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Procurement of Human Tissues for Research Banking in the Surgical Pathology Laboratory: Prioritization Practices at Washington University Medical Center

Sandra A. McDonald,1 Rebecca D. Chernock,2 Tracey A. Leach,2 Ajaz A. Kahn,2 James H. Yip,2 Joan Rossi,3 and John D. Pfeifer2

Academic hospitals and medical schools with research tissue repositories often derive many of their internal human specimen acquisitions from their site’s surgical pathology service. Typically, such acquisitions come from appropriately consented tissue discards sampled from surgical resections. Because the practice of surgical pathology has patient care as its primary mission, competing needs for tissue inevitably arise, with the requirement to preserve adequate tissue for clinical diagnosis being paramount. A set of best-practice gross pathology guidelines are summarized here, focused on the decision for tissue banking at the time specimens are macroscopically evaluated. These reflect our collective experience at Washington University School of Medicine, and are written from the point of view of our site biorepository. The involvement of trained pathology personnel in such procurements is very important. These guidelines reflect both good surgical pathology practice (including the pathologic features characteristic of various anatomic sites) and the typical objectives of research biorepositories. The guidelines should be helpful to tissue bank directors, and others charged with the procurement of tissues for general research purposes. We believe that appreciation of these principles will facilitate the partnership between surgical pathologists and biorepository directors, and promote both good patient care and strategic, value-added banking procurements.

Introduction

Barnes-Jewish Hospital is a large tertiary-care teaching hospital affiliated with Washington University School of Medicine, with ~55,000 surgical pathology specimens annually. These specimens represent a complex variety of common and rare tumors and other diseases of interest for tissue procurement, and nondiseased tissues useful as controls. Consequently, appropriately consented tissue discards from this service are a valuable and commonly accessed resource, which support the Tissue Procurement Core (TPC) at Washington University School of Medicine. The TPC contains ~400,000 diseased and normal specimens that are derived from a wide variety of clinical trials and collection protocols, of which one is the general banking protocol from surgical pathology described here. The TPC supports a wide variety of translational and other research programs at the School of Medicine on a request-driven basis. Quality control measures (among them histologic review, and DNA and RNA quality assessments) are used when specimens are disbursed, to assure that banked specimens have an acceptable degree of integrity and readiness for use. During the past year, ~2000 new fresh-frozen tissue specimens were accessioned into the biorepository from the general surgical pathology laboratory.

Tissue banking procurements in the surgical pathology setting come from surgical resections for diseased tissue, whose quantity exceeds that needed for diagnosis and patient care. Nondiseased tissue may be included in the resected specimen, and this is an opportunity for banking also. Real-time specimen documentation and transport mechanisms in place at Barnes-Jewish Hospital help maximize the proportion of surgical specimens that go from the operating room to the laboratory’s banking process in 30 min or less, and thus can be snap-frozen with minimal degradation, and maximum preservation of labile nucleic acids. Surgeons are asked to send nonbiopsy specimens fresh whenever possible (ie, without formalin in the container), to preserve the option for banking. On arrival, specimens are prioritized for dissection jointly by the transport personnel and full-time pathologists’ assistants, to further increase efficiency, and minimize the time lapse. Since the actual transport time is

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usually well under 30 min, the prioritization mechanism and pathologists’ assistant support ensures that even with time for examination, margin ink, and so on, many specimens can be sectioned and banked within 30 min.

Tissue for banking is typically taken when the specimen is “grossed in” (ie, specimen examined and dissected in the gross room, a macroscopic description given, and sections from key foci taken for microscopic evaluation). Essentially, each tissue area or component within every specimen is subjected to a 3-way decision: to sample for diagnosis (surgical pathology), to sample for research banking, or to do neither, in which case the tissue is typically placed in formalin and stored for several weeks before being discarded. A 2-methylbutane —50°C cryobath (Shandon Lipshaw Inc, Pittsburgh, PA) is present in proximity, so that freshly procured tissue for banking may be frozen rapidly, with minimal time delay relative to its receipt in the laboratory. Cryobath-frozen specimens are then transported in regular intervals on dry ice to the tissue repository lab, by technologists from that lab.

