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The procurement, storage, and quality assurance of frozen blood and tissue biospecimens in pathology, biorepository, and biobank settings

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Abstract

Well preserved frozen biospecimens are ideal for evaluating the genome, transcriptome, and proteome. While papers reviewing individual aspects of frozen biospecimens are available, we present a current overview of experimental data regarding procurement, storage, and quality assurance that can inform the handling of frozen biospecimens. Frozen biospecimen degradation can be influenced by factors independent of the collection methodology including tissue type, premortem agonal changes, and warm ischemia time during surgery. Rapid stabilization of tissues by snap freezing immediately can mitigate artifactually altered gene expression and, less appreciated, protein phosphorylation profiles. Collection protocols may be adjusted for specific tissue types as cellular ischemia tolerance varies widely. If data is not available for a particular tissue type, a practical goal is snap freezing within 20 minutes. Tolerance for freeze-thaw events is also tissue type dependent. Tissue storage at −80°C can preserve DNA and protein for years but RNA can show degradation at 5 years. For −80°C freezers, aliquots frozen in RNAlater or similar RNA stabilizing solutions is a consideration. It remains unresolved as to whether storage at −150°C provides significant advantages relative to −80°C. Histologic quality assurance of tissue biospecimens is typically performed at the time of surgery but should also be conducted on the aliquot to be distributed because of tissue heterogeneity. Biobanking protocols for blood and its components are highly dependent on intended use and multiple collection tube types may be

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CONFLICTS OF INTEREST
We have no conflicts of interest to declare.

needed. Additional quality assurance testing should be dictated by the anticipated downstream applications.

Keywords

Biorepository; biobank; frozen; tissue; procurement; biospecimen

1. Introduction

The world population has seen exponential growth and is projected to increase from the current 7.2 billion to 9.6 billion by the year 2050 [1]. With this sizeable expansion in the human population, there will be a correspondingly large increase in biomedical biospecimens. In the United States alone, the number of biospecimens is estimated to have tripled over a decade to reach approximately 600 million in 2010 [2]. Furthermore, there has been a rapid evolution of increasingly affordable “next-generation” technologies that permit global or targeted evaluation of the genome, epigenome, proteome, and metabolome of tissues and cells and that are critical to personalized medicine- the tailoring of targeted therapies for each patient. Frozen tissue is the favored biospecimen for modern testing because it produces a high yield and high quality of nucleic acids and proteins that the more common formalin-fixed paraffin embedded (FFPE) tissue cannot match [3]. Until now, collection of frozen biospecimens has largely been the preserve of research programs but “next-generation” testing is moving rapidly into daily clinical care suggesting that frozen tissue collections may become routine when cancer or certain disorders are suspected. Some technologies, with compromises, are modified to test FFPE tissues and room temperature storage modalities are under development. Nevertheless, for the immediate future, pathology departments and biobanks will likely have to store and disseminate increasing numbers of frozen biospecimens. Patient biospecimens can broadly be categorized as tissue, blood, or other fluids. These are sometimes processed to produce derivatives such as cells, nucleic acids or proteins, and then stored. Blood and fluids also may be processed to separate out cellular components before freezing. It is this panoply of biospecimens that need to be collected and stored under optimal conditions.

Ultra-low temperature frozen tissue (−80°C to −190°C) and formalin-fixed paraffin embedded tissue each has advantages and disadvantages [4–6]. Histology of frozen tissue is often adequate for quality assurance though inferior to FFPE tissue for detailed microscopic analyses. However, unlike FFPE tissue, the DNA and RNA from frozen biospecimens are generally high molecular weight and without cross-linking - suitable for a wide variety of purposes. Frozen tissue yields DNA and RNA ideal for current approaches such as whole genome amplification, whole genome sequencing, and cDNA microarray analyses [3, 7]. In frozen tissue, proteins are uniquely well preserved including intact enzymatic activity which is lost with FFPE specimens [8]. Infectious organisms in frozen tissue may remain viable so universal precautions are necessary in handling frozen biospecimens. At ultra-low temperatures, biospecimens can be stored for years to decades. However, studies have noted RNA fragmentation after five years despite storage at −70°C or −80°C [9, 10]. Frozen storage has other drawbacks. Many medical centers, outside of the major academic centers, do not have the personnel or infrastructure for frozen biospecimen procurement and storage. Biomolecules can degrade with increasing freeze-thaw cycles. Storage costs for frozen specimens are much more than for room temperature specimens. Loss of temperature control can spoil irreplaceable specimens. A freezer failure, apparently undetected by redundant alarm systems, damaged one third of specimens in a national autism brain bank and numerous other brain specimens [11]. Storing samples in liquid nitrogen (LN_2) poses rare but serious risks including LN_2 burns, LN_2 supply tank explosions, and suffocation from

LN_2 leaks in an enclosed space [12]. Finding adequate contiguous and practical space for banks of freezers or liquid nitrogen vats is extremely challenging at major medical and research institutions.

