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Review

Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids

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Clinical and molecular medicines are undergoing a revolution based on the accelerated advances in biotechnology such as DNA microarrays and proteomics. Answers to fundamental questions such as how does the DNA sequence differ between individuals and what makes one individual more prone for a certain disease are eagerly being sought in this postgenomic era. Several government and nonprofit organizations provide the researchers access to human tissues for molecular studies. The tissues procured by the different organizations may differ with respect to fixation and processing parameters that may affect significantly the molecular profile of the tissues. It is imperative that a prospective investigator be aware of the potential contributing factors before designing a project. The purpose of this review is to provide an overview of the methods of human tissue acquisition, fixation, and preservation. In addition, the parameters of procurement and fixation that affect the quality of the tissues at the molecular level are discussed. ***(Am J Pathol 2002, 161:1961–1971)***

Profiling techniques such as DNA microarrays, tissue microarrays, and two-dimensional gel electrophoresis have been successfully used to provide information on the genes, proteins, metabolites, and other molecular features related to specific disease conditions.[1] Several novel genes and their products have been identified in human cancers by screening archival tissue samples using these methods.[2,3] Molecular diagnostic testing is requested more often in certain clinical conditions, eg, test for T- or B-cell clonality in early cutaneous lymphomas.[4,5] The need for molecular pathology to arrive at a conclusive clinical diagnosis will increase in the future. Hence, it is important for both clinicians and prospective investigators to be cognizant of the factors that affect the molecular quality of clinical and research specimens and measures to overcome them. The purpose of this review is to provide an overview of the methods of human tissue acquisition, fixation, and preservation. In addition, the parameters of procurement and fixation that affect the quality of the tissues at the molecular level are discussed.

Human Tissue Procurement Organizations

Biomedical science researchers frequently indicate that their investigations have been hampered by the lack of availability of human tumor tissue. In 1984, the United States Congress mandated that greater access to human tissues become available to scientists involved in cancer research. In response, the National Cancer Institute funded the Cooperative Human Tissue Network in 1987.[6] The Cooperative Human Tissue Network specializes in the prospective procurement, preservation, and distribution of various human tissues including normal, diseased, benign, and malignant tissues for institutional review board approved investigators.[7] The tissues supplied to researchers are not tissues specifically removed for research but are excess tissue over and beyond that essential for routine diagnosis and staging purposes. In addition, the patient confidentiality is strictly maintained.[8] Other tissue banks developed and funded by the National Cancer Institute under the Cancer Therapeutic Evaluation Program[9] include several clinical trial cooperative organizations. Several academic medical institutions have developed an internal tissue procurement service as a resource for researchers.[10] All tissue procurement services and biomedical specimen banks adhere to the regulatory requirements of local institutional review boards that follow the federal regulations administered by the Health and Human Services Office for Human Risk Protection (*http//:www.nih.ohrp.gov*). Recently an international society of biological and environ-

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mental repositories (ISBER, *http//: www.isber.org*) has been established to assist in the development of standards in methodology, management, and education. ISBER also serves as an informational resource for groups involved in procurement systems and repositories.

Tissue Procurement Methods

In general, the molecular biologist or the bioinformatician working on molecular profiling data sets seldom realizes the importance of sample acquisition and processing issues that may compromise their observations significantly. However, it is apparent that changes in the molecular profile of cells may occur as a sample is being removed from the patient, as it waits to be processed, and during processing itself.[2,11–14] Hence, a prospective investigator must consider factors involved in sample processing and tissue acquisition before embarking on a project.

Nature of the Clinical Samples

Depending on the nature of the disease and the therapeutic strategy, four types of biological material can be made available to the investigators for molecular studies: 1) tissue removed from the patient, either as a resection during surgery or as a small biopsy; 2) cytology samples, including cells from fine needle aspiration biopsy or body fluids; 3) blood, plasma, and serum products; and 4) autopsy specimens. Caution should be exercised when considering autopsy specimens for molecular studies because during the postmortem time there is extensive degradation of mRNA and protein in the tissue. In general, the longer the postmortem time, the poorer the molecular quality of the tissue.[13]