Comprehensive summaries of gross evaluation and dissection practices exist for surgical specimens, yet these rarely if ever reflect the point of view of biorepositories, who naturally impose coexisting demands on the same tissue resource, and who have interests and objectives that are often distinct from those of diagnostic surgical pathology (Table 1). Because the latter is a complex science with its own organ-specific standards of practice, the involvement of appropriately trained personnel is required for good procurement decisions (ie, where to take samples from, and how much to take). Doing so will promote good patient care, by ensuring that sufficient tissue remains for diagnosis, while also enhancing the probability that the areas selected for banking will be useful for research. For pathology trainees, learning good tissue bank practices will enhance their general surgical pathology skills, and help them learn to prioritize competing needs for tissues—a challenge likely to figure prominently in research and practice environments of the future.

At our institution, we have found it helpful to deliver these gross room principles in a seminar offered to relevant personnel in anatomic pathology (including new resident trainees rotating on the service), as well as in an easily accessible written protocol. Pathologists’ assistants, residents, and faculty pathologists contribute to specimen evaluation and the procurement process, and decisions regarding diagnostic needs and tissue banking. Senior surgical pathologists and tissue repository scientists are also available to provide supervision and guidance as needed. In many cases, the involvement of pathologists with subspecialty expertise (a common resource at larger institutions such as ours) contributes further value.

In this article, we summarize organ- and tissue-specific guidelines that reflect our surgical pathology-based tissue banking practice, with the expectation that the guidelines will be useful to other groups and institutions as an introduction to the field, or for comparison of best practices. The emphasis here is on gross evaluation at the time the surgical specimen arrives in the laboratory, when the decision for tissue banking is made. Microscopic evaluations that contribute to the science of tissue banking have been separately described. The relevance of the various points to other laboratories will of course depend on the focus and objectives of one’s tissue repository. The principles here mainly apply to the collection of tissue for general future banking endeavors, where tissue that would otherwise be discarded is banked, and then disbursed in a de-identified fashion to investigators. Tissue procurements driven by a clinical trial protocol, or as the sole objective of a specified research study, can deviate somewhat from these practices, depending on the stipulations in the protocol.

At our institution, there is an emphasis on translational studies requiring well-preserved snap-frozen tissue. Repositories that support mostly paraffin tissue-based work, such as immunohistochemistry for protein localization, may be able to employ less stringent procedures (especially relating to collection timeframes and the need for unfixed snap-frozen tissue) than those described here.

**Discussion**

There are several broad principles that characterize the practice of general tissue banking at our institution. The most important is that tissue for clinical diagnosis has the highest priority. Thus, only tissue that is absolutely not needed for clinical diagnosis should be taken for general banking. Once a procured sample is accessioned into the biorepository, it leaves the College of American Pathologists–certified environment, thereby hindering the ability to retrieve the tissue for clinical diagnosis later. Another consideration is that banked tissue is often subsequently chemically digested, such as for DNA or RNA isolation, and so its microanatomic structure may be lost by downstream procedures and not be able to be reconstructed. Thus, it is important to make the right procurement decisions in the grossing room initially.

Also, selection of the most optimal sample location (ie, most representative of the disease process, having the least hemorrhage or necrosis, etc.) yields the best downstream results, and thus enhances the positive impact on translational and other research programs. Again, as with the first principle, it is important to make the right decisions initially, since procedural reversals such as the retrieval of tissue may pose obstacles. For example, because surgically resected tissue not submitted for banking or sectioned for diagnosis is typically stored in formalin, it loses the research value that it once had as fresh tissue.

A third overarching principle is that good, consistent communication is needed between the clinical staff (including surgeons) and the surgical pathology laboratory. This is critically important for all aspects of good pathology practice—including information on surgical specimens that facilitates good diagnostic and procurement decisions.

In discussions with resident trainees and grossing room personnel, we have found it helpful to emphasize the general contraindications—both absolute and relative—that exist for banking tissues from surgical pathology specimens. As a corollary, any situation not fitting a contraindication is then generally understood to be one where tissue can be procured for banking.

