Tissue Handling and Specimen Preparation in Surgical Pathology

Issues Concerning the Recovery of Nucleic Acids From Formalin-Fixed, Paraffin-Embedded Tissue

Stephen M. Hewitt, MD, PhD; Fraser A. Lewis, PhD; Yanxiang Cao, PhD; Richard C. Conrad, PhD; Maureen Cronin, PhD; Kathleen D. Danenberg; Thomas J. Goralski, PhD; John P. Langmore, PhD; Rajiv G. Raja, PhD; P. Mickey Williams, PhD; John F. Palma, PhD; Janet A. Warrington, PhD

• ***Context.*—Expression profiling by microarrays and real-time polymerase chain reaction–based assays is a powerful tool for classification and prognostication of disease; however, it remains a research tool, largely reliant on frozen tissue. Limiting the utility of expression profiling is the isolation of quality nucleic acids from formalin-fixed, paraffin-embedded tissue. The collection, handling, and processing of tissue directly impacts the biomolecules that can be recovered from it. High-quality nucleic acids can be obtained from formalin-fixed, paraffin-embedded tissue, but greater attention to all steps in the process of tissue handling and preparation is required.**

***Objective.*—To summarize the current state-of-the-art of preanalytic factors in tissue handling and processing as they impact the quality of RNA obtainable from formalin-fixed, paraffin-embedded tissue. The goals are to provide recommendations that will improve RNA quality for expression profiling from formalin-fixed, paraffin-embedded tissue and highlight areas for additional research. Tissue is an analyte and it must be handled in a standardized fashion to provide consistent results.**

***Data Sources.*—The literature was reviewed. Consultation with industry and academic leaders in the use of RNA for expression profiling was obtained to identify areas for additional research.**

***Conclusions.*—Development of RNA-based assays from formalin-fixed, paraffin-embedded tissue is feasible. Greater attention to tissue handing and processing is essential to improve the quality of biospecimens for the development of robust RNA-based assays. Standardization of procedures and vigorous testing of alternative protocols are required to ensure that these assays function as designed.**

(*Arch Pathol Lab Med.* 2008;132:1929–1935)

Nucleic acid–based diagnostics are of great utility in molecular medicine but have been slower to reach the clinic than had been anticipated. The success of these platforms as biomarkers of disease is dependent on their reduction to an assay that is reproducible and widely applicable.[1,2] One hurdle in the introduction of nucleic acid–based diagnostics is the nature of the specimen from which the nucleic acids are obtained. Most hospitals and clinics lack the infrastructure to store and archive frozen tissue for isolation of RNA and DNA.[3] For tissue-based nucleic acid assays to enter the clinical setting, nucleic acids must be obtainable through the current practices of diagnostic histopathology. The current practice of specimen preparation is diverse and lacks strict standardization or well-defined standard operating procedures. As a result, significant diversity is found in the quality of the analyte source, namely, formalin-fixed, paraffin-embedded (FFPE) tissue.

This report summarizes the challenges the field faces and provides guidance on how to accomplish the goal of standardization. By providing this information to investigators in the clinic, pathology laboratory, and research environments, the collection of tissue in clinical trials, as well as in routine medical care, can be improved. The goal is to elevate the quality of FFPE tissue obtained in clinical trials as the basis for the discovery and validation of new biomarkers. Central to these efforts is the appreciation that tissue is no longer used only for microscopic interpretation of histopathology, but also for molecular assays of both nucleic acids and proteins. With the introduction of molecular assays, tissue becomes an analyte, whose spec-

Accepted for publication July 10, 2008.

From the Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Md (Dr Hewitt); Histopathology and Molecular Pathology, the Leeds Teaching Hospitals NHS Trust and Pathology and Tumour Biology, the University of Leeds, Leeds, United Kingdom (Dr Lewis); Applied Biosystems, Foster City, Calif (Drs Cao and Conrad); Genomic Health, Inc, Redwood City, Calif (Dr Cronin); Response Genetics, Inc, Los Angeles, Calif (Ms Danenberg); Expression Analysis, Inc, Durham, NC (Dr Goralski); Rubicon Genomics, Inc, Ann Arbor, Mich (Dr Langmore); the Molecular Devices Corporation, Sunnyvale Calif (Dr Raja); Roche Molecular Systems, Pleasanton Calif (Dr Williams); Veridex, LLC, a Johnson & Johnson Company, San Diego, Calif (Dr Palma); and Affymetrix Inc, Santa Clara, Calif (Dr Warrington).

Dr Warrington is an employee and shareholder of Affymetrix Inc. The other authors have no relevant financial interest in the products or companies described in this article.

Reprints: Stephen Hewitt, MD, PhD, TARP Lab, Advanced Technology Center, National Cancer Institute, MSC 4605, Bethesda, MD 20892-4605 (e-mail: genejock@helix.nih.gov).

ification of quality is an element of the validated assays performed with it.