Sample Acquisition

Molecular changes may begin even before surgery because of the chemical nature of the anesthetic used and may accrue as the tissue is being removed and during prefixation time.[15] To minimize these changes, the surgeon and the pathologist must function in coordination for faster and efficient collection. Whenever possible, a representative portion of the tissue (in consultation with the pathologist) should be snap-frozen in the operating room. However, in our experience this will not be possible for most resected tissues. Developing an optimal protocol for obtaining resected or biopsy tissue poses the greatest challenge for a tissue procurement team. Several issues that must be considered include: 1) logistic issues of predicting the precise time of surgical removal of a tissue and the availability of sufficient technical and nontechnical personnel. 2) The prefixation time between tissue resection and processing (discussed below. 3) The type of anesthetic used may influence the molecular profile of the tissue, eg, some anesthetics affect the phosphorylation state of cellular signaling pathways.[16,17]Anoxic changes begin in the tissues with the clamping-off of the blood supply. Individual surgical procedures as dictated by the nature of blood supply to the organ may affect the duration of *in situ* anoxia. 4) When separate specimens are removed at different times during a surgical resection, the time interval between removal and processing for each specimen must be consistent (and short). 5) Other factors such as the changes in the local pH and environmental stress may cause new genes to be transcribed at any point during the procedure. Blood and cytology samples are more amenable for molecular studies, because the process of acquisition is short (if needed under local anesthetic) and the cells can be flow sorted or processed rapidly for molecular analysis.[18] However, it is not always possible to obtain cytological samples from tissues. Furthermore, cell-cell and cell-matrix interaction studies require whole tissues.

Tissue Processing

The pathologist receiving the tissue has three immediate options: freeze the tissue, keep it fresh, or stabilize the tissue in a fixative. Each generates a different type of specimen for analysis

Frozen Samples: Tissues can be preserved by freezing them directly at −80°C in a suitable medium or by immersing in liquid nitrogen preferably in a medium such as isopentane. Histological sections from frozen tissues are routinely reviewed by pathologists as intraoperative diagnostic procedures to guide the surgeon. Frozen tissues procured for research may be homogenized for recovery of DNA, mRNA, and proteins. Rapid freezing of tissues often can cause freezer burn at the periphery that may contribute to changes in the molecular profile of the tissue.[19,20]

Fresh tissues: Fresh tissue samples may be either dissociated for the production of cell lines or homogenized for recovery of DNA, mRNA, and proteins. Cytological samples should be immediately smeared onto a glass slide, air-dried, and fixed in an alcohol-based fixative. Peripheral blood lymphocytes and cells from lymphoma tissue can be flow sorted immediately or processed for cytology as above.[18] Plasma and serum separated from whole blood are frozen and stored at either −20°C or −80°C. In a recent annual meeting of the ISBER (2002) it was reported that whole cells maintained at −132°C or lower exhibited the best long-term viability with minimal or no enzymatic degradation (personal communications).

Fixation and Its Effect on Tissue Nucleic Acids

Fixation

As early as 400 BC Hippocrates discussed the biological effects of mercury and alcohol as a fixative.[21,22] However, curiosity about the histological structure of the tissues began only with the invention of the microscope. Even then, the early microscopists were preoccupied with improving their scopes and cared little about the specimens, for anything visible was impressive by the sheer novelty, once attention was directed toward it by a

lens.[21,22] A century and nanomicron magnifications later, the importance of the quality of the specimen for accurate validity of the data were realized. A systematic study of the fixatives began in the latter half of the 19th century. However, it must be noted that fixation by itself introduces a major artifact. The living cell is in a fluid or a semifluid state, and fixation involves some chemical modification of tissue proteins and constituents, a necessary event to prevent their loss during tissue processing. Much attention was focused on developing fixatives that would preserve cells and tissue constituents in as close a life-like state as possible while allowing them to undergo further preparative procedures without change.[23] With the advent of technology for developing monoclonal antibodies that would recognize specific antigens the staining techniques were perfected for *in situ* identification of proteins using tissue sections. Several reviews have discussed extensively the effects of fixation and fixatives on immunohistochemical detection of antigenic proteins.[24,25] The focus of this article is to discuss in detail the fixation and storage of human tissues for optimal preservation of proteins and nucleic acids for diagnostic and research purposes.

Increasing knowledge of the biochemical pathways and the genetic alterations underlying several disease processes have led to the identification of specific molecular targets as diagnostic and prognostic markers.[2] This molecular approach requires optimal preservation of proteins, RNA, and DNA in diagnostic specimens. Progress toward clinical application of potentially useful markers is hampered by the established routine fixation methods that fail to conserve the structure of nucleic acids and proteins in tissues and the limited ability to extract sufficient high-quality RNA or protein from fixed tissue. Several prefixation, intrafixation, and postfixation factors are integrally involved in the maintenance of the *in vivo* status of the human tissue *ex vivo* (Table 1).