In developing standard operating procedures for our biobanks, we were able to identify studies and focused reviews of specific topic on frozen biospecimens but not an up to date unified overview of experimental data pertaining to procurement, storage, and quality assurance. We used PubMed, Google, and Google Scholar engines to search with the following keywords and combination thereof: biobanking, biorepository, biospecimen, frozen, tissue, blood, procurement, container, quality control, and quality assurance. Studies were included if peer-reviewed and in English. Reference lists from these identified articles were reviewed for other relevant publications. In this paper, we will provide an overview and update on the fragmented and incomplete data regarding how best to procure and preserve frozen biospecimens for genomic, epigenomic, transcriptomic, proteomic, and metabolomic studies. We will highlight several important questions. How quickly must we freeze a biospecimen? Is the ubiquitous −80°C freezer really adequate to stabilize biospecimens or is −150°C storage superior? How much does freeze-thawing, as occurs with removing specimens from freezers repeatedly, affect biospecimen degradation? What is the basis for the quality assurance methodologies currently in use? In the context of these discussions, we will discuss infrastructure considerations for frozen biospecimen banking. Lastly, we make an argument for development of new room temperature storage strategies to replace frozen biospecimens.

2. Considerations in the procurement of tissue and blood biospecimens

Procurement of biospecimens, until the last decade, was a relatively unstudied area with only “best guess” approaches possible [13]. Recently, the International Society for Biological and Environmental Repositories (ISBER), the National Cancer Institute (NCI), and other organizations have drafted best practices for collection and storage of human biospecimens (frozen tissue, blood, urine, saliva and any other human samples) [14, 15]. Standard operation procedures (SOP) for collection and storage of either fixed or unfixed human samples have been suggested but these are rapidly evolving as more data accrues [16–20].

2.1 Procurement of tissue

Human tissues are typically obtained from surgeries and autopsies. A variety of factors such as the post-mortem interval or length of surgery may impact the amount of tissue ischemia and degradation prior to collection. For autopsy derived tissue, premortem or agonal changes may have a greater effect on molecular integrity than post-mortem handling [21]. Secondary to loss of blood and oxygen supply, gene expression and protein phosphorylation patterns as well as other cellular properties may be artifactually affected by degradation of cellular contents or by intracellular responses [21–23]. For practical purposes, warm ischemia time is usually measured as the amount of time that tissue is at ambient room temperature after being resected from the human body but prior to being stabilized- typically frozen or fixed in formalin. It should be kept in mind that warm ischemia also progressively occurs in the lesional tissue during the surgical resection itself which can last from a couple of hours to sometimes all day. The areas of earliest incision endure the longest ischemia times as the blood supply is cut off first (see Figure 1). Warm ischemia time during surgery is not often tracked as the operating room environment is extremely busy and focused on a technically safe and successful surgery for the patient. To bypass this issue, some centers obtain biopsies (representative samples) specifically for freezing down prior to performing the main resection. Some biobanks send a technician into the operating room with a liquid nitrogen container to reduce ischemia time typically for specific research patients, but this

expensive approach is not widely undertaken. Often tissue is left at ambient temperature until it is delivered to a pathologist who apportions tissue for clinical and biobank samples. More centers are now placing tissue sample containers on wet ice in the interim. Keeping the tissue biospecimen on ice until it can be frozen down can limit cellular changes and retard degradation. This cold ischemia time can be measured and is defined as the period that tissue is kept on ice or in a 4°C refrigerator after resection but before formalin fixation or freezing (see Figure 1). Freezing of tissue specimens is typically performed by placing a cryovial of tissue in liquid nitrogen or on dry ice with subsequent storage in –80°C freezers or liquid nitrogen [24].

RNA is often considered a labile molecule that is prone to degradation; however, there is surprisingly contradictory data. To assess RNA degradation, most studies use the RNA integrity number (RIN), a numerical representation of electrophoretic measurements of whole RNA integrity [25]. A study on colon samples did not show a decrease in RNA integrity with warm or cold ischemia for up to 4 hours [26]. RNA was also stable in breast tumor samples stored at room temperature for up to 24 hours prior to freezing [27] and in tonsil tissue after overnight storage (16 hours) at room temperature [28]. In contrast, several other studies have shown a progressive deterioration in RNA integrity with increasing time between tissue excision and freezing [29–33]. While RNA degradation is one measurement, the direct effect on gene transcription studies has also been studied [27,32,34–36]. These indicate that there are time dependent effects on gene transcription in cancer samples from diverse organs though intratumoral variation may exceed warm ischemia effects [36].

In terms of protein phosphorylation, rapid freezing (<5 minutes) in liquid nitrogen appears to optimize phosphorylation status and minimize post-mortem proteolysis in heart tissue samples [37]. Walker and his colleagues showed an increase in troponin I phosphorylation and a decrease in troponin T and myosin light chain 2 phosphorylation in tissues that were kept in cardioplegia when compared to samples that were immediately frozen in liquid nitrogen. Espina and colleagues similarly have highlighted increased phosphorylation of some proteins and decreased phosphorylation in other proteins within the same samples. The advocate stabilization of tissue samples with solutions containing phosphorylase and kinase inhibitors or freezing within 20 minutes [23]. In terms of protein preservation, there is variability for individual proteins. In muscle biopsies, vascular endothelial growth factor protein expression tended to be higher in specimens subjected to a 30 or 60 minutes cold ischemia time compared to those frozen within 15 minutes of tissue removal [38]. Significant reduction in immunohistochemistry (IHC) staining for progesterone receptor in breast cancer samples occurs after 2 hours for non-refrigerated samples [39]. Similarly, for Her2neu testing, the American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) guidelines on breast cancer currently recommend a cold ischemia time of <1 hours [40].