The Paraffin Embedded Working Group is a self-identified group of researchers with the common goal of improving the quality and methodologies for the extraction of nucleic acids from paraffin-embedded tissue. The group is sponsored by Affymetrix (Santa Clara, Calif) as a part of its Standards Program, dedicated to advancing international efforts in the development of standard controls and best practices. Members of the group are from academia, government, and industry worldwide. Their recommendations focus on the specification of the analyte, but do not address the isolation and quality metrics that are incumbent for FFPE tissue to be routinely used as a source of RNA for RNA-based analysis in a clinical setting.

Nucleic acids from FFPE tissue can be used in discovery and validation of biomarkers. The reproducible artifact of formalin fixation applies not only to histology and cytology, but also to the preservation of DNA, RNA, and proteins. Currently, appreciation of quality is subjective; however, for quantitative assays, quality must be objective and quantifiable. From the clinical laboratory perspective, no assay can be validated until the analyte has been specified. Currently, the specifications for fixation and handling of FFPE tissue are inadequate and result in widely variable and poorly understood differences in the recovery of biomolecules for diagnostic assays. This problem plagues not only nucleic acid–based assays, but also protein-based assays, including immunohistochemistry, as researchers strive to develop predictive assays.

Our goal is to educate the biomedical community in an effort to improve the quality of RNA that can be recovered from FFPE tissue. In designing a clinical trial, it is no longer acceptable to state that ''tissue will be collected by 'standard protocol''' when in fact the protocols are not standard between hospitals and do not meet the demands of biomedicine. Although pathologists and pathology laboratories are central to improving and assessing the quality of nucleic acids obtained from FFPE tissue, the biomedical community at large must play a role in ensuring that quality is maximized.

Our recommendations fall into 3 broad categories: (1) recording of information so that data can be compared, (2) defining best practices to improve quality and reproducibility, and (3) identifying areas that require additional research. Only the combined efforts of those who design assays, design clinical trials, and care for patients will move RNA-based assays forward in the areas of research and diagnostics. These recommendations are only one step on the path to redefining FFPE tissue as an analyte for molecular assays. Implementation of the recommendations contained in the report is not trivial; however, these can be carried out and tested in clinical trials. The following is a step-by-step analysis of the process of tissue collection, fixation, and processing as it relates to FFPE tissue.

THE PROCESS OF TISSUE COLLECTION AND HANDLING

From the Patient to the Laboratory: Issues Impacting RNA Quality Before Specimen Acquisition by the Pathology Laboratory

The first steps in the process of sample handling are challenging to control, but impact the quality of the specimen. These include the times of anesthesia administration, ligation of vessels, and specimen removal from the patient.[4–6] These factors cannot be strictly controlled because they impact patient care; however, a detailed recording of these times are an important metric of tissue quality as they affect the quality of the resultant biomolecules. The most commonly described impact on tissue quality is the ''warm ischemia time'' from when the blood supply is ligated until the specimen is received by the pathologist for procurement. Ischemia time can vary from minutes to hours depending on the organ, the surgical approach, the surgeon, nursing staff, and standard operating procedures of the institution. Studies have demonstrated that both RNA and protein changes occur during this time.[4] The magnitude of these changes is poorly understood. Efforts to shorten, and, at a minimum, to record this time will allow investigators to determine which genes are potentially unreliable biomarkers.

We recommend that all protocols include the recording of the times for the administration of anesthesia, ligation of the vascular supply, and removal of specimen from the patient's body. These times should be included as part of the record of submission of the surgical specimen to the pathology department. For biopsies, time of specimen removal should be recorded and communicated to the pathology department.

Tissue Fixation and Tissue Dissection and Preparation

Microscopic examination of ''permanent sections'' obtained from fixed and embedded tissue is the ''standard of care.'' Frozen sections, although used for intraoperative consultation, are not sufficient for definitive diagnosis. Preparation of microscopic slides for histopathologic examination requires the preservation of the tissue through chemical means by a process called *fixation.* Fixation is the first step in tissue processing and is essential.

The manner in which the specimen is prepared has a dramatic impact on the results.[7] The failure to appreciate and standardize these steps poses problems for the researcher who works with the resultant RNA. The fixation step entails 3 elements: thickness of tissue, volume of fixative, and time. Failure to optimize all 3 of these elements results in underfixation or overfixation of the tissue.[8,9] Incompletely fixed or overfixed tissue introduces a significant source of variability. Both overfixation and underfixation result in degradation of the specimen after paraffin infiltration, and they hinder diagnosis by the pathologist by altering histomorphology and immunoreactivity.[5] Nucleic acids obtained from both underfixed and overfixed tissues are suboptimal for molecular analysis. Both of these conditions result in shorter nucleic acid fragments upon isolation (F.A.L., M.C., and S.M.H., oral communication, March 2004).

