THE USE OF ULTRARAPID FREEZING AND FREEZE SUBSTITUTION TO VERIFY VITRIFICATION AND/OR ICE FORMATION IN VASCULAR TISSUE

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The expanding field of cryobiology, in particular that of the study of vitrification for long term storage of tissues for transplantation, has demonstrated that ice is damaging to smooth muscle tissue.[1,2] Consequently conventional methods such as ultrarapid freezing and freeze substitution are becoming routine protocols to determine the quality of cryopreservation. This article introduces the scientific community to the PS-1000 cryofixation unit (Delaware Diamond Knives, Wilmington, DE) which provides both ultrarapid freezing and a means of validation of freeze substitution methods. When any tissue is frozen or vitrified for clinical use it is imperative to know the structural and functional integrity of these tissues. Ice formation within the extracellular matrix and dehydration of multi-cellular tissues, using conventional cryopreservation, is the principal reason why these methods frequently prove to be ineffective. Regardless of the method used to cryopreserve tissue ice will be formed during the cooling process; therefore, the avoidance of ice during cooling (vitrification) is the most desirable way, albeit the most challenging, to cryopreserve any biological system. To convert water from a liquid to a solid state involves a complex series of events involving cooling rate, presence of solutes and pressure. In multi-cellular biological systems the physiochemical changes are poorly understood after many years of research. To better understand the principles of freezing and thawing one should refer to the papers of Dubochet[3] and Franks.[4]

Ultrarapid Freezing: Electron microscopists have employed various methods of ultrarapid freezing (plunge, jet, spray, high-pressure and metal-mirror). Questions involving immunocytochemistry and x-ray microanalysis often require the use of one or more of these methods. Metal mirror fixation (slamming) is the preferred method of cryofixation for most subsequent protocols, especially if vitrification is to be achieved of relatively large samples. When using this method, the use of ultra-pure copper is the metal of choice for achieving maximum heat transfer along with rapid cooling rates. It is also the preferred, and probably the only, method to check the accuracy of freeze substitution protocols since both vitrified and frozen tissue will be present in the cryopreserved sample. The chilled copper block, devoid of scratches or imperfections, ensures maximal preservation (vitrified or near vitrification) of tissue to a depth of at least 15 µm, which can serve as a control, for both fixation and the substitution process. For most applications requiring ultrarapid freezing the PS-1000 portable fixation unit shown in Figure 1 can be used. Figure one is a representation of how to achieve *in situ* fixation of cardiac tissue in an animal model and may be applied to most tissues. Other *in vitro* preparations such as biopsies (both in and out of the surgical suite) and tissue cultures can be fixed using this cryofixation unit.

Freeze Substitution: The history of the use of freeze substitution for light microscopy dates back to the 1940's and 50's.[5] Cryobiologists often refer to freeze substitution as cryosubstitution. Freeze substitution is used in protocols in which chemical fixation may be detrimental such as immunocytochemistry and x-ray microanalysis. Freeze substitution has been extensively used in our laboratory to determine the presence and location of ice and its possible impact on cellular structure in both vitrified and slowly frozen vascular and cardiac tissue.[6,7] Many laboratories have used methods of substitution, which incorporated acetone as the ice dissolution solvent. In our studies we have shown that methanol achieves the 'solvent/water' ratio needed for substitution better than that of acetone; this is especially true when working with large hydrated samples. The method used in our laboratory is as follows: (A) frozen or vitrified samples are placed in vials containing approximately 20 ml of substitution media (methanol/osmium) which had been frozen in liquid nitrogen or pre-cooled substitution media (-90° C) was transferred to a vial containing the frozen or vitrified tissue, (B) the vial is placed in an aluminum heat sink at 90° C which assures a constant temperature of these vials, (C) the substitution media is changed over a period of five days followed by gradually increasing the temperature to -20° C and held for twenty-four hours. The samples were then warmed to 4° C for two hours and finally room temperature and (E) the tissue is infiltrated with epoxy resin and sectioned for light and/or electron microscopy. This method is designed to provide a true representation of ice formation or lack of within these cryopreserved tissues.