2.2. Procurement of blood

Blood is one of most common biospecimens used in clinical and translational research. It includes a variety of fractions such as plasma, serum, white blood cells, and red blood cells. How blood is collected is dictated by the intended testing as no single collection method will meet all needs. The type of the preservatives and additives in blood collection tubes can be changed based on the specific application [16, 41]. For some assays in clinical biochemistry, serum is useful; therefore a collecting tube containing a clot accelerator such as silica or thrombin is needed. Anticoagulated blood (consisting of plasma, buffy coat, and RBCs) is preferred for DNA or RNA based assays. There are several types of anti-coagulant which are used for different purposes. Citrate-stabilized blood yields higher quality RNA and DNA and also more lymphocytes for culture compared to other anti-coagulants [16]. Ethylenediaminetetraacetic acid (EDTA)-coated collection tubes are suitable for a wide

range of DNA based and protein assays [42], but because it increases sister chromatid exchanges and decreases mitotic index, it is not adequate for cytogenetic studies [16]. Heparin, another anti-coagulant, affects T-cell proliferation and binds to many proteins, but it is often used for metabolomic studies [16, 18]. Other stabilizers like protease inhibitors may be added for specific purposes but they have potential toxicity and lability during long-term storage and can interfere with future assays. The UK biobank, that has a massive blood collection program, collects as many as 5 different tubes; 2 EDTA tubes for plasma, buffy coat, red cells; Lithium heparin tube for plasma; Serum-separating tube (with silica clot activator) for serum; acid citrate dextrose tube for dimethyl sulfoxide (DMSO) blood; Tempus tube for whole blood (RNA) [42].

Collected blood can remain whole or be divided into its fractions. Serum and plasma can be used for analyte studies, including proteins, lipids, and small molecules, and as a source of cell-free nucleic acids [41]. Cell concentrates are used in functional studies, flow cytometry, culture experiments, or as a source for cellular nucleic acids (only white blood cells are nucleated and thus transcriptionally active) [41, 43]. Blood components are labile in nature, and their integrity requires timely processing. The allowable time varies by the component of interest and their stability. Live cells are stable at room temperature for up to 48 hours, but must be either cultured or cryopreserved in liquid nitrogen at −150°C in order to remain viable [16]. Elapsed time between collection and processing with the samples remaining at room temperature or even at 4 °C (>24 hours) can result in protein alterations, so immediate separation of plasma from cells is required to limit degradation [44, 45]. It has been shown that a 24 hour time lag between sample collection and processing is acceptable and it does not adversely affect the quality of extracted DNA by storing the blood sample at 4°C [46]. Delayed processing of tissue is correlated with RNA degradation as 5′ RNA tags increase with lengthier processing times for both plasma and buffy coat samples. These changes may be a result of cellular anoxia or cell death following collection [47]. Tsui et al. demonstrated that more than 3 hours of delay in RNA extraction post-collection causes a significant increase in transcript levels when samples are left at room temperature [48]. Ideally, the samples should be fractionated and aliquoted immediately after collection, with the aim that all samples are cryopreserved not more than 24 hours after collection [42].

3. Optimal storage temperatures for frozen biospecimens

3.1. Storage of frozen tissue

Storing biospecimen at −20°C was commonly used in the past for all type of samples. In recent decades, ultra-low temperatures (−80°C and −150°C) became standard for long-term storage as it became clear that tissue degradation did occur at −20°C. Many centers currently use −80°C though some authors recommend liquid nitrogen as superior in part because there is less desiccation [49]. It has been suggested that storage at below −137°C may be the optimal temperature as −137°C is the glass transition temperature (Tg) of water, below which biochemical activity that might degrade intracellular contents are thought to be inert. Temperatures below −137°C can be achieved by some electrical freezers (−150°C) or by liquid nitrogen storage. With liquid nitrogen, vapor phase storage (−150°C) is generally preferred over liquid phase (−196 °C) because of the risk of contamination by errant floating tissues fragments in liquid nitrogen. As the name implies, in vapor phase containers, biospecimens are stored above the liquid phase nitrogen but surrounded by the vapor (gaseous) phase. Despite excellent biospecimen stability in liquid nitrogen [49, 50], there are handling hazards with liquid nitrogen that have driven the use of −150°C electrical freezers. There is little long-term storage data on these relatively new Ultra-low temperature freezers but they share with the vapor phase nitrogen containers low temperatures that theoretically ameliorate temporary temperature fluctuations related to opening freezer doors or storage vats.

Effects of a range of storage temperatures on specimen stability have been assessed. After 7 years of storage, human liver specimens stored at −25°C had visibly degraded, showing evidence of ice crystal formation and visual inhomogeneity whereas specimens stored at both −80°C and −150°C showed no visible changes [51]. In terms of biomolecule integrity, the quantity and quality of brain autopsy tissue RNA as measured by optical density (OD) 260/280 ratios and reverse transcription polymerase chain reaction (RT-PCR) was not affected by storage for 15 years at −70°C [52]. However, some other studies showed reduced RNA integrity in specimens stored at −70°C or −80°C for 5 years or more [9, 10]. Whether this decreased RNA integrity is due to freeze-thaw events or ineffective temperature maintenance of the refrigeration units rather than an intrinsic biospecimen instability at that temperature is unknown. In contrast to RNA, DNA yield and integrity remains unchanged in long-term storage at −80°C [10]. The proteome of frozen tissue may remain intact for years if stored at or below −70°C [53]. McLeay and colleagues showed that quantifiable epidermal growth factor receptor activity in breast cancer specimens is independent of storage temperature (liquid nitrogen, −70°C, −20°C) [54]. It also has been shown that specimen trace element composition (Rb, Fe, Zn) in liver tissues is unaltered by 17 years of storage at −150°C in a liquid nitrogen vapor cooled freezer or a −80°C freezer [55]. For long-term storage, specimens should be stored at least at −80°C. In theory, −150°C storage may mitigate the effects of temperature fluctuations when opening freezer doors. While there are theoretical advantages, additional peer reviewed data is needed regarding whether −150 °C storage (with the added cost) is beneficial for biospecimen quality particularly since there is data to suggest that RNA degradation does occur at −80°C.