Table 1 summarizes the key issues, which are described in more depth below. Also, this table summarizes the often competing viewpoints of surgical pathologists and tissue banking personnel regarding these issues, and (in our view) what represents the optimal resolution for them.
**Table 1. Contrasting Viewpoints of Surgical Pathology and Tissue Banking for Gross Specimens**

<table>
<thead>
<tr>
<th>Situation/issue</th>
<th>Surgical pathology viewpoint</th>
<th>Tissue banking viewpoint</th>
<th>Optimal reconciliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsies and other small cases (liver, gastrointestinal and skin biopsies, melanoma excisions, etc.)</td>
<td>Usually necessary to submit and process all available tissue for diagnosis</td>
<td>Some cases might have research value, but others are too small for long-term banking value.</td>
<td>Except in unusual circumstances (eg, research protocol), do not submit these tissues for research banking.</td>
</tr>
<tr>
<td>Surgical margins of resection</td>
<td>Necessary to preserve these areas for diagnosis and potential clinical significance</td>
<td>Surgical margins contain “adjacent normal” tissue that might be valuable for comparison purposes; however, other adjacent normal tissue may exist.</td>
<td>Diagnosis has priority; surgical margin areas should not be submitted for banking. “Adjacent normal” tissue, if desired, should be taken from other locations.</td>
</tr>
<tr>
<td>Specimens with obscuration of disease/nondisease interface</td>
<td>Sampling these areas for microscopic examination may be an interesting and important part of the pathologist’s evaluation and diagnosis.</td>
<td>Depending on objectives, these areas are less likely to be valuable since they may contain a lower cellularity % for the disease entity of interest.</td>
<td>A histologic quality assurance section can be done to clarify the relationship of gross to microscopic pathology, and the usefulness of the tissue for research.</td>
</tr>
<tr>
<td>Grossly visible areas of necrosis, hemorrhage, and adipose tissue</td>
<td>May be important to document their presence in the diagnostic process, but they are usually not of primary interest</td>
<td>These areas are rarely of research value.</td>
<td>Do not bank tissue from these areas, except in unusual circumstances.</td>
</tr>
<tr>
<td>Chemotherapy- or radiation-treated tumors</td>
<td>Very important to document residual, viable neoplasm if it is there</td>
<td>Tumors in this situation can be valuable opportunities for research banking because of the insight they offer in treatment resistance.</td>
<td>Only sample tissue for research banking if a sufficiently sized, grossly viable tumor mass is present, in excess of what is needed for diagnosis.</td>
</tr>
<tr>
<td>Specimens with collection time delays or preservational deficits</td>
<td>Specimens with &gt;30 min of warm ischemia time are still useful for surgical pathology evaluation.</td>
<td>Warm ischemia times of &gt;30 min are generally most problematic for tissues for which RNA isolation/evaluation is planned.</td>
<td>Decision about banking tissues with varying levels of warm ischemia time depends on research objectives. RNA integrity measurements can help.</td>
</tr>
<tr>
<td>Extensive tissue sampling needed to corroborate a previous biopsy diagnosis</td>
<td>For some disease entities, it is necessary to extensively sample resected tissue to confirm or further evaluate a previous biopsy diagnosis.</td>
<td>Disease entities of this nature are often interesting ones for research banking.</td>
<td>Tissue for diagnosis has priority; one should be very cautious about submitting tissue for research banking in this situation. Generally, previously banked tissue cannot be returned to diagnostic pathology.</td>
</tr>
<tr>
<td>Preservation/evaluation of key anatomic landmarks (capsular involvement, extranodal tumor extension, deepest tumor invasion, laryngeal landmarks, etc.)</td>
<td>Submission and evaluation of tissue from these areas is important for diagnosis, prognosis, and patient management.</td>
<td>Diseased tissue from these areas might be interesting for research banking purposes, though useful tissue can often be taken from other regions that are not critical for diagnosis.</td>
<td>Avoid sampling tissue for banking from these important foci, and take tissue from elsewhere if available. This requires good knowledge of surgical pathology principles. The optimal resolution here is situation dependent. Best research uses for tissues are downstream applications that are not hindered by decalcification procedures.</td>
</tr>
<tr>
<td>Bony lesions requiring decalcification</td>
<td>Decalcification is often needed before sections can be taken and evaluated (eg, osteosarcomas)</td>
<td>Decalcification treatments may be problematic for the downstream research value of the tissue, especially for samples that require quick snap-freezing and sectioning.</td>
<td></td>
</tr>
</tbody>
</table>