Formalin penetrates tissue at an average rate of 1 mm/h,[6] but this rate can vary depending on tissue type. Methods to speed this process have been suggested,[10] but they lack feasibility for widespread use. There is large diversity in the size of surgical specimens. Needle biopsy specimens are small and fix rapidly compared to larger, denser tissue samples, including excisional biopsy specimens and removed organs. Most needle biopsy specimens are about 1.5 mm in diameter and 20 mm in length, whereas excisional specimens range from 1 cm in diameter to larger diameters for specimens weighing 1 or more kilograms. The greatest challenge is the fixation of large surgical

specimens, frequently the ''organ-type'' specimen. Only a small portion of these specimens is subjected to microscopic examination. It is essential that these large specimens be immediately dissected and sectioned into appropriately sized pieces of tissue to ensure they are properly fixed. Too often, the entire specimen awaits dissection and sectioning or is fixed in toto, which results in less than optimal fixation. The length of time a tissue remains in fixative has become an issue in sample preparation for surgical pathology.[11] Standard fixation times are a minimum of 5 hours for needle and endoscopic biopsy specimens and 12 or more hours for sections from larger specimens. These times are required for complete fixation of the specimen.[6] There is unjustified pressure to decrease the time from when tissue is removed until its final diagnosis, which puts the quality of histomorphologic diagnosis at risk. Reports have described microwave and ultrasound fixation with a variety of fixatives to speed the fixation of tissue.[6,10] The effects on the recovery of biomolecules from specimens exposed to microwaves are not well documented and require further investigation. Alternatively, overfixation, the prolonged exposure of tissue to formalin, inhibits the recovery of nucleic acids. The conditions that lead to overfixation are less well understood; however, fixation for more than 36 hours decreases the quality of biomolecules from FFPE tissue.[12]

The volume of fixative varies widely during the handling of surgical specimens. The minimum formalin:tissue ratio is 10:1. Biopsy specimens, especially needle and endoscopic, are placed in relatively large volumes of 10% formalin.[6] The volume of fixative is a problem for large surgical specimens, especially those weighing more than 100 g. Optimally, sections should be taken from the fresh specimen and allowed to fix after dissection. Unfortunately, it is a common practice to fix many specimens overnight to facilitate their dissection or because they arrive late at the pathology department. The lack of sufficient fixative presents the same problems as do thicker specimens, both for the diagnosis and the recovery of biomolecules from the specimen. Optimal fixative:specimen ratios are only of benefit if the tissue is sectioned properly.

We recommend that biopsy specimens be placed immediately in fixative and that larger specimens be dissected and sectioned as rapidly as possible to shorten the ''warm ischemic time'' between removal of specimen from the patient and fixation. The length of time from specimen removal to fixation and the time spent in fixative should be recorded. Fixation times should be standardized to ensure adequate fixation of the specimens: for biopsy specimens, 6 to 18 hours, and for surgical specimens, 12 to 36 hours.

Formalin: The Details

The most common fixative is 10% formalin, which is 3.7% formaldehyde in water. Formalin is a 2-phase fixative, with an initial alcohol fixation phase, followed by a cross-linking phase mediated by aldehydes. Although a number of alternative fixatives have been used that provide similar microscopic features for diagnosis,[13,14] formalin has remained the preferred fixative, with a decrease in the use of alternative fixatives. This dominance is driven by utility and cost. Many alternative fixatives are poor biomolecule preservatives (acid-containing fixatives) or else contain toxic heavy metals (B5 fixative).[15] Efforts have been made to use alcohol-based fixatives,[14,16,17] most notably 70% ethanol, but fixation artifacts have prevented adaptation of this approach for all specimens.[18]

Some researchers have advocated splitting a specimen into 2 sections and preserving it, either by freezing or fixation and embedding, to optimize the recovery of biomolecules. This approach is impractical. Tumors are not homogenous masses and are often impossible to identify by gross examination of the unfixed specimen. Without direct microscopic examination of the tissue specimen from which nucleic acids are isolated, issues of contamination and/or analysis of inappropriate tissue will limit applications of this strategy.[19] This fact is complicated by the desire to pursue molecular analysis on smaller lesions, especially biopsy material. Without complete microscopic examination of the specimen, the risk for inappropriate/incomplete diagnosis exists. Many biopsy samples are too small for reasonable division or for repeated sampling in biopsies of the breast and prostate.

The last decades have seen the introduction of buffered formalin preparations. Several different buffers are used, including calcium carbonate, magnesium carbonate, citrate, Tris, and phosphate buffers (pH $\sim$ 7.0).[20] Formalin lacking buffer has a limited shelf life and degrades rapidly. Data on the different buffers is limited; however, phosphate-based buffers appear to be superior to other common formulations for RNA recovery (J.-Y. Chung, PhD, unpublished data, June 2008). Neutral buffered formalin (NBF) has a longer shelf life. Tissue fixed with NBF yields consistently better quality RNA although good quality nucleic acids are obtainable from ''freshly'' prepared 4% paraformaldehyde. Buffers slow, but do not prevent, the degradation of formalin, and degraded formalin is believed to contribute to the poor quality of nucleic acids obtained from FFPE tissue. Degradation of NBF is dependent on storage conditions, including light and temperature.