3.2. Storage of blood

Depending on which analyte, marker, or molecule is the focus of interest, the temperature for short and long-term storage may vary. Typically, collected blood samples are processed as soon as possible to avoid any drop in biomolecule yield and to minimize potential degradation [16]. It has been shown that DNA can be extracted with acceptable yield and quality from blood samples that are stored at room temperature, 4°C, and at −20°C for a maximum of 1 month [56]. Prolonged storage causes lysis of the erythrocytes and, with severe hemolysis, it is expected that some of the leukocytes will have lysed as well, which will result in loss of DNA during the leukocyte harvesting step in DNA extraction [56]. In this respect, when processing cannot be undertaken immediately, blood samples should be frozen at −80°C to improve DNA yield [57]. While extracted DNA from blood samples is stable at 4°C for several weeks, at −20°C for months, and at −80°C for years [58], RNA is more labile and degrades quickly at temperatures higher than −80°C. It has also been shown that the integrity of microRNA (miRNA) is maintained for years in plasma samples stored at −80°C highlighting that not all RNA species are equally susceptible to degradation [59]. Serum and plasma contain a large number of soluble molecules, including many proteins that have varied stability. Immunoglobulins in plasma are considered stable, even at room temperature for few days, whereas other proteins are labile and remain stable at 4 °C only up to 24 hours [45]; therefore, requiring very low temperature to stay intact for long-term storage (−80°C) [16]. No degradation of protein and other molecules was seen in plasma samples stored at −80°C or liquid nitrogen for up to 6 years [42, 60].

3.3 Types of storage containers

Available literature provides mostly experience-based recommendations regarding containers. Screw-cap cryovials typically made from polypropylene and polystyrene are recommended for long-term, low temperature storage [61]. Sealing cryovials with membranes or overwraps can mitigate contamination in liquid nitrogen [62]. Wrapping frozen tissue slices in aluminum foil can minimize desiccation [63]. Storage of brain slices

in sealed plastic bags is typical [64]. Detailed, formal comparative studies on the effect of different storage containers on the quality of frozen tissue biospecimens are lacking.

4. Freeze-thaw cycles and their effect on biospecimen quality

Repetitive thawing and freezing may occur when the same tissue portion is repeatedly sampled for a variety of analyses over time. It may occur with temperature fluctuations related to power outages, freezer mechanical failure, or frequent door openings when removing samples.

4.1. Freeze-thaw effects on frozen tissue

Since RNA is believed to be the most vulnerable molecular component of unfixed tissue, studies have addressed the impact of freezing and thawing on RNA integrity. Two studies found that repeated freezing and thawing of ovarian and brain tumor samples causes no alterations in RNA quality, gene expression profile, or protein expression [65, 66]. Jochumsen et al. showed that ovarian tumor samples can be frozen and thawed at least three times without compromising the RNA integrity and genetic expression profile [66]. A study done by Botling et al. indicated that minimal degradation of tonsillar tissue RNA integrity occurred only after 6 freeze-thaw cycles. Each thaw event was 5 minutes suggesting that short thawing time can minimize degradation [67]. Several other studies have found that many repeated freeze-thaw cycles do lead to significant decreases in RNA integrity particularly for autopsy brain tissue [36, 68, 69]. In some cases, as few as 2 thaw events were sufficient to reduce RNA quality. The differences between those reports can be due to the inherent differences in the nuclease content and lability of biomolecules in different tissue types and also thawing conditions. These findings suggest that freeze-thawing should be avoided where possible by appropriate aliquoting. Where not possible, placement of samples on dry ice or wet ice during sampling should be considered. Sherwood and colleagues found that aliquots stored in RNAlater (Qiagen, Valencia, CA) produced better quality RNA than snap-frozen samples and were more resistant to freeze-thawing [68].

4.2. Freeze-thaw effects on frozen blood

DNA, RNA, and protein stability in blood samples are also affected by freeze-thawing. In one study, a single freeze-thaw episode (from −70°C storage) decreased the yield of DNA by 25% [70] but did not significantly change the messenger RNA (mRNA) concentrations in plasma or serum [48]. In contrast, Kopreski et al. found that freezing and thawing of serum promoted rapid degradation of mRNA and showed that, after the third freeze-thaw cycle, amplifiable RNA could no longer be detected [71]. Baumann et al. reported that the best reproducibility of proteomic patterns is with a single thawing of frozen serum samples and that repeated freeze-thaw cycles lead to significant changes of serum and plasma proteomes [72]. Vascular endothelial growth factor (VEGF) levels in plasma significantly changed with the number of freeze-thaw cycles [38, 73]. Hsieh and colleagues demonstrated that repeated freezing at −80°C and thawing on ice for up to ten times did not present any obvious changes in low-molecular-weight serum and plasma proteomes [45]. The mixed finding in these studies may reflect differing analytes and thawing parameters (e.g. length of thaw events varied) evaluated. As a general rule, we believe that freeze-thawing of blood samples should be minimized and this can be accomplished in part by aliquoting or by extracting upfront then storing relatively stable derivatives such as DNA.

