



A novel method for freezing and storing research tissue bank specimens[☆]

Steve D. Loken^{a,d}, Douglas J. Demetrick^{a,b,c,d,*}

^aDepartment of Pathology and Laboratory Medicine, Calgary, Alberta Canada T2N 4N1

^bDepartment of Oncology, Calgary, Alberta Canada T2N 4N1

^cDepartment of Biochemistry and Molecular Biology, Calgary, Alberta Canada T2N 4N1

^dUniversity of Calgary and Calgary Laboratory Services, Calgary, Alberta Canada T2N 4N1

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Summary Preserving small pieces of frozen tissue for possible future ancillary studies (“tumor banking”) usually involves either placing a small piece of tissue in a cryovial and snap freezing it in liquid nitrogen or embedding and freezing the tissue in a block of cryopreservation medium, such as optimal cutting temperature (OCT) compound. The **cryovial storage method** leaves an irregularly shaped piece of tissue frozen to the side of the plastic vial, where it is exposed to air, subject to desiccation (“freezer burn”), and difficult to remove, but the vials are easy to store. The **OCT method** results in good morphological preservation, but yields a large awkwardly stored block from which it may be difficult to locate and recover small specimens. We have **proposed a novel method of storing tissue bank specimens**, the **“capsule-freeze” method**, which combines the advantages of OCT specimen preservation with those of cryovial specimen storage. Using this method, a tissue specimen is “snap-frozen” in OCT within a size “00” VCap pharmaceutical capsule, then the capsule is stored within a 1.5-mL cryovial. The specimen is harvested by simply cutting a slice out of the capsule and sealing the cut ends with OCT before their return to the freezer. The slice is then embedded en face within an OCT block before frozen sectioning. Morphological preservation is excellent, and the capsules are very easy to store. Occasional cracking that we found with the use of gelatin capsules is greatly diminished with the use of **VCaps cellulose-walled capsules**. The OCT can be easily removed by rinsing in cold 70% ethanol solutions. This method of tissue storage is ideal for the small specimens that are now commonly archived in these days of tissue sparing surgery.

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1. Introduction

A great need exists for resources of well-preserved specimens of normal or neoplastic human tissues that can be used for biomedical research. “Banks” of these specimens can contain blood or blood products such as buffy coat preparations, small tissue specimens, cytological aspirates,

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* Corresponding author. Department of Pathology and Laboratory Medicine, The University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta Canada T2N 4N1.

E-mail address: demetric@ucalgary.ca (D.J. Demetrick).

or even noncellular specimens such as serum or urine, which are stored under the premise that qualified medical investigators with scientific and ethical approval may use the specimens at a later time. Such specimen banks can be used for numerous discovery applications such as the identification and structural characterization of human genes, expression analysis, or discovery proteomics. Biological specimens, when coupled with standardized treatment and outcome data, may be essential resources for the identification, characterization, and validation of biomarkers predictive of disease treatment.

Many such biorepositories have been established, often directed to particular types of specimens [1-6], although lately, large banks have been established as national resources [7,8]. Ethical arguments concerning accrual and use of such specimens are rife, with patient rights to privacy and “ownership” of their tissue specimens being balanced by the social good of research resulting from such repositories [9-12]. Several countries have enacted regulations or laws establishing expectations concerning accrual, storage, and disposition of such specimens [11,13,14]. Although the ethical landscape surrounding research tissue banks has been evolving and novel uses for these materials are increasing, the process of harvesting and storing human tissue specimens for research purposes has not changed materially in more than a decade [2,15-17].

Various approaches to banking research tissue specimens have been described [2,15-17]. Ideally, specimens are harvested and frozen in as short a time after tissue devascularization as possible. Specimens are often stored in freezers at -80°C or, preferably, within nitrogen liquid or vapor freezers. The method of storage of the specimens is varied, but most facilities use either simple tissue storage within cryovial tubes [16] or tissue embedding in cryosection molds using cryopreservation media such as optimal cutting temperature (OCT) compound. The former method, which is more commonly used, results in specimens that are easy to label and store in sample boxes or racks, but small specimens tend to desiccate during storage even with “O-ring”-sealed cryovials or may be difficult to remove from the wall of the cryovial without specimen thawing. The latter method results in excellent morphology, but removal of small specimens from the excess cryopreservation medium may be difficult, and the blocks are somewhat inefficient to store, especially within a typical liquid nitrogen freezer. Freezing the tissue in cryopreservation medium within a cryovial results in a plug of tissue and cryopreservation medium that is almost impossible to remove without difficulty and even with partial thawing. We propose a novel method of tissue freezing and storage that combines the ease of storage using cryovials, with the excellent morphology resulting from cryopreservation medium. A pharmaceutical capsule-based system of preserving frozen tissue was developed, and two different capsule materials were compared for durability under freezing and frozen storage conditions.