Other elements and compounds can be added to formalin, including mercury compounds, zinc, EDTA, ethylene glycol tetraacetic acid (EGTA), and acids. These should be avoided as they can alter the ion balance in the tissue and may even impair reverse transcriptase and *Taq* polymerase function. The addition of acids for decalcification can result in the nicking of nucleic acids, thus reducing the lengths of fragments recovered. Both EDTA and EGTA have been reported to slow nuclease activity and can be used as decalcifying agents; however, their benefits on the recovery of nucleic acids are not well documented[19] and both can impact the analysis of phosphoproteins.[21] RNAlater (Ambion, Austin, Tex) improves RNA recovery; however, it cannot be mixed with formalin and additional time and extensive handling is required when it is used with fixed tissues.[22,23]

A challenge underscored by the current literature is the failure of researchers to adequately investigate the impact of alternative fixatives and the impact of additives to existing fixatives on histologic appearance or on the biomolecules isolated. For optimal comparisons, NBF should be used as a reference for histologic appearance and snap-frozen tissue as a reference for biomolecule quantity and quality.

We recommend that NBF be used as the routine fixative and that the buffer used be recorded. Additional research is required to determine the effects of different buffering agents. Formalin cannot be regarded as an unimportant

reagent and its quality should be determined and monitored.

Tissue Processing From Fixative to Paraffin

The process of embedding tissue with paraffin impairs the recovery of biomolecules but appears to have less impact on their quality, as determined by the process of handling and fixation.[12] The general processing steps include sequential *dehydration* from an aqueous environment to an alcohol environment (most often ethanol), subsequent replacement by xylene (or xylene substitute) in a process referred to as *clearing*, and replacement of the xylene with paraffin (*impregnation*). Typically, this process is completely automated, but it lacks standardization and depends on the instrumentation, specimens, and reagents used. Quality of reagents, time, and temperature[6] impact sample integrity. The duration for the complete process can vary from less than 4 hours to more than 12 hours. It is crucial that reagents be of high quality and be replaced on a regular basis. The alcohols and xylenes used in processing become diluted with carry-over from prior steps; as a result, tissue processed at a later time may not be completely dehydrated. The impact of time is similar to that of poor quality reagents or exhausted reagents. It is essential that tissue be completely dehydrated during processing, as residual water will not be replaced by paraffin, thus making the tissue susceptible to degradation.[6] Incomplete dehydration of tissue appears to be compounded by poor fixation. The mechanism is probably related to incomplete coagulation of proteins; as a result, water gets trapped within the tissue. Data concerning alternative reagents and a comparison to the common protocols are lacking. Controlled studies of alternative alcohols and clearing agents have not been carried out for the recovery of nucleic acids. Studies on protein suggest that differences do exist that require modification of downstream protocols.

We recommend that detailed records of processing procedures be maintained. Details about the times, temperatures, presence of vacuum and instrument type, as well as the reagents should be included. Accelerated tissue processing protocols require adequate studies to measure their impact on biomolecule recovery and stability. Reagent quality and replacement should be monitored. Studies comparing alternative reagents and processing conditions should be carried out and reported.

The Impact of Paraffin on Fixed Tissues

The paraffins used in impregnation and embedding vary and are chosen to meet the demands of the individual laboratory. Paraffins have different melting points and textures that impact the sectioning characteristics of the final blocks. Not only are a diversity of paraffins used, their exact compositions are often proprietary and/or contain beeswax of marked variation.[24] Synthetic paraffins with low melting temperatures (55°C–63°C) are typically used in the United States and Western Europe. These formulations may contain latex, dimethyl sulfoxide, and proprietary ''plasticizers'' that modify texture and malleability.[6] Beeswax, containing pollen and other contaminants, is routinely used in Eastern Europe, Africa, and South America to modify the melting temperature and improve the malleability of poor-quality paraffins. These paraffins interfere with the recovery of biomolecules.[25] The use of higher-melting-temperature paraffins results in decreased and inadequate deparaffinization and reduction in the amount of nucleic acids recovered.[23,25] The next step in tissue handling is embedding, the process of surrounding the paraffin-infused tissue with paraffin so that it can be easily sectioned on a microtome. Embedding has not been identified as a key factor impacting the quality of extracted biomolecules; however, there has been concern about the use of overly hot paraffins in the embedding process and their potential for degrading tissue.[6]

Low-melting-temperature paraffins are recommended for the impregnation of tissue. The type of paraffin should be recorded. Avoid the use of additives such as beeswax.