5. Quality control and quality control tools

While we have discussed biospecimen quality in prior sections of this review, we will discuss in greater depth the question of how to evaluate the quality of frozen biospecimens

5.1. Histologic quality control of tissue- Is there adequate lesional tissue of interest?

Histologic quality control is commonly performed on cancer samples. For molecular studies of tissue, histologic quality control, in the past, included evaluation for the percentage by area of the tissue involved with tumor. However, area measurements can be deceptive. A high density of tumor cells in a small area may sometimes yield a high and adequate percentage of tumor DNA. Conversely, involvement of a large area by rare scattered tumor cells may yield a very low percentage of tumor DNA. It is therefore preferable to estimate tumor percentage by the ratio of tumor nuclei to all nuclei. As standard Sanger sequencing can detect 20% mutant alleles, forty percent is sometimes chosen as a minimum quality control threshold because mutant alleles may be heterozygous [74]. However, it may be wise to choose a higher percentage than 40% given difficulties in precisely estimating tumor nuclei percentages. At our center, some investigators request a 70% tumor nuclei threshold for acceptable specimens. Recent technologies permit detection mutant alleles of as little as 2% and may obviate some of the current concerns [75]. When samples do not meet the criteria, non-neoplastic areas can sometimes be dissected off to adequately enrich for tumor tissues. The Cancer Genome Atlas (TCGA) project mandates 80% tumor nuclei for glioblastoma multiforme (GBM) studies [76] and 60–70% tumor nuclei for other cancers. The TCGA project also excludes tissues that have greater than 20–50% necrosis. These fairly strict criteria were necessary for the TCGA as they intended their biospecimens for a large, broad, and comprehensive set of analyses. For some assays, we suggest that simply using the extent of necrosis as a criterion can unnecessarily exclude cases as viable tumor nuclei content is often high [77]. For example, immunohistochemistry can be highly informative even in specimens with 50% or more necrosis. For some assays, dissection to remove extensive areas of necrosis before use is one possibility. We may accept tumor samples with simple coagulative necrosis but exclude areas of necrosis where there are abundant inflammatory cells such as neutrophils as their genomes may confound DNA analyses. Ultimately, the acceptable percentage of tumor nuclei and of necrosis will depend on the specific downstream applications. In clinical trials where patient enrollment is often challenging, stringent criteria indiscriminately applied may reduce statistical power for a study and overly lax criteria can lead to data confounded by excessive non-neoplastic components.

5.2. Assessing DNA quality

An often used method for rapid evaluation of the yield and purity of the nucleic acid extracted from cells or tissue is to determine the optical density (OD), a spectrophotometric reading, at the 260 nm and 280 nm wavelengths. Nucleic acids absorb ultraviolet (UV) light due to the heterocyclic rings of the nucleotides and higher OD readings reflect greater nucleic acid concentrations [78]. The wavelength of maximum absorption for DNA is 260nm; an OD 260/280 ratio greater than or equal to 1.8 is generally accepted as pure for nucleic acids. This ratio is only an indication of purity of nucleic acids in relation to the absence of protein and does not necessarily reflect the integrity of the nucleic acid [79]. If the ratio is lower than 1.8, it may indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm [79] or it can be due to the effect of various solvents used to dissolve nucleic acid. A low salt buffer (e.g. Tris or TE buffer) gives very reproducible readings of the 260/280 ratio when compared to water [80]. The NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) that uses only 1–2μl of sample, far less than the typical spectrophotometer, is now widely used to assess OD. Another common method that can be used for assessing DNA integrity is agarose gel electrophoresis. On a 1–1.5% agarose gel, intact genomic DNA appears as a compact, high-molecular-weight band with no or scanty low-molecular-weight smears [79, 81]. Usability of the DNA from a tissue for downstream molecular applications can also be assessed by amplifying a specific sequence by polymerase chain reaction (PCR). DNA with no or reduced PCR product

indicates poor quality and tissue degradation; however, it is likely that the tissues assessed may be mostly acellular [81]. Jewell and colleagues suggest that the integrity of DNA and RNA extracted from human tissues can be assessed by simple electrophoresis alone [81]. DNA can also be evaluated for the presence of crosslinking or for appropriate methylation of specific genes. These studies are not done routinely but may be considered for specific needs.

5.3. Assessing RNA quality

Intact RNA is important for the evaluation of gene expression but RNase enzymes can quickly degrade the RNA. The resultant fragmented RNA can compromise gene expression evaluations [36, 82, 83]. Once RNA is extracted from a biospecimen, optical density (OD) measurement at multiple wavelengths provides a basic assessment of quantity and quality of RNA samples. Spectrophotometric readings at various wavelengths can quickly provide basic information about an RNA sample: 240 nm (background absorption and possible contaminants like EDTA); 260 nm (nucleic acid content); 280 nm (protein content); and 320 nm (background absorption and possible organic compounds such as phenol, sugar or alcohol) [84]. An OD 260/280 ratio greater than 1.8 is usually considered an indicator of acceptably pure RNA relatively free of protein [85]. The OD 260/240 and OD 260/320 ratios inform on contaminants other than protein [82]. Contamination with DNA, protein [82], or buffer solutions with different pHs can change this ratio [80]. As for DNA, the NanoDrop spectrophotometer can be used to assess RNA OD readings while using minimal sample volumes.