2. Methods

Size “00” pharmaceutical capsules (Now Foods, Bloomington, Ill) were obtained commercially. One capsule type (gelatin capsules) is gelatin-based, whereas the other (VCaps) is cellulose-based and composed predominantly of hydroxypropyl methylcellulose. Anonymous samples of 4 standardized tissue types (skeletal muscle, breast, kidney, and liver) were obtained from autopsies or surgical specimens not needed for diagnostic use. Size “00” pharmaceutical capsules were partially filled with 4 drops of OCT (EMS, Hatfield, Pa) freezing medium immediately before addition of the tissue specimen. A standardized piece of tissue (approximately $3 \times 3 \times 5$ mm) was placed within the capsule followed by 2 to 3 more drops of OCT. The capsule was then immediately frozen in liquid nitrogen. This was repeated 9 times for each capsule type with each of the 4 tissue types (total 40 specimens for each capsule type). In addition, 10 “empty” capsules of each type, filled only with OCT freezing medium, were created. After freezing, the capsules were removed from the liquid nitrogen for immediate use or stored within 1.5-mm cryovials (Nunc, Roskilde, Denmark) at -80°C until use. Photographs were taken using a Nikon digital camera.

After storage, the capsules were examined for freezing damage. From intact capsules, 2- or 3-mm cross-sections of the capsules were cut with a scalpel or sharp knife and embedded in OCT blocks, ensuring that the top of the “slice” was slightly below the surface of the OCT. The remaining portions of capsule were resealed with a drop of OCT, placed within a plastic cryovial, and then returned to the freezer at -80°C . Frozen sections cut with a conventional cryostat were stained with hematoxylin-eosin or other stains, dehydrated through graded ethanol solutions into xylene, and then mounted with Permount (VWR Scientific, Toronto, Ontario, Canada). Microscopic sections were evaluated using a Zeiss Axioskop. Statistical evaluations were conducted using commercial biostatistics software (Prism v4.02). The methodology is summarized in Fig. 1.

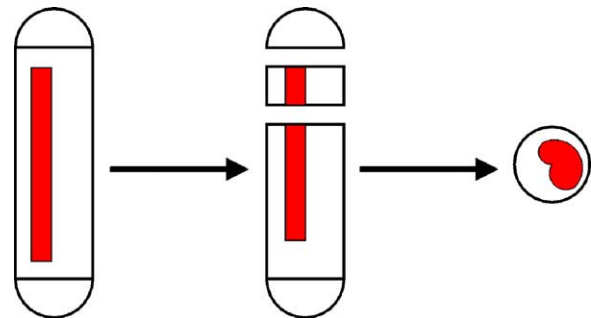


Fig. 1 Schematic diagram of tissue-storing protocol. A small tissue sample is placed within an OCT-filled capsule and “snap-frozen” in liquid nitrogen. When sections are needed, slices of the capsule may be cut, with the remaining tissue sealed with a drop of OCT.

3. Results and discussion

The method of freezing the specimens proved to be very simple (Fig. 2). Care was needed to ensure that the gelatin capsules were not wetted long (5–8 seconds), or they would quickly become soft and difficult to handle. The VCaps capsules seemed to be somewhat more resistant to this problem. Initially, we noted that the gelatin capsules also seemed quite prone to freezing damage, so we undertook an evaluation of gelatin versus cellulose capsules using the 4 different types of tissue specimens and standardized freezing methods. The gelatin-based capsules showed significantly more freezing-induced capsule damage than did the cellulose-based capsules (1-tailed $P < .0003$ using the χ^2 test). The cracks ranged from tiny holes that leaked small amounts of OCT medium to large fractures that broke the capsules into two pieces (see Fig. 3). Multiple freeze-thaw cycles increased the likelihood of capsule damage. Although these cracks were irritating, they were usually minor and uncommon with the VCaps capsules unless the capsule was filled with OCT alone (an unlikely practical concern). Of course, with the capsules being stored within cryovials, even capsules cracked into two pieces yielded useful tissue specimens, with the degree of desiccation