Block Storage and Sectioning

After a piece of tissue has been correctly fixed and processed, the block requires proper storage. The conditions associated with proper storage are simple and best described as those of an office-like environment. Humidity and temperature control are necessary, as storage in warehouse-like conditions leads to decreased recovery of biomolecules. Damage and/or destruction of paraffin blocks by insects or rodents can occur (S.M.H. and M. Robinowitz, MD, oral communication, September 2004).

Once tissue is embedded with paraffin, it appears to be stable for extended periods. Some investigators have noted a reduction in the quantity of nucleic acids recovered from older tissues, on the order of 5% to 50% for each decade of age.[26] It is unclear whether this reduction is a function of the time the tissue was embedded, the original quality of tissue processing, or the incremental changes in reagents and processes used in fixation. Many groups have found improved RNA recovery by disposing of the first sections of the block and using deeper sections for the isolation of RNA. Many laboratories routinely work with 20-year-old material without any problem,[26] and it is not unusual to encounter material that is older than 50 years. In fact, nucleic acids have been recovered from pathology museum specimens dating back to the early 20th century (Leeds) and to the 1918 flu epidemic.[27] The same is true for recovery of protein epitopes.

Sectioning of the block can increase the risk for impairing the quality of biomolecules, especially the recovery of nucleic acids. Contamination of specimens with a different tissue source has been identified by polymerase chain reaction, although strong data is lacking in this regard.[28] The impact of small amounts (<1%) of contaminants on an expression array are unknown; however, it is doubtful they would lead to spurious results. The consensus recommendation of many investigators is that disposable microtome blades be replaced when sectioning different specimens.

Storing unstained microscope slides is much more complex. The literature has extensively demonstrated that precut sections used for immunohistochemistry can degrade, even after a short time.[25,29,30] There has been controversy on the mechanism of this degradation, which is probably a combination of hydration effects and oxidation.[25,31] Degradation is markedly worse for poorly fixed and processed tissue, probably because of residual water in the tissue.[6] Refrigeration will not prevent degradation.[31] Investigators have advocated a variety of means for storage of cut sections. The most common method, dipping the slide in molten paraffin, is problematic because the paraffin is challenging to remove. Other investigators store slides under gaseous nitrogen, which displaces both humidity and oxygen. This method requires special equipment and is

Table 1. General Recommendations to the Biomedical Community at Large

- Additional research into the impact of tissue handling must be carried out to assess both general effects as well as tissue-specific differences.
- Additional evaluation of different buffering agents in formalin and their impact on the recovery of nucleic acids should be investigated.
- Alternative fixation protocols should be investigated to determine the impact on biomolecules and the equivalency of recovered nucleic acids.
- Investigations should be made into the effects of xylene substitutes and their impact on proteins and nucleic acids.
- Alternative processing systems should be used with caution, as their impact on the quality of nucleic acids is unknown. These processing conditions should be specifically documented. We call on manufacturers to broadly investigate the impact these instruments and processes have on biomolecules. We caution that tissue obtained with these systems cannot be analyzed by using the same parameters as those for other formalin-fixed, paraffin-embedded tissue until their impact is documented.

Table 2. Recommendations on the Annotation, Handling, Fixation, and Embedding of Tissue

- The times of the administration of anesthesia, ligation of the vascular supply, and removal of specimen from the patient's body should be included in the record of submission of the surgical specimen to the pathology department. For biopsies, time of specimen removal from the patient should be recorded and transmitted to the pathology department.
- Tissues should be fixed in buffered formalin, with quality-control procedures in place to prevent the use of degraded fixative. The type and concentration of buffer and the date the formalin was prepared should be recorded.
- Specimens must be dissected appropriately to allow adequate penetration of fixative. Tissue must be properly sectioned before processing, must weigh ≤1 g, and must be no larger than 1.5 × 1.5 × 0.4 cm.
- Adequate volumes of fixative are required (a minimum fixative: tissue ratio of 10:1 with 10 mL of fixative for every gram of tissue). Tissue must have sufficient time to fix completely: 5 hours for small biopsy specimens (<0.25 g), and 12 or more hours for larger sections (0.25 to 1.0 g of tissue specimen/section). Duration of fixation should be recorded, in 2-hour increments for biopsy specimens, and 4-hour increments for excisional specimens. Overfixation (>36 h) should be avoided.
- Tissue must be processed by using appropriate cycles to complete the dehydration process. Large specimens should be separated from biopsy specimens, if necessary. Processing times and protocols should be documented. Appropriate reagent replacement on automated tissue processors is essential, and the schedule should be documented.
- The use of low-melting-temperature paraffins is encouraged. Contamination with beeswax should be avoided. When other/unknown paraffins are encountered, it should be so noted. Type of paraffin used and melting point should be documented.
- Paraffin blocks should be stored in a controlled-temperature environment, protected from excessive humidity, dryness, and light. Optimal storage of cut slides is difficult to achieve. When long-term storage is essential, slides should be stored in gaseous nitrogen or under vacuum. For slides obtained from another facility, it is recommended that fresh sections be requested and used as rapidly as possible.
- Pathology laboratories must document with greater detail the reagents used and the conditions for tissue fixation and processing in the standard operating procedures of the laboratory.

not without hazard.[29] Some investigators store slides under vacuum. In a worst case situation, slides must be stored in enclosed slide cases, away from dust and direct light. Because it is a common practice to provide cut slides rather than the original block, storage and handling of cut sections is not a trivial issue.