Although OD readings are useful for determining the approximate amount and purity of nucleic acid, they give no indication as to the physical integrity (lack of fragmentation) of the sample. The traditional method to assess RNA integrity is the ratio of 28S to18S ribosomal RNA (rRNA). When total eukaryotic RNA is run on an agarose gel, there are two distinct bands corresponding to 18S and 28S ribosomal RNA. In intact RNA, the 28S rRNA band should be approximately twice as intense as 18S rRNA band [86, 87]. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will have a lower ratio. Highly degraded RNA will appear as a very low molecular weight smear and genomic contamination will be visible as a very high molecular weight band. The 18S and 28S rRNA species are derived from the same precursor molecule, meaning that there should be the same number of copies of both molecules in the cell. For humans, the 28S rRNA has 5034 bases and the 18S rRNA has 1870 bases [88], so the exact ratio is 2.7 for total human RNA [89]. In practice, a 28S/18S ratio of 2 or higher is considered to reflect high quality RNA [84, 90]. The intensity of the rRNA bands however can be affected by electrophoresis parameters, the quantity of RNA electrophoresed, and the saturation level of ethidium bromide [86]. Also, this approach relies on the subjective interpretation of gel images and typically uses 0.5–2 μg of total RNA- consuming substantial amounts of what can be limited samples [84, 91]. The 28S/18S ratio is not ideal for assessing RNA quality for microarray hybridization or quantitative real-time PCR (qRT-PCR) [82, 92].

A relatively recently developed and now widely used RNA quality measurement is the RNA Integrity Number (RIN) that is obtained with a microfluidics-based capillary electrophoresis approach needing only small samples of 1–4 μL. A software algorithm generates the RIN number from the whole electrophoretic trace in the electropherogram of the RNA sample [82]. RIN values range from 10 (completely intact) to 1 (highly degraded). This numbering system is a user-independent, automated, and reproducible procedure [84]. RIN values can differ depending on tissue type and composition. Single cell populations such as white blood cells tend to have high RIN scores (9.36±0.13) [82] while tissues with high connective tissue content have lower RIN scores [83]. Kiewe and colleagues found that RIN scores were superior to the 28S/18S ratio in the prediction of microarray quality [93]. Depending on

stringency of criteria, their data indicated that RINs of 7.15 or 8.05 or below were not suitable for microarray analyses. Similarly, Raman and others exclude samples from microarray analyses if RIN scores are below 7.0 [92]. qRT-PCR of long amplicons (400bp) amplicons should be performed on samples with RINs not less than 5.0 [82]. However, qRT-PCR of short amplicons (75–200bp) is feasible essentially independent of the RIN score [82]. Nevertheless when possible, high RIN scores above 8.0 are preferable for qRT-PCR [83].

In addition to rRNA, other major RNA species include messenger RNA (mRNA) and microRNA (miRNA), which constitutes a class of small, noncoding cellular RNAs that function as post-transcriptional regulators of gene expression. The stability of mRNA differs from that of rRNA. Messenger RNA can remain intact in microwaved tissue, despite complete degradation of rRNA [94]. Similarly, in blood samples miRNA can remain stable even in severely degraded RNA samples (RIN 1.9) [95]. Published data has demonstrated that ribosomal ratio would reject many more samples than necessary and has a poor correlation to the expression value of the house keeping genes (such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hypoxanthine-guanine phosphoribosylt transferase (HPRT) gene) [25]. Some specific mRNAs can be preferentially degraded [96, 97]. A RIN score, while currently the most practical and best global RNA assessment tool, should not be taken as a perfect arbiter of RNA quality.

5.4. Assessing protein quality

Investigation of biospecimen proteins can yield useful information on pathological pathways or biomarkers related to a particular disease. Therefore, assessing protein integrity is important since variation in pre-analytical conditions may result in proteolysis, protein degradation, or other proteome-associated changes [38, 72, 98]. There is not currently a cost effective, facile, and comprehensive way to assess protein quality in frozen biospecimens [53]. Histologic evaluation of tissues, which is often performed at the time of procurement, can provide a preliminary screen for degraded tissue. There is blurriness of nuclear and other cytologic details if specimens are not well preserved. Another crude approach is to simply assess protein yield. Protein concentrations can be quantified by the Bicinchoninic acid assay (BCA assay) [99], Lowry protein assay [100], or Bradford protein assay among others [101]. In these colorimetric assays, a measurable color change of the sample solution is proportional to total protein concentration. A poor yield of protein relative to tissue samples of the same type would suggest tissue degradation. Numerous additional modalities to assess protein quality can be attempted but are not warranted on all specimens on a routine basis. Depending on the target audience of the biobank, some of these modalities may be used periodically to evaluate a subset of samples to ensure that they will meet the needs of your users. Specific immunohistochemical stains can be performed to evaluate for specific antigens including those of phosphoproteins. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) study stained with Coomassie Blue can be crudely informative of proteome integrity. In this procedure, SDS which is an anionic detergent linearizes proteins so that proteins separate by size during electrophoresis. We have used this approach to compare frozen brain tumor specimens with paired tissues preserved by other methods [7]. Western blotting can be used to identify specific proteins including phosphorylated proteins. A number of other more sophisticated strategies including mass spectrometry may be employed [53]. Mass spectrometry can identify peaks representing proteins that reliably degrade with poor sample handling and also peaks that represented proteins that did not degrade, which serve as internal controls [102]. These additional targeted quality evaluations are probably best left to the investigators who use the samples as the necessary quality testing depends on the intended application.

