prepared replica membranes containing immunogold particles in the immunostained specimens before quick-freezing (Ohno et al., 1993; Terada et al., 1997). On the other hand, after dissolving the biological components not attached to the replica membranes, another replica immunostaining procedure enabled us to visualize three-dimensionally the target molecules in cells and tissues on the replica membranes (Fujimoto, 1995). We have already reported the *in vivo* immunolocalization of a membranous protein, caveolin, in smooth muscle cells of the living mouse intestine, with a combination of the “*in vivo* cryotechnique” with immunostaining on the replica membranes (Fig. 8a,b) (Takayama et al., 1999). Moreover, replica immunoelectron microscopy was applied to the examination of the extracellular localizations of soluble molecules, such as hyaluronic acid (a kind of proteoglycan), in the rat mandibular condylar cartilage at the electron microscopic level (Zea-Aragon et al., 2004b). Therefore, the combination of our “*in vivo* cryotechnique” with the replica immunostaining enables us to examine the most native intra- or extracellular distribution of biological molecules in cells and tissues of living animals.

As described in the previous section, a small specific gene sequence is enough to detect the intracellular localization of the translated target protein to be inserted with certain fluorescent probes. Furthermore, by using another photo-conversion protocol to make the DAB a reactive complex after attaching fluorescent probes, it may be applicable for a future ultrastructural study using electron microscopy (Fig. 8c,d). How clearly such transgenic proteins can be visualized as osmium deposits on the replica membrane is an interesting question to identify in the *in situ* molecules with their original ultrastructures. By changing the ratio of the evaporation intensity with platinum and carbon, and following the treatment of DAB reaction deposits with osmium tetroxide to increase their electron density (Fig. 8d), it may be possible to visualize directly the localization and molecular structure of transgenic proteins on the replica membranes (Fig. 8c). Moreover, for transgenic mice, in which some proteins are artificially modified to interact with fluorescent probes, a series of preparation protocols, such as the “*in vivo* cryotechnique” - freeze-fracturing - replica membrane preparation - reaction with fluorescent probes, also applicable in living cells due to its membrane permeability, - photo-conversion with DAB - osmium tetroxide treatment, may enable us to

visualize the native protein localization *in vivo* in living animal organs in the near future.

### Concluding remarks

The “*in vivo* cryotechnique” can be combined with various light and electron microscopic procedures, which is an essential first step to maintain the normal blood circulation of various organs in living animals. It is also possible to visualize the native structures for morphological analyses and examination of the biological components *in vivo*. Although some of the preparation procedures in this article are hypothetical and are now being tried, the future application of the “*in vivo* cryotechnique” in broad biological fields will enable us to approach the simultaneous analysis of the native morphology of functioning cells and the intracellular localization of their components. The time will soon come when we succeed in bringing about a “morphology renaissance” with the “*in vivo* cryotechnique” during the 21st century.

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