Control Tissue (Metal-Mirror Fixation): Figures 2 and 3 illustrate vascular tissue which had been cut open to expose the luminal surface and rapidly frozen using the PS-1000 cryo fixation unit. Tissues were subsequently freeze substituted via the standard protocol described above. The image in figure 2 shows the lumen (L) and the three layers of the jugular vein. The tunica intima

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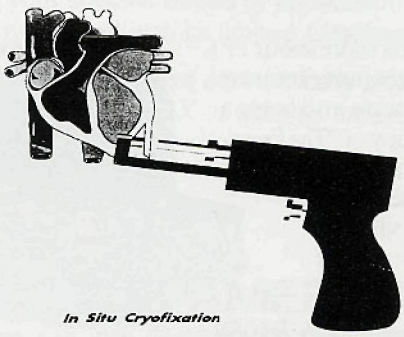

Figure 1: Metal Mirror cryofixation unit (PS-1000) which is used for both *in vivo* and *in vitro* fixation

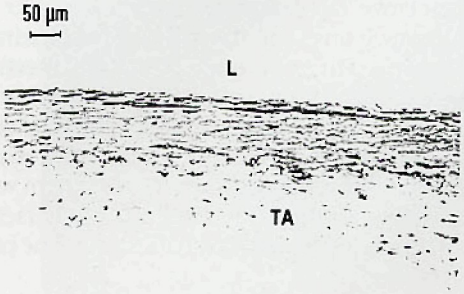

Figure 2: Control jugular vein, which has been rapidly frozen using the PS-1000, and subsequently freeze substituted.

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(endothelial cells), media (smooth muscle) and approximately one-half of the tunica adventitia (TA) appear to be well preserved; the lower portion of the TA contains large ice artifacts. Light microscopy reveals the intact blood vessel, which clearly defines the depth of freeze, which is approximately 20-30 µm. At higher magnifaction (figure 3) the cellular structure of the endothelia (arrow), smooth muscle and fibroblasts are clearly discernible without obvious ice crystal damage. By using fresh tissue, which has been rapidly frozen, one is able to judge the quality of the freeze substitution process and to better understand the tissue architecture during processing. Much of the histology in our studies is focused at a light microscopic level such that smaller ice crystal domains (nanometer scale), if present will not be discernible. Most of our work consists of using cryoprotectants for vitrification and/or slow controlled rate freezing. The process of cryoprotecting these tissues involves a gradual infiltration of the tissue with a solution containing a high concentration of cryoprotectants followed by rapid cooling (vitrification) to -135 °C or using a standard protocol of controlled slow cooling (1°C/min) to -80°C, followed by storage in the vapor phase of liquid nitrogen.

Controlled Cooling and Vitrification of Jugular Veins: Figure 4 shows a section of jugular vein, which has been gradually infiltrated with a cryoprotectant and subsequently control-rate frozen at 1°C/min as described previously. The tissue was freeze substituted in methanol/osmium substitution solution.

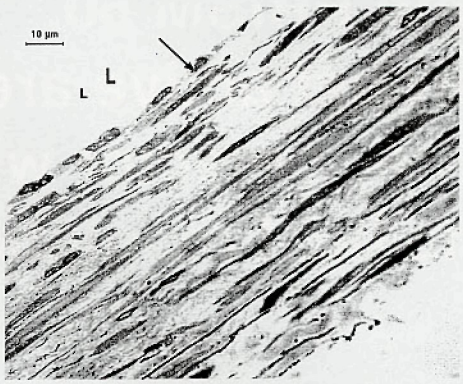

Figure 3: High magnification of freeze substituted control tissue that has been ultrarapidly frozen and clearly shows the well-preserved architecture of this tissue.

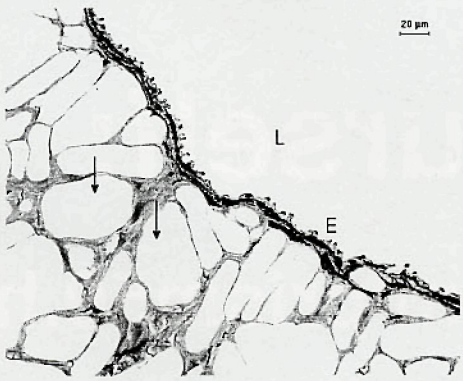

Figure 4: Jugular vein, which has been cryoprotected, control rate frozen and freeze substituted. Large ice domains are seen within the tunica adventitia.