6. Pathologists, sample selection, storage costs, and space issues

The pathologist or cytopathologist is critical for establishing the presence and type of lesional tissue at the time of collection and at disbursement [64, 103–105]. They are also responsible for ensuring adequacy of samples for clinical use prior to releasing tissue samples for research. The role of a pathologist in biobanking is well reviewed elsewhere [105]. Pathologists are the biobankers of choice and institutional support for pathologist time and effort is essential [105]. How much sample should be stored? At University of California, Los Angeles (UCLA), the National Neurological AIDS Bank (NNAB) freezes half the entire brain for each decedent. The freezer requirements per patient at the NNAB are far more than those for the UCLA Alzheimer Disease Research Center (ADRC) where multiple, stereotypical areas of the autopsy brain are sampled. For the ADRC, a specific region is sampled as four 1 cm×1cm×0.5cm aliquots that are conveniently individually disbursed minimizing freeze-thawing concerns. Clearly, storing half a brain preserves more tissue that can support more studies but the latter requires far less freezer and floor space at our medical center which is scarce as is true of many medical centers. Over approximately 15 years, the NNAB has filled 5 Ultra-low temperature freezers with tissue while the ADRC has filled 2 freezers even though the former collects tissue from 20 decedents per year and the latter about 50 per year. In the future, given widespread cost constraints, selective sampling may be required to lessen long-term storage expenditures.

Infrastructure costs for a freezer room should include access to emergency backup power and adequate cooling as the freezers will generate substantial heat. A biohazard hood in the freezer room is ideal to minimize unnecessary movement of samples. Consider also the type of cooling you will need in light of the biospecimen types that you will store. Each −80°C freezer can hold significantly more specimens than its −150°C counterpart where compressor and other mechanical requirements are particularly high and where the actual storage space for containers may be as little as 17.3% of the total freezer volume (0.23 m^3 storage capacity; 1.34 m^3 total volume- Sanyo MDF-C2156VANC, Sanyo North America, Wooddale, IL, USA). Space needs for a freezer exceed the base footprint of the freezer as there is a need for adequate spacing in between units to limit overheating from heat emissions of adjacent freezers. In addition, space for back-up liquid nitrogen tanks to be connected to −150 °C freezers should be considered. We provide a comparison of costs for refrigerator and freezer units at different temperature points (see Table 1). Costs and environmental impacts increase as specimens are kept at colder temperatures.

7. Conclusions

Several points can be taken home. There is variability by organ and by cell type as to the tolerance for ischemia. Developing the best procurement protocol may require testing for your specific need. The optimal general approach is near instantaneous freezing of tissue samples. However, given the practical difficulties in the clinical setting, placing the specimen on ice immediately and then freezing the specimen within 20 minutes will mitigate artifactual gene expression and phosphorylation aberrations. If biospecimens are to be used mainly for DNA and protein, current data suggests that long-term −70°C or −80°C storage is satisfactory but that RNA can degrade by 5 years. Given this degradation, freezing small aliquots in RNAlater specifically for gene expression needs in parallel to the usual frozen specimen is a consideration. Peer reviewed data regarding long-term liquid nitrogen storage or −150°C freezer storage is sparse but there are theoretical advantages and anecdotal information to suggest that all derivatives including RNA are well preserved. In terms of quality control, histologic evaluation is essential for tissue to ensure that lesional material is present. This is typically done at the time of surgery by intraoperative frozen section. Given tissue heterogeneity, a number of biorepositories including ours also performs histologic

quality control of the specific aliquot that is provided to the researcher. Quality control for DNA, RNA, and protein might be performed periodically on a subset of cases in a biorepository. Collection of quality control data from end-users as they prepare derivatives for their studies can minimize using up of valuable biospecimens. In view of the costly logistical challenges of storing and using frozen biospecimens, room temperature alternatives for storing whole tissue biospecimens while preserving high quality DNA, RNA, and protein contents are greatly needed. Long term ambient room temperature storage of purified DNA or RNA is already feasible but it is room temperature whole tissue storage that can profoundly change the landscape of biobanking and biospecimen-based research.

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ABBREVIATIONS

FFPE	formalin-fixed paraffin embedded
LN2	liquid nitrogen
ISBER	International Society for Biological and Environmental Repositories
NCI	National Cancer Institute
RIN	RNA integrity number
IHC	Immunohistochemistry
EDTA	Ethylenediaminetetraacetic acid
DMSO	dimethyl sulfoxide
TG	glass transition temperature
OD	optical density
miRNA	micro RNA
VEGF	vascular endothelial growth factor
GBM	glioblastoma multiforme
TCGA	The Cancer Genome Atlas
UV	ultra violet
rRNA	ribosomal RNA
mRNA	messenger RNA
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPRT	hypoxanthine-guanine phosphoribosylt transferase
RT-PCR	reverse transcription polymerase chain reaction
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
BCA	bicinchoninic acid assay

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UCLA	University of California, Los Angeles
NNAB	National Neurological AIDS Bank
ADRC	Alzheimer Disease Research Center

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HIGHLIGHTS

1. Frozen biospecimens contain high quality nucleic acids and proteins for testing.
2. Tissue type and heterogeneity affect biospecimen quality and degradation.
3. Storage at −80°C preserves DNA and proteins well but RNA can degrade.
4. Minimize resampling or freeze-thaw events by aliquoting.
5. Quality assurance approaches can be tailored to the projected biospecimen use.