Many researchers cut paraffin sections and place the ribbon of paraffin directly in a microcentrifuge tube. This approach prevents microdissection and can inhibit efficient deparaffinization. When some form of microdissection is required, the sections must be applied to a solid support, typically by floating the section in a water bath to obtain an unwrinkled section. Water baths immediately introduce water, the enemy to preservation for paraffin-embedded tissue. Despite concerns that water baths may become contaminated with RNases, this adverse effect has yet to be demonstrated. The thickness of the section used for recovery of nucleic acids appears to have little impact, and is more a matter of convention. The thickness of sections typically varies from 4 to 20 μm.

We recommend that fresh sections be cut from blocks for the isolation of nucleic acids. Blocks require storage in a temperature- and humidity-controlled environment. Previously cut sections should be avoided. Disposable microtome blades should be used to prevent cross-contamination.

CONCLUSIONS

The isolation of RNA from FFPE tissue is feasible. Unfortunately, current practices in the biomedical community vary and lack the standardization required for sensitive molecular interrogation. Fundamentally, tissue must be handled in a standardized fashion, similar to how blood and other body fluids are used in routine clinical assays. Analysis of tissue has been called *cellular chemistry*, and a specimen must meet a specification for the analysis to be consistent and reliable. Formalin-fixed, paraffin-embedded tissue is not currently handled in a fashion consistent with the protocols for analytes used in molecular analysis. Educating the community, including pathologists, clinicians and researchers, is essential for bringing RNA-based assays into the clinical setting (Table 1). Cooperation is needed to advance the design of protocols to maximize both research and patient benefit.

The quantity and quality of nucleic acids obtained from FFPE samples are inferior to those from frozen tissue, but with appropriate controls, equally important, utility can be obtained and validated. The recommendations presented here are congruent with those of other groups examining the handling of tissue for molecular assays and can be implemented without great effort by anatomic pathology departments in the United States or Western Europe. The general recommendations on tissue handling and fixation do not represent a departure from current recommendations (Table 2). Although the steps for recording information are new (Table 3), they should not be problematic to implement with the advent of computerized medical care, even if a reporting standard must be developed. Implementation carries a cost. Therefore, new economic models must be sought that will compensate for the cost associated with the improved quality of biospecimens as an element of improved personalized diagnostics. There is no point in investing in predictive assays if

Table 3. Parameters to be Reported for Results Obtained With Nucleic Acids Recovered From Formalin-Fixed, Paraffin-Embedded Tissue

- Fresh specimens
  - Date and time the specimen was removed from the patient
    - If devitalization of specimen (removal of blood supply) occurred more than 15 min before its removal from the patient, this additional information should be recorded
  - Date and time the specimen was received in the pathology department
  - Date and time the specimen was examined and prepared for fixation and processing by the pathologist or pathology assistant
- Biopsy specimens
  - Date and time biopsy was performed and specimen was placed in fixative
  - Date and time biopsy specimen was received in the pathology department
- All specimens
  - Fixative: type, concentration, and buffering agents
  - Total duration of fixation
  - Details of tissue processing conditions
    - Reagents and times spent in each reagent on the tissue processor
    - Type of tissue processor
    - Presence of heat, vacuum, or other elements of tissue processing (microwave, ultrasound)
    - Date tissue block was prepared
    - Conditions of block storage
    - Presence or absence of precautionary measures to avoid contamination by RNases during sectioning
  - Date the slide was prepared

the quality of the analyte is such that the assay is irreproducible.[32,33]

Different analytic approaches use different specifications for the quality of RNA suitable for analysis. Fundamentally, these issues concern transcript copy number and RNA integrity. Efforts are underway to better quantify these differences and to provide guidance on these issues.[34] Different endogenous factors impact RNA quality in tissue because of the presence of degradative enzymes, especially in pancreas, liver, and stomach. This fact emphasizes the importance of time in tissue handling and processing.[33] Further complicating these issues are the inherent complexity of tissue and the desire/need of some investigators to perform cell-type analysis, which requires some means of microdissection or in situ analysis.

Although frozen tissue is the gold standard for the isolation of biomolecules, the proposal of Medeiros et al[35] for creation of tissue banks with storage at −80°C is impractical for routine clinical care. Storage in mechanical freezers at −80°C is inadequate to maintain RNA integrity for long periods.[23] Storage and retrieval in vapor-phase liquid nitrogen freezers are expensive and technically complicated. These approaches cannot be implemented without significant cost to community hospitals and clinics, where most specimens are obtained. We anticipate that the discovery of new biomarkers from frozen tissue will continue, but that widespread use of these new biomarkers will require their application to FFPE samples. One challenge is anticipating which samples will be required for molecular analysis and in which time frame. Utilization of frozen tissue banks to address these molecular diagnostic issues is difficult at the current time, when less than 0.1% of specimens are subjected to molecular diagnostics. It is impossible to predict the diagnosis of a specimen or its ultimate requirement for a molecular diagnostic analysis before it receives a histopathologic diagnosis.