**Biopsies and other small cases where most or all tissue must be processed for diagnosis**

Most diagnostic biopsies, including gastrointestinal, gynecologic, brain, bone marrow, and liver biopsies, fall in this category. The reason is usually that all of the typically scarce tissue biopsy must be evaluated histologically to provide an accurate diagnosis. As a practical matter, these biopsies often have limited utility for long-term research banking due to their small volume, even aside from diagnostic considerations.
A related situation is tumors or disease types where, due to a combination of relatively small specimen or lesion size, and the need to detect focal but important findings that affect the diagnosis, it is standard practice to submit and evaluate all or most of the tissue of interest for diagnosis. Testicular germ cell tumors are one example; here, depending on the primary lesion’s size, it is often necessary to section and process all available tumors for diagnosis, so as to detect the various possible components of germ cell tumor (seminoma, mature and immature teratoma, yolk sac tumor, and others). These subtypes may exist in a wide variety of combinations, the specifics of which may influence diagnosis and prognosis.8,9 Primary cutaneous malignant melanomas are another example; here, unless the excised tumor tissue is very large, it is often necessary to entirely process and evaluate it, to evaluate tumor depth and thickness, growth pattern, histologic features, and approach to margins—all of which contribute to the diagnosis, and assist in prognosis and management.7,8 Key findings in these categories could be present in only a small part of the specimen, and one cannot risk that they might reside in the sample submitted for research banking. By extension, this principle also applies to any excised skin lesion for which malignant melanoma is part of the differential diagnosis.8

Finally, incoming tissue samples may sometimes be intended for a special study, such as immunofluorescence, culture, or flow cytometry. Often these samples arrive in special fixatives and/or with specific instructions. This represents an obvious further contraindication to banking.

Surgical margins of resection

Evaluation of surgical margins is a critical component of surgical pathology practice, especially in oncology, for many different specimens. We have described malignant melanomas, but in addition, margin evaluation is part of the examination of breast excisional biopsies, head and neck resections, vulvectomy, and gastrointestinal tract and liver resections, just to name a few categories.5 Because of the potential influence of margin evaluation on prognosis or subsequent clinical management, one should obviously not sample margin tissues for banking. In some cases, it is necessary to return to the gross specimen and re-sample margins after an initial microscopic evaluation, thus raising the importance of prioritizing these areas for diagnostic purposes. It is important that the surgeon inform the pathology personnel (through specimen orientation, marking sutures, face-to-face communication, or all of these) what areas constitute surgical margins, so that these foci are not inadvertently sampled for banking.

The surgical margin of gross specimens typically needs to be inked (on the exterior, grossly visible surface), before examination of the tissue’s interior and the taking of sections for diagnosis and banking. This is so that the margin surface(s) can be identified in histologic sections later on. The ink will also help one avoid margin areas when tissues for banking are chosen. The application of such specimen ink is most commonly done on receipt of a specimen into the surgical pathology laboratory.

Specimens with no grossly visible lesion, or where diseased and nondiseased areas cannot be clearly delineated grossly

This is a relative contraindication related to the common objective of procuring tissues that are enriched for the disease of interest. Samples where disease and nondiseased tissue cannot be clearly delineated grossly (especially for tumor vs. nonmalignant areas) are of less value since, if sampled purely according to the gross appearance, they are less likely to have a high cellular proportion for the entity of interest. Sometimes the gross pathologist will have good choices available as to where to sample tissue, but in other cases, gross obscuration of the disease/nondisease interface is an intrinsic part of the pathology. Examples of the latter include the alveolar growth pattern of bronchioloalveolar carcinoma coexisting with pneumonic consolidation,10,11 and prostatic carcinoma, which often shows discrepancies between the gross appearance and the actual extent of tumor.11 At our institution, for radical prostatectomies done for malignancy, we take one representative section from each lobe of the prostate for banking, rather than trying to choose areas based on the gross appearance.

For any grossly ambiguous specimen, one can do a microscopic evaluation, either at the time of procurement or later, and thereby judge the usefulness of the submitted area for banking or research. Such quality assurance procedures,4 especially estimating the % cellularity for the tumor of interest (ie, the percentage of nuclei in a section that are from neoplastic cells), are in fact a common practice in our tissue repository. A convenient situation arises when a frozen section for diagnostic purposes is taken adjacent to the area in question, thus providing a real-time indication as to the usefulness of that same area for banking. Tissues for which admixture of pathologic cells (or any entity of interest) and background cells is unavoidable may often be enriched for the former through laser capture microscopy.12

Some tumors, notably exocrine pancreatic adenocarcinoma, produce a diffuse fibrotic appearance and a low tumor % cellularity because of the intense desmoplasia that they commonly elicit.12 Also, a few disease processes (Hashimoto’s thyroiditis14 and some hemolymphoid tumors15) may involve entire organs (eg, thyroid and spleen) diffusely and relatively uniformly. These entities are useful for tissue banking, and represent an exception to the guideline that a distinct mass lesion or circumscribed abnormality should be seen before procuring “abnormal” tissue for banking.

Grossly visible areas of primarily necrosis, hemorrhage, or adipose tissue

Unless they are of primary scientific interest (which is uncommon), these areas likely will contribute little scientific value, since they are typically paucicellular and/or contain degraded cells and nucleic acids. Therefore, they should not be sampled for banking. Here, gross pathology skills are important in delineating regions affected by these processes. This may seem an obvious point, but in our experience, samples retrieved from the archives of some commercial tissue vendors sometimes show considerable amounts of these adverse features, including necrosis.4

An interesting subcategory consists of chemotherapy- or radiation-treated tumors with resulting treatment effect. Here, tissue should not be submitted for banking if all that is visible in the excised specimen is necrotic tissue with no grossly visible tumor mass—microscopically visible residual tumor might be missed, and the specimen might have little research value anyway. However, if sufficiently sized, viable residual neoplasm is present, it can represent a valuable opportunity for tissue banking, because it can yield molecular insights into treatment effect and/or resistance.16,17
Specimens with collection time delays or preservational deficits

Caution should be used in banking tissues as fresh-frozen specimens that have been delayed >30 min past their procurement time in the operating room, though specimens with longer times can be banked and the delay noted in the database. This is because of the adverse effects that delayed processing of fresh tissue may have, particularly on RNA quality, and gene expression profiles. Naturally, surgical pathology services with good documentation of collection and transport times are better able to determine tissue acceptability based on such criteria. At our institution, we also discourage the use of tissue for banking that has been freeze-thawed, or frozen slowly (eg, fresh tissue placed directly into a −80°C freezer), because of potential adverse effects on both histologic and molecular preservation. For some tissues and situations, RNA has been demonstrated to be stable after delays of a few hours at room temperature before processing; however, differences in RNA stability across various tissue types limit the applicability of these studies. Quality assessment procedures, such as those for RNA integrity, may be helpful if one is considering using frozen banked tissue for which the collection or preservation conditions were questionable.

Specimens where extensive tissue sampling is needed to corroborate a previous diagnosis, or to look for invasive tumor within established atypia or in situ neoplasia

Practically speaking, a common surgical pathology objective is to confirm the presence and extent of disease in a surgical specimen, and compare the findings to a previous biopsy on which the decision for surgery was based, either in whole or in part. For some disease entities, this may require generous sampling and histological processing of surgical specimen tissue, with relative contraindications to tissue banking. Examples include ductal breast carcinoma _in situ_, lobular breast carcinoma _in situ_, and atypical endometrial hyperplasia, which are often diagnosed in biopsy specimens. Not only must the pathologist generously sample the surgically resected tissue to document the extent of the biopsy-diagnosed disease, but the pathologist must also look for occult invasive malignancies that are etiologically related (for the entities above, these respectively are invasive ductal breast carcinoma, invasive lobular breast carcinoma, and endometrial adenocarcinoma). Such invasive foci are clinically significant, but might be occasional or rare within a given specimen. In some cases, it is necessary to return to the gross specimen and submit more tissue for evaluation, based on negative microscopic findings in a prior set of sections. Thus, sampling for diagnosis in these situations may need to be extensive, and procurements for tissue banking should proceed with significant caution, if at all. The size of the resected specimen will influence this decision to some extent.

Preservation of other landmarks important for surgical pathology evaluation

Surgical pathology evaluation is replete with organ- and site-dependent landmarks that affect the pathologic stage, influence the prognosis or advisability of further surgery, or exert other consequences. It is important to avoid sampling these landmark foci for banking, but instead preserve them for clinical diagnosis. A complete discussion of all potentially relevant topics is beyond the scope of this article; however, the tissue repository director who works with a surgical pathology service should be familiar with the more common situations discussed below.