Table 1. Fixation Parameters for Optimal Tissue Preservation

- Group I: Prefixation parameters
  - 1. Constant factors
    - a. Nature of the anesthetic
    - b. Duration of anesthesia
    - c. Anoxic injury *in situ*
  - 2. Variable factors
    - a. Prefixation time
- Group II: Intrafixation parameters
  - 1. Properties of the fixatives
    - a. Chemistry and mechanism of action
    - b. Tissue penetration
  - 2. Condition of fixation
    - a. Temperature
    - b. Duration
    - c. pH
    - d. Osmolarity
    - e. Concentration
    - f. Size of the specimen
    - g. Volume of the fixative
- Group III: Post-fixation parameters
  - 1. Storage parameters
    - a. Duration
    - b. Temperature
    - c. Condition (vacuum/nonvacuum packed)
  - 2. Nature of the biological factor to be analyzed
    - a. Proteins
    - b. Enzymes
    - c. Lipids
    - d. Nucleic acids
    - e. Mucopolysaccharides
    - f. Biogenic amines
    - g. Glycogen

Prefixation Parameters

Ideally, tissues should be fixed immediately and completely from the living state. In animal experiments, this can be achieved by *in vivo* perfusion. This is not possible for human tissues for obvious reasons. Most tissues are removed surgically, the tissue is relatively anoxic for some period because of anesthesia and the placement of surgical clamps and ligatures to stop bleeding.[14,26] The transient anoxia, the changes in local pH, and other environmental factors may lead to increased transcription of some genes. However, the variable factors of prefixation are primarily determined by the nature of the surgery and as such cannot be manipulated.

Prefixation Time

By definition the prefixation time refers to the time lapse from the surgical excision of the specimen to the fixative.[15] Significant biochemical alterations occur in tissues within 10 minutes after anoxia.[11,27] Hence, the prefixation time should be kept at the minimum to minimize RNA and protein degradation, especially for tissues with high levels of RNases and proteases, eg, pancreas, gall bladder, and skin.[28] Any surgical procedure *ex vivo* including grossing of the specimen and sectioning to smaller pieces should be performed immediately to prevent loss of enzymes, mitochondrial damage, decrease in histological mitosis, and possible transcription of apoptotic factors because of the ensuing anoxia.[29] A loss of 30 to 50% of mitotic figures because of delay in fixation by 2 to 6 hours has been reported.[30] This progressive fall in mitotic index has been implicated to introduce errors in the grading of breast carcinoma. Some measures to avoid extended prefixation period include careful analysis of the specimen delivery service and reorganization if needed to reduced the transit time and greater education of all staff stressing on shorter transit time.

Intrafixation Parameters

Fixatives

Fixed and archived tissues are a huge resource of DNA for molecular biological studies in cancer research, screening for genetically based diseases, and developmental biology studies. With the expansion of polymerase chain reaction (PCR) and other techniques of nucleic acid analysis for clinical diagnosis, knowledge about the effects of fixatives on the integrity and utility of the preserved DNA and RNA is increasingly important.[1] Many approaches to fixation and types of fixatives have been developed and tested throughout the years. The mech-

anisms by which fixatives act may be broadly categorized as dehydrants, heat effects, cross-linkers, and effects of acids and combinations of these. Agents that combine with proteins are called additives and those that precipitate proteins are called coagulants. At this time, it is accepted that no one fixative fulfils all of the aims of cell or tissue preservation: namely prevention of autolysis and preservation of physical and chemical properties of the tissue.[31]

Formaldehyde

Ferdinard Blum has been credited as the first person to use formaldehyde as a tissue fixative.[32] To date, formaldehyde as a 10% neutral buffered formalin is the most widely used universal fixative because it preserves a wide range of tissues and tissue components. However, attempts to extract usable DNA from formalin-fixed tissues for molecular biological studies have been variably successful.[33–35] Even short-term treatment of sections with formalin have been shown to significantly reduce the DNA solubility.[36] Although considerable evidence suggests that formaldehyde induces DNA degradation, few studies have reported yield of high-molecular weight DNA from formalin-fixed tissues.[37]