We believe that by better defining the process, setting specifications, and approaching tissue as an analyte for molecular analysis, the quality and reproducibility of RNA recovered from FFPE tissue will be enhanced, enabling nucleic acid–based biomarker discovery and validation. Working groups, such as ours, have already had an important impact on improving the quality of expression analysis and have provided a clear path toward its integration into clinical care.[34,36] Numerous groups have examined the processing of clinical tissue samples, and our recommendations are, in general, congruent with their findings. The goal is a single set of standards that provides a high-quality specimen for molecular analysis. Other groups that are actively examining these issues include the College of American Pathologists, the Clinical and Laboratory Standards Institute, as well as other ad hoc groups interested in immunohistochemistry. The National Cancer Institute is addressing these issues through the Office of Biorepositories and Biospecimen Research (http://biospecimens.cancer.gov, accessed June 24, 2008). New guidelines for tissue collection in clinical trials for breast cancer have been codeveloped by the Breast International Group and the National Cancer Institute.[37] One possible benefit derived from these efforts is the creation of references and standards that will allow investigators to better compare new approaches in tissue handling and preparation, as well as the impact of new isolation and analytic methods. Already some progress has been made by the MicroArray Quality Control project in comparing analytic methods.[34]

We encourage a standardized approach for reporting these metrics and hope it will be adopted and in widespread use. Inclusion of these checklists in clinical trial design will further our knowledge and hopefully lead to validated biomarkers of disease. Specification of tissue handling, fixation, and processing beyond the ''standard protocol'' will enhance quality. The biomedical community will benefit from additional recommendations based on research results demonstrating the quality of nucleic acids recovered from FFPE tissues. Issues regarding data analysis and subsequent recommendations specific to FFPE tissue–based assays need to be addressed. Many groups are currently working on overcoming the challenges posed by expression assays from FFPE tissue, and we anticipate that the body of knowledge on this important topic will continue to grow.

The quality of nucleic acids isolated from FFPE tissue will never match that of their counterparts recovered from fresh/frozen tissue; however, that is not the goal. The goal is to isolate nucleic acids that will provide the analyte for a new family of medical diagnostics.[37] This is feasible although many questions remain to be addressed. For instance, how to analyze FFPE-derived RNA remains an open question.

We thank Berkeley Breathed for inspiration and guidance in development of this manuscript. The authors also thank Max Robinowitz, MD; Cecil Fox, PhD; Till Braunschweig, MD; Yvonne Gathright; Joon-Yong Chung, PhD; Kimberly Tuttle; Langston Lim; Andrew Hruszkewycz, MD; Jean-Claude Zenklusen, PhD; and Robert Star, MD, for useful discussions.

References

1. Sullivan PM, Etzioni R, Feng Z, et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst.* 2001;93:1054–1061.
2. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med.* 2004;351:2817–2826.
3. Eiseman E, Bloom G, Brower J, Clancy N, Olmsted SS. *Case Studies of Existing Human Tissue Repositories: "Best Practices" for a Biospecimen Resource for the Genomic and Proteomic Era.* Santa Monica, Calif: Rand Corporation; 2003.
4. Dash A, Maine IP, Varambally S, et al. Changes in differential gene expression because of warm ischemia time of radical prostatectomy specimens. *Am J Pathol.* 2002;161:1741–1748.
5. Miyatake Y, Ikeda H, Michimata R, et al. Differential modulation of gene expression among rat tissues with warm ischemia. *Exp Mol Pathol.* 2004;77:222–230.
6. NCCLS. *Quality Assurance for Immunocytochemistry.* Approved guideline. Wayne, Pa: NCCLS; 1999. NCCLS document MM4-AC.
7. Prento P, Lyon H. Commercial formalin substitutes for histopathology. *Biotechnol Histochem.* 1997;72:273–282.
8. Rosai J. *Ackerman's Surgical Pathology.* 8th ed. St Louis, Mo: The CV Mosby Co; 1996.
9. De Marzo AM, Fedor HH, Gage WR, et al. Inadequate formalin fixation decreases reliability of p27 immunohistochemical staining: probing optimal fixation time using high-density tissue microarrays. *Hum Pathol.* 2002;33:756–760.
10. Chu WS, Furusato B, Wong K, et al. Ultrasound-accelerated formalin fixation of tissue improves morphology, antigen and mRNA preservation. *Mod Pathol.* 2005;18:850–863.
11. Macabeo-Ong M, Ginzinger DG, Dekker N, et al. Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analyses. *Mod Pathol.* 2002;15:979–987.
12. Goldstein NS, Hewitt SM, Taylor CR, Yaziji H, Hicks DG, and the Members of Ad-Hoc Committee On Immunohistochemistry Standardization. Recommendations for improved standardization of immunohistochemistry. *Appl Immunohistochem Mol Morphol.* 2007;15:124–133.
13. Wiedorn KH, Olert J, Stacy RA, et al. HOPE—a new fixing technique enables preservation and extraction of high molecular weight DNA and RNA of >20 kb from paraffin-embedded tissues: Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect. *Pathol Res Pract.* 2002;198:735–740.
14. Gillespie JW, Best CJ, Bichsel VE, et al. Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am J Pathol.* 2002;160:449–457.
15. Tbakhi A, Totos G, Pettay JD, et al. The effect of fixation on detection of B-cell clonality by polymerase chain reaction. *Mod Pathol.* 1999;12:272–278.
16. Titford ME, Horenstein MG. Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology. *Arch Pathol Lab Med.* 2005;129:502–506.
17. Goldmann T, Flohr AM, Murua Escobar H, et al. The HOPE-technique permits Northern blot and microarray analyses in paraffin-embedded tissues. *Pathol Res Pract.* 2004;200:511–515.
18. Naganuma H, Murayama H, Ohtani N, et al. Optically clear nuclei in papillary carcinoma of the thyroid: demonstration of one of the fixation artifacts and its practical usefulness. *Pathol Int.* 2000;50:113–118.
19. Simone NL, Bonner RF, Gillespie JW, et al. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. *Trends Genet.* 1998;14:272–276.
20. Coolidge BJ, Howard RM. *Animal Histology Procedures.* Bethesda, Md: US Dept of Health, Education, and Welfare, Public Health Service, National Institutes of Health; 1979. NIH publication 80-275.
21. Hewitt SM. Design, construction, and use of tissue microarrays. *Methods Mol Biol.* 2004;264:61–72.
22. Florell SR, Coffin CM, Holden JA, et al. Preservation of RNA for functional genomic studies: a multidisciplinary tumor bank protocol. *Mod Pathol.* 2001;14:116–128.
23. Chung J-Y, Braunschweig T, Hewitt SM. Optimization of recovery of RNA from formalin-fixed, paraffin-embedded tissue. *Diagn Mol Pathol.* 2006;15:229–236.
24. Fricain JC, Rouais F, Dupuy B. A two-step embedding process for better preservation of soft tissue surrounding coral implants. *J Biomed Mater Res.* 1996;33:23–27.
25. Fergenbaum JH, Garcia-Closas M, Hewitt SM, et al. Loss of antigenicity in stored sections of breast cancer tissue microarrays. *Cancer Epidemiol Biomarkers Prev.* 2004;13:667–672.
26. Cronin M, Pho M, Dutta D, et al. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. *Am J Pathol.* 2004;164:35–42.
27. Taubenberger JK, Reid AH, Krafft AE, et al. Initial genetic characterization of the 1918 "Spanish" influenza virus. *Science.* 1997;275:1793–1796.
28. Bernsen MR, Dijkman HB, de Vries E, et al. Identification of multiple mRNA and DNA sequences from small tissue samples isolated by laser-assisted microdissection. *Lab Invest.* 1998;78:1267–1273.
29. DiVito KA, Charette LA, Rimm DL, et al. Long-term preservation of antigenicity on tissue microarrays. *Lab Invest.* 2004;84:1071–1078.
30. Jacobs TW, Prioleau JE, Stillman IE, et al. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J Natl Cancer Inst.* 1996;88:1054–1059.
31. Wester K, Wahlund E, Sunderstrom C, et al. Paraffin section storage and immunohistochemistry: effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. *Appl Immunohistochem Mol Morphol.* 2000;8:61–70.
32. Hewitt SM, Takikita M, Braunschweig T, Chung J-Y. Promises and chalenges of predictive tissue biomarkers. *Biomarkers Med.* 2007;1:313–318.
33. Hewitt SM, Dear J, Star RA. Discovery of protein biomarkers for renal diseases. *J Am Soc Nephrol.* 2004;15:1677–1689.
34. MAQC Consortium. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol.* 2006;24:1151–1161.
35. Medeiros F, Rigl CT, Anderson GG, et al. Tissue handling for genome-wide expression analysis: a review of the issues, evidence and opportunities. *Arch Pathol Lab Med.* 2007;131:1805–1816.
36. The Tumor Analysis Best Practices Working Group. Expression profiling—best practices for data generation and interpretation in clinical trials. *Nat Rev Genet* 2004;5:229–232.
37. Leyland-Jones B, Ambrosone C, Bartlett J, et al. Recommendations for collection and handling of specimens from group breast cancer clinical trials, from onsite collection through shipping to the central bank. *J Clinic Oncol.* In press.