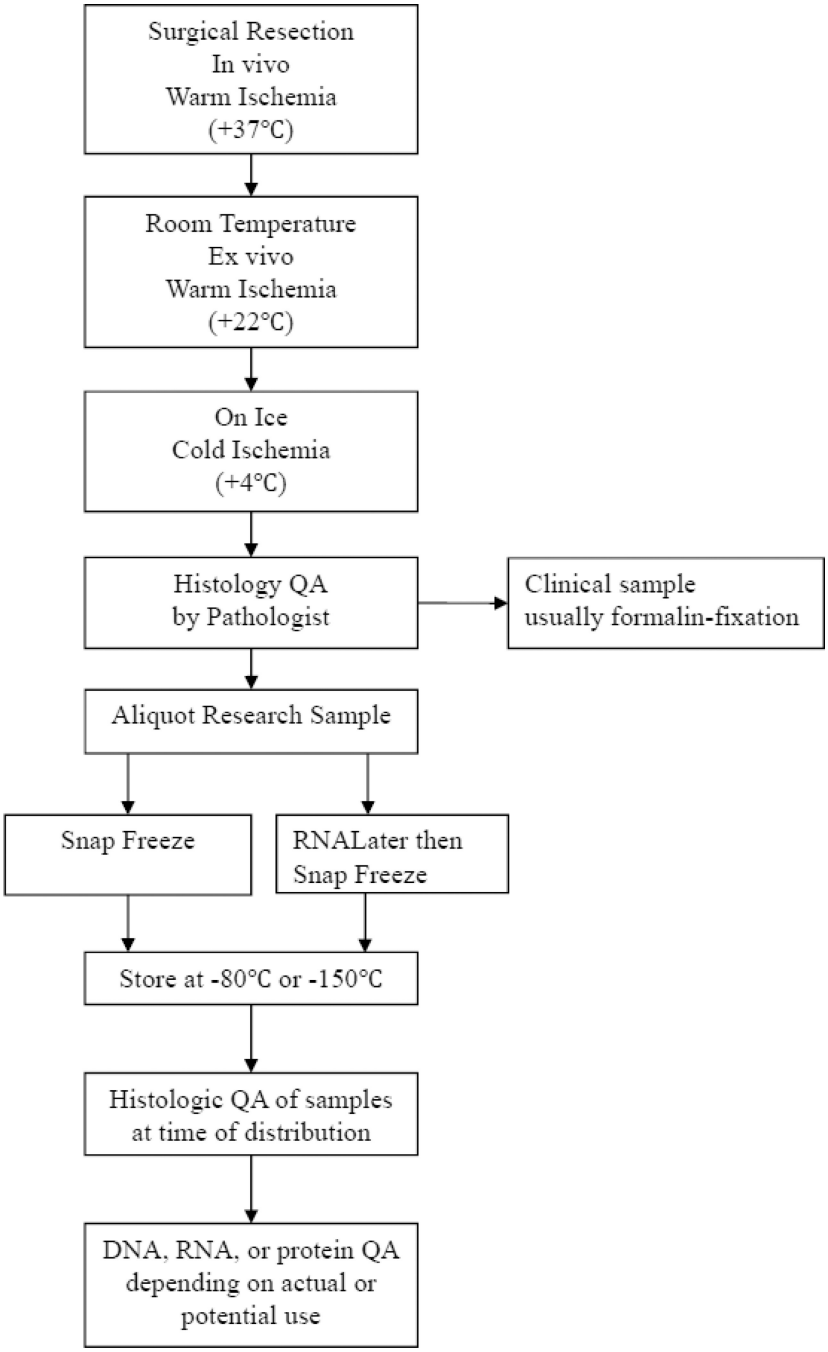

Figure 1.
Surgical tissue frozen biospecimen workflow

Table 1

Refrigerator and freezer capital and operational costs

Freezer	+4 °C	−20 °C	−80°C		−150°C	
	Refrigerator A	Freezer B	Freezer C	Freezer D	Freezer E	Freezer F
Purchase price*	~3300 USD	~ 2100 USD	~9700 USD	~7000 USD	~24000 USD	~20000
Effective capacity	20 Cubic feet (566 Liters)	20 Cubic feet (566 Liters)	25.7 Cubic feet (728 Liters)	13.4 Cubic feet (379 Liters)	10.3 Cubic feet (291 Liters)	8.2 Cubic feet (231 Liters)
Annual energy costs**	419 USD	450 USD	538 USD	680 USD	2499 USD	1485 USD
Annual liquid Nitrogen costs*	NA	NA	1088USD	1088USD	1088USD	1088USD
Annual CO_2 costs*	NA	NA	28 USD	28USD	NA	NA
Energy consumption***	11.5 kWh/day	12.4 kWh/day	15.1 kWh/day	19.1 kWh/day	70.2 kWh/day	40.8 kWh/day
Energy consumption per cubic feet	0.57 kWh/day/ft^3	0.62 kWh/day/ft^3	0.58 kWh/day/ft^3	1.4 kWh/day/ft^3	6.8 kWh/day/ft^3	5.0 kWh/day/ft^3
Carbon emission******	7.1 kg CO_2/day 2592 kg CO_2/yr	7.7 kg CO_2/day 2810 kg CO_2/yr	9.37 kg CO_2/day 3422 kg CO_2/yr	11.88 kg CO_2/day 4329 kg CO_2/yr	43.6 kg CO_2/day 15911 kg CO_2/yr	25.3 kg CO_2/day 9247 kg CO_2/yr

* The costs are approximate and may vary significantly

** Assuming about 10 UScents/kWh (U.S. Energy Information Administration national electricity retail price average [106])

*** One kilowatt hour (kWh) is equivalent to 3.6 million joules

**** Using a conservative ratio of 1.37 lbs (0.621 kg) CO_2 per kWh [107] used by the U.S. Energy Information Administration