Capsular involvement for follicular thyroid masses and other lesions. Thyroid masses are a common surgical specimen and an attractive candidate for tissue banking. However, for encapsulated follicular masses—a key subtype—tissue banking should proceed with great caution to avoid the capsule, since detection of capsular invasion affects the diagnosis and prognosis of the lesion, and thus the capsule must be entirely submitted or at least extensively sampled. Thorough diagnostic sampling of thyroid follicular masses is important also because of other changes, including vascular invasion and tumor histopathology, that affect diagnosis and/or prognosis. Diagnostic sampling is also important for the documentation of capsular penetration for renal tumors, and for certain encapsulated adrenal and salivary gland tumors; therefore, tissue banking efforts should avoid sampling these areas.

Extranodal extension of tumor. Metastatic tumors, including those in lymph nodes, are good candidates for tissue banking because of the potential insight they offer into cancer biology. However, extranodal extension of such tumors (ie, penetration of tumor through the lymph node capsule into surrounding soft tissue) is a key prognostic anatomic landmark for various organs and sites. Therefore, such areas need preferentially to be sectioned for clinical diagnosis (to the extent they can be identified grossly) rather than included in a tissue banking sample. Larger lymph nodes containing metastatic tumor may have enough tissue for the requisite surgical pathology sections, including the lymph node capsular areas needed to assess the presence of extranodal extension, while also allowing a banking specimen from excess tissue elsewhere.

Areas of deepest tumor invasion. This is a key landmark that affects staging and prognosis for a variety of tumors, including skin, gastrointestinal tract, urinary bladder, and endometrium. Thus, areas of deepest invasion should be sectioned for diagnosis and documentation purposes, and not used for tissue banking procurement. This of course requires careful gross dissection skills in identifying and sectioning the relevant areas.

Stalk in colonic adenomatous polyp. Adenomatous polyps of the colon are a very common surgical pathology specimen. Sometimes they are large enough to be tempting as a tissue banking specimen. However, the pedunculated stalk of these polyps should not be inadvertently sampled for banking, since incipient cancers in an otherwise benign-appearing polyp can be detected in this area. Such a finding may establish a need for close clinical follow-up and/or more aggressive management, especially in situations where the invasive tumor is deep within the stalk, shows lymphvascular invasion, or reaches the stalk’s surgical margin.

Laryngeal dissections. Though far from the only site in this category, the larynx deserves mention because of the large number of anatomic landmarks, for which tumor involvement (if present) needs to be sectioned for staging and documentation purposes: false and true cords, aryepiglottic folds, ventricles, subglottic extension, paraglottic and preepiglottic
space and cartilage invasion, and others. Thus, tissue taken for banking should not obscure the demonstration of these features in sections taken for surgical pathology evaluation.

**Bony tumors requiring decalcification**

Some tumors produce bone as an intrinsic part of their growth, either due to their primary lineage (eg, osteosarcoma) or as an unusual metaplastic feature of an otherwise soft, cellular tumor (eg, metaplastic breast carcinoma). Depending on the tissue’s resulting hardness and nature of the intended studies, taking samples for banking may be problematic—especially if immediate snap-freezing and subsequent tissue sectioning is desired, since decalcification steps are typically needed to soften the tissue sufficiently for research procedures. The usefulness of such tissue for fixed paraffin-tissue banking can be compromised by the possible denaturation or loss of proteins of interest from the decalcification procedure.

**Conclusion**

Consideration of surgical pathology practice requirements, especially in a tissue- and situation-dependent fashion, will enhance the quality of both clinical diagnosis and the utility of appropriately consented specimens that are procured and accessioned into a research tissue repository from the surgical pathology laboratory. Tissue bank directors should establish good working relationships with surgical pathologists, and understand the key principles and challenges of the latter group’s practice. The points summarized in this article may be useful as an introduction to the field, as a substrate for strategic operating protocols, or as information for trainees. Optimal implementation requires the involvement of trained pathology personnel within the laboratory. The contraindications listed here notwithstanding, there are many useful tissue banking specimens that can be procured from a typical surgical pathology service, and we encourage tissue banks to fully utilize such sources when possible. We invite comparison of our best practices with those at other institutions.

**Author Disclosure Statement**

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**References**


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