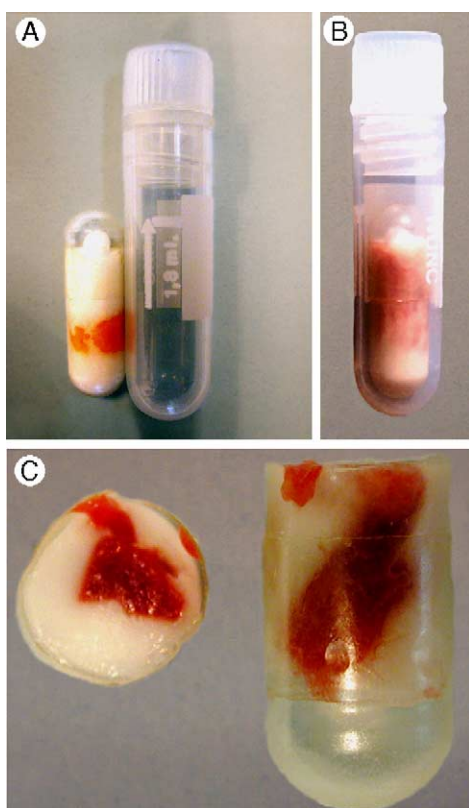


Fig. 2 Capsule-freeze method of tissue storage. A, Cellulose capsule filled with tissue and frozen OCT next to a cryovial. B, The capsule fits easily within the cryovial for convenient storage. C, When needed, slices may be cut from the capsule for histological or other studies. The remaining tissue remains safely embedded within the OCT capsule.

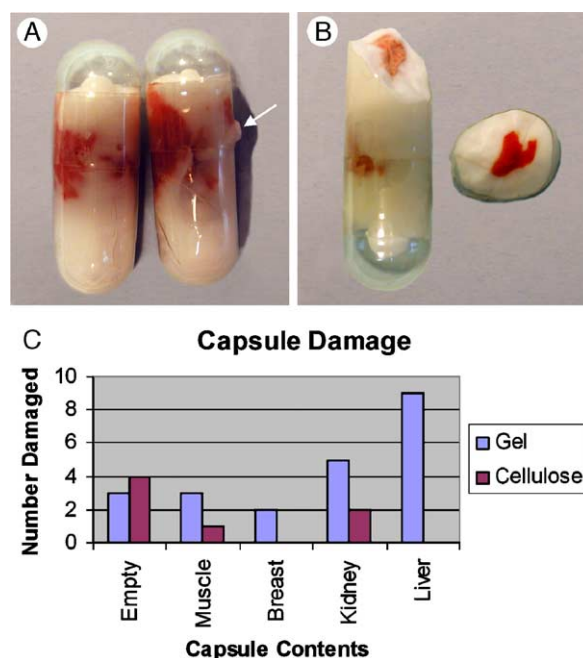


Fig. 3 Artifacts associated with the capsule-freeze technique. A, One type of minor damage to gelatin capsule, showing leakage of OCT that occurred during freezing (arrow). B, Major damage occurring to gelatin capsules when large cracks form and sections of tissue and capsule break off during the freezing process. C, A graphic representation of multiple replicates of 4 different tissue types frozen in either gelatin (gel) or VCaps (cellulose) capsules. With each tissue type and OCT alone, 10 capsules were evaluated, for a total of 50 capsules of each capsule material. Although not immune to freezing damage, the cellulose VCaps capsules tend to be far more resistant to cracking than the gelatin capsules, except in the case of capsules filled with OCT alone.

expected to be the same as or less than that often observed in specimens stored in cryovials without cryopreservation medium. The slices of the capsules had to be cut quickly to prevent excessive thawing of the OCT, which can wet and damage the capsule. To no great surprise, evaluation of the frozen section morphology from specimens of the capsules showed essentially identical morphology as tissues frozen in cryomolds with OCT (not shown). Although the OCT capsule-embedded specimens would be expected to yield less freezing artifact than those from simple cryovial storage, we did not test this hypothesis. We recognize that storage of tissue in cryopreservation medium has not been recommended by some investigators [18]. Nonetheless, this method is still used commonly. Anecdotally, we have found that RNA isolated from OCT-preserved samples has been quite usable in reverse transcription polymerase chain reaction (PCR) and Northern blot assays, and after trimming of the tissue with a scalpel, OCT can be removed by a simple rinse of the frozen tissue in cold (4°C) 70% ethanol solutions, in case of concerns regarding inhibition of PCR by carryover of OCT into the sample.

In summary, we found that the capsule-freeze method of preserving frozen tissue is an easy-to-use protocol. The

tissue is easily accessible for performing frozen sections for histological staining or other ancillary studies (eg, PCR analysis). In our evaluation, the morphology and immunostaining characteristics are identical to that obtained with conventional OCT embedding, which is to be expected because our small specimens are also embedded in OCT. Interestingly, the capsule material usually disappeared during the aqueous steps of the section processing.

The size "00" capsules store within 1.5-mL cryovials, which can also be labeled and stored in partitioned boxes with minimal space requirement. As we have shown, cellulose-based VCaps capsules are superior to gelatin-based capsules. The cellulose capsules are less rapidly dissolved by the OCT freezing medium during specimen harvesting and experience less freezing-induced cracking. The combination of simplicity of specimen freezing and storage, with the excellent preservation of the samples over alternate methods, suggests that our "capsule-freeze" technique is an improvement to existing research tissue banking methods.

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