The lumen (L) is shown along with the tunica intima, which shows a ridge of endothelial cells (E). The tunica media, directly beneath the tunica intima is rich in smooth muscle cells. The ice domains (arrows) within the tunica adventitia have expanded this region and these domains are abutting the external elastic membrane producing a 'compaction' of the t. media. Figure 5 shows the structure of a slowly frozen jugular vein followed by a rewarming and chemical fixation. The lumen (L) is seen with protrusion of rounded endothelial cells. The t. media (TM) and t. adventitia (TA) appear to have retained a relatively normal appearance. When vascular tissues were vitrified and subsequently freeze substituted there were no obvious ice domains or signs of ice damage as illustrated in figure 6. The lumen (L) can be seen in this cross sectional slice of tissue, the smooth muscle cells (M) appear to have contracted during handling of tissue and the TA appears to have a normal appearance with loosely arranged connective tissue. Figure 7 shows the structure of jugular vein, which had been vitrified and rewarmed. The lumen (L) and tunica intima, with rounded endothelia, can be seen along with smooth muscle cells (M) and TA. The vitrification process caused limited structural changes within this tissue. Figure 8 is an electron micrograph (original magnification x3,000) of vitrified and freeze substituted jugular vein. The lumen (L) is shown with a flattened tunica intima. The smooth muscle cells appear to be somewhat shrunken, with frequent vacuolization. Small vacuoles (<1 µm) are present and there is a possibility that they in-fact may be small ice domains.

Controlled Slow-Cooling and Vitrification of Cardiac Tissue (Heart Valve Leaflet): Figure 9 shows the structure of a freeze-substituted heart valve leaflet, which had been cryoprotected, and frozen slowly (1°C/min) using a conventional method used widely

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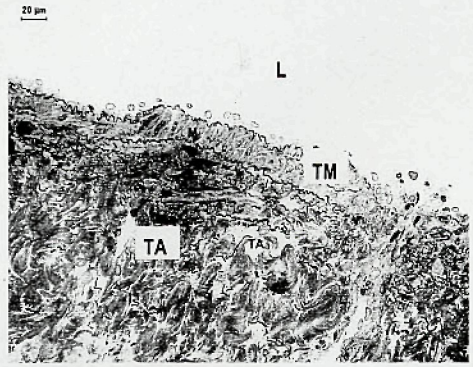


Figure 5: Cryoprotected and control rate frozen jugular vein, which was rewarmed and chemically fixed. Rounded endothelial cells are protruding into the lumen.

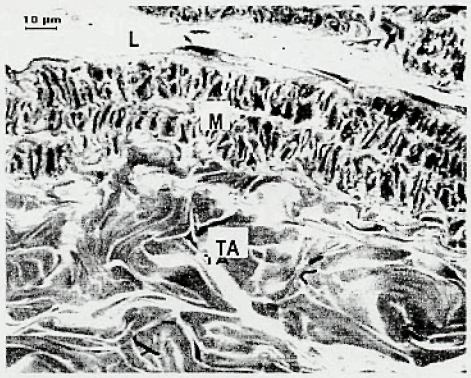
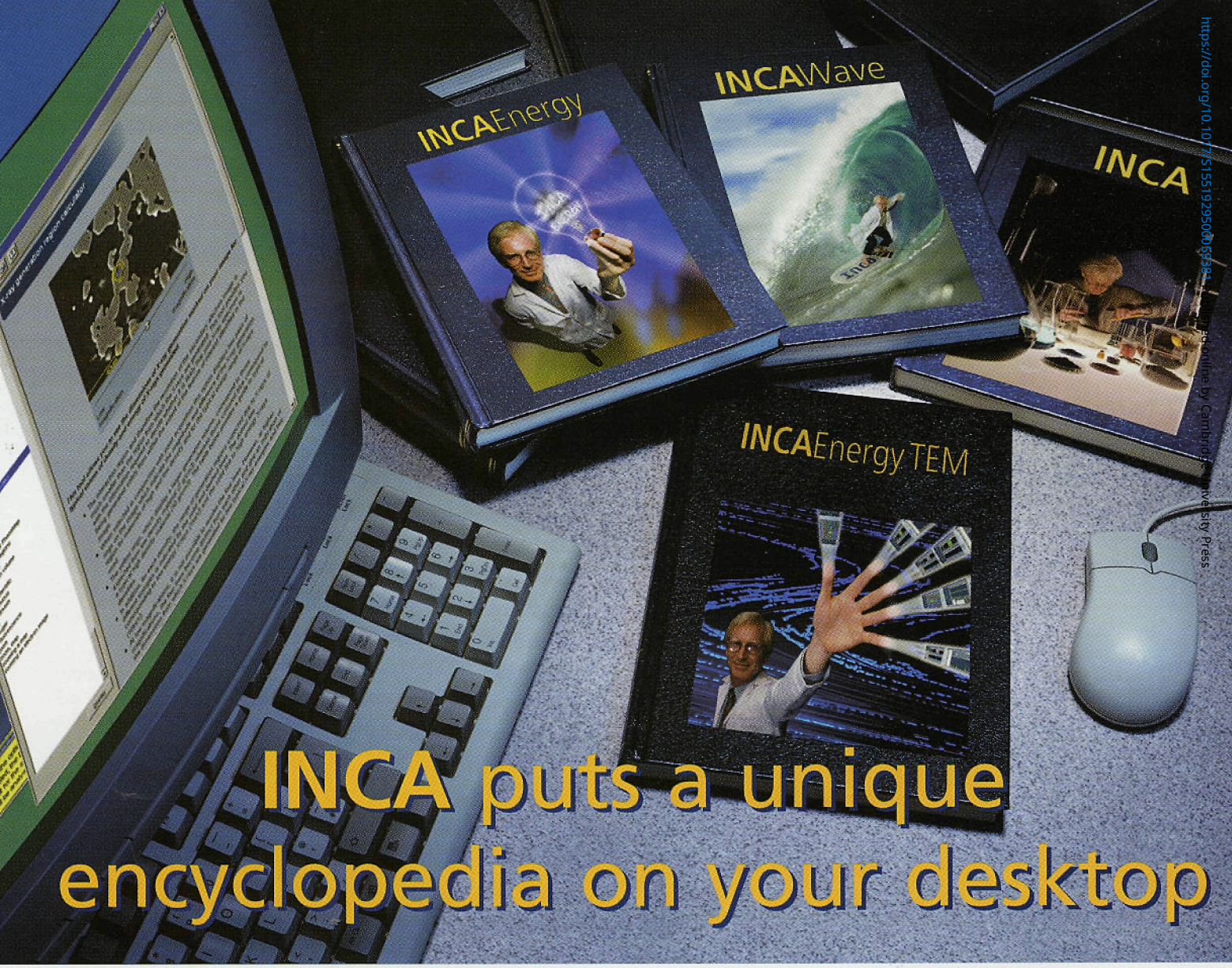


Figure 6: Jugular vein that has been vitrified and freeze substituted and exhibits no obvious ice damage or ice domains.



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in clinical practice. All heart valves frozen using conventional cryopreservation consistently demonstrated extracellular ice formation. It can be seen clearly in Fig 9 that ice domains were present within all three regions, fibrosa (F), spongiosa (S), and ventricularis (V) as well as the hinge region of the valve leaflet. The most striking observation was that the ventricularis consistently exhibited smaller ice crystals than that of the other two regions. In marked contrast, Figure 10 shows the structure a vitrified heart valve leaflet after cryo- substitution and revealed no ice formation at the light microscopic level, but occasional cytoplasmic vacuoles were observed. The fibrosa (F) and spongiosa (S) appear intact along with a clearly defined endothelial layer (arrow).

Summary:

Crystal size in relation to cooling rates must be considered in all cryopreserved. When working with 'bulk' samples the cooling rate throughout the whole object may be inadequate to produce uniformly small crystals without the use of cryoprotectants. One must ask himself what it is he or she is trying to achieve by freezing the specimen prior to the use of cryoprotectants or metal mirror fixation; is it a structural study, analytical study, viability study or a combination? It is important to remember that 'true' vitrification may not be possible, as yet, via many of these procedures. However, small microcrystalline structures may be of little or no consequence if they are below the resolution of the analytical tools being employed. When any method of cryo fixation is used, it is important to include a control that has both vitrified and frozen tissue within the sample. This can be achieved by using metal-mirror fixation with a system such as the PS-1000 and will act as both a structural and functional control for the substitution process. ■

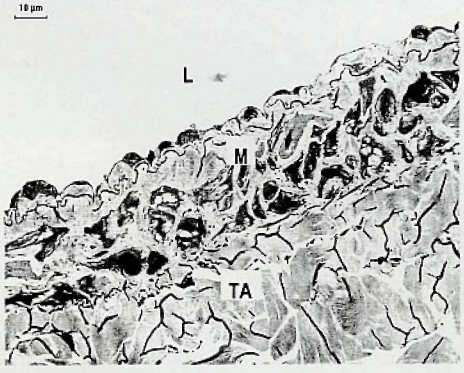

Figure 7: Jugular vein that had been vitrified and rewarmed. The structural architecture is similar to that of control tissue.

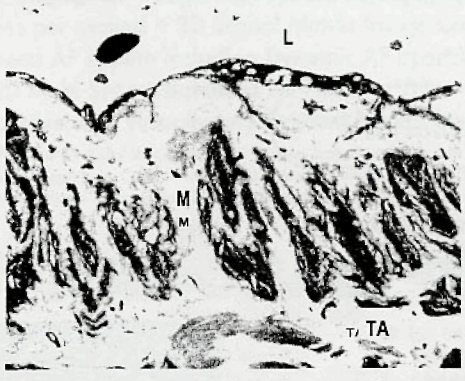

Figure 8: Electron micrograph of vitrified and freeze substituted jugular vein. The endothelial and smooth muscle cells appear shrunken. Vacuolization (<1 µm) may be small ice domains.

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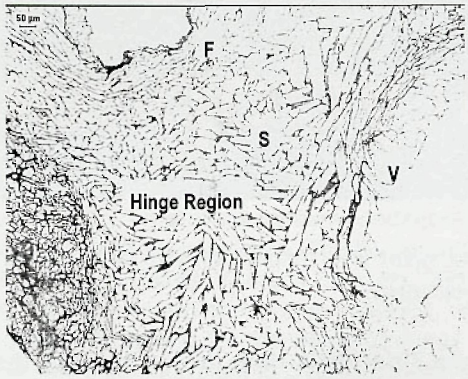

Figure 9: Heart valve leaflet, which has been cryoprotected, control rate frozen and freeze substituted. Extracellular ice domains can be seen throughout all three regions.

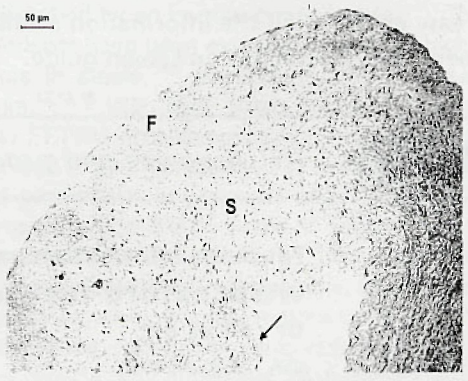

Figure 10: Vitrified heart valve leaflet, which has been freeze substituted. The structural architecture is of normal appearance and shows no evidence of any ice domains compared to control rate frozen tissue

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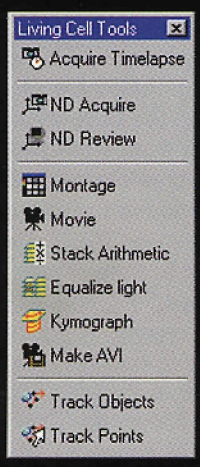

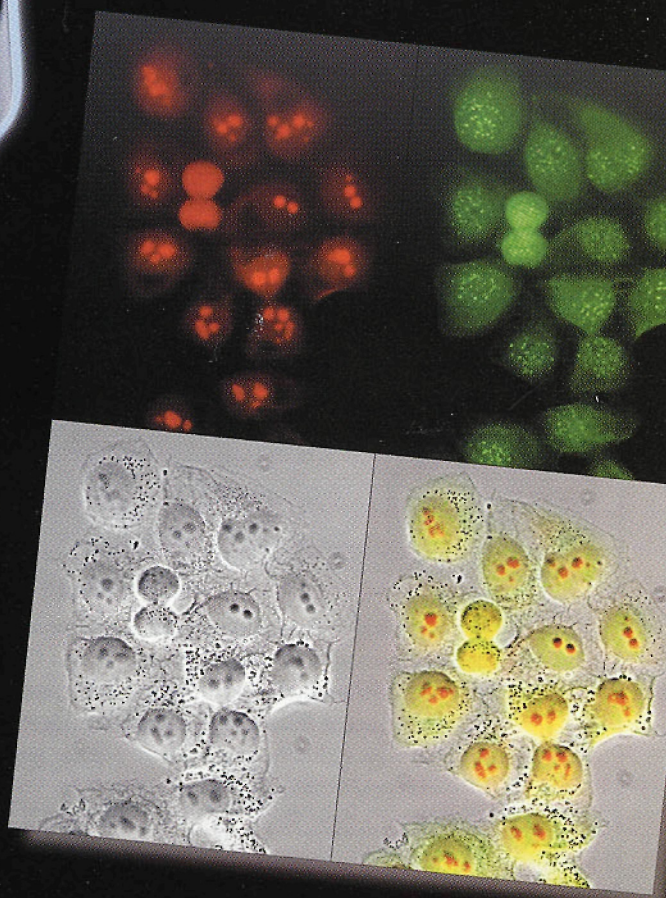

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