Interactions of Formaldehyde with DNA

Studies of chemical reactions between formaldehyde and nucleic acids have demonstrated that several basic reactions are similar to those observed in formalin-protein interactions.[29] The formaldehyde initiates DNA denaturation (interchain hydrogen bonds break and bases unstack) at the AT-rich regions of double-stranded DNA creating sites for chemical interaction. There are four interactions of formaldehyde with DNA: 1) The first is an addition reaction. Formaldehyde is added to the nucleic acid base to form a hydroxymethyl (methylol) group (-CH2 OH). 2) The second is a slower electrophilic attack of *N*-methylol on an amino base to form a methylene bridge between two amino groups.[38] 3) Formaldehyde treatment can generate AP (apurinic and apyrimidinic) sites via hydrolysis of the N-glycosylic bonds, leaving free pyrimidine and purine residues. AP sites have a highly unstable cyclic carboxonium ion that hydrolyzes rapidly to yield 2-deoxy-D-ribose.[36] 4) Formaldehyde may also cause slow hydrolysis of the phosphodiester bonds leading to short chains of polydeoxyribose with intact pyrimidines.[36]

When compared to the DNA isolated from frozen tissues, formalin-fixed tissues exhibit a high frequency of nonreproducible sequence alteration. It has been speculated that the artifacts may be because of formalin cross-linking cytosine nucleotides on either strand. As a result, in PCR the Taq-DNA polymerase fails to recognize the cytosine and incorporates an adenine in the place of a guanosine, creating an artificial C-T or G-A mutation.[29] Furthermore, damaged DNA are known to promote jumping between templates during enzymatic amplification permitting Taq-DNA polymerase to insert an adenosine residue at the end of a template molecule, then jump to another template and continue the extension producing an artificial mutation that is subsequently amplified. Such mutations are more likely when fewer cells are used and isolated random mutation occurring in an early PCR cycle is amplified to detectable level. Up to 1 mutation artifact per 500 bases have been recorded.[29] The actual frequency of errors is then a cumulative error including the reported *Taq* polymerase (normal) error frequency and the errors because of DNA damage and/or cross-linking depending on the degree of dilution.[39] Although conformational sequencing of independent amplification can distinguish between artifacts and true mutations, it has been cautioned that nonrecognition of such artifacts may have profoundly influenced mutation databases in formalin-fixed material.[29]

Effect of Formaldehyde on RNA

While Rupp and Locker[40] first reported Northern hybridization of formalin-fixed samples, von Weizasacker and colleagues,[41] amplified endogenous mRNA from archival tissues. However, extraction of useful RNA from formalin-fixed paraffin-embedded tissue is often compromised because of incomplete lysis leading to poor extraction efficiency and high content of RNases in some tissues.[42] Although multiple modifications have been introduced in the general procedure, most mRNA amplification is limited to small fragments. In addition, the DNase treatment with subsequent phenol/chloroform purification often reduces the final RNA yield considerably and the sensitivities in transcript detection are much lower when compared to mRNA detection from frozen tissues.[43] It has been suggested that the RNA may be degraded during fixation or made resistant to extraction because of cross-linking with proteins or otherwise chemically modified causing the poor yield.[44]

As with DNA, formaldehyde reacts with RNA forming an *N*-methylol (*N*-CH2OH) followed by an electrophilic attack to form a methylene bridge between amino groups. Recently Masuda and colleagues,[44] demonstrated these two reaction using oligo RNA and showed that the reactivity of the bases are of the order A/C≫G>U, suggesting that the tertiary amino group is the primary target for formalin attack. Nearly 40% of adenines in synthetic RNA acquire monomethylol addition after incubation in buffered formalin for 61 hours at 4°C. Because adenine is the most susceptible for the electrophilic attack, it is likely that the poly(A) tail of fixed mRNA will be heavily modified.[45] Hence, oligo (dT) would not anneal well to the poly(A) tail and not extend cDNA synthesis by proper reverse transcription. In addition, the RNA extracted from fixed tissues being partially degraded may not contain both the poly(A) tail and the target area of the PCR amplification. It has been suggested that random-priming in cDNA synthesis may provide a better yield than batch isolation of mRNA using oligo (dT).[44]

In the cellular RNA, the methylol addition impedes reverse transcription and cDNA synthesis, which can be

partly reversed by heating.[44] The semireversible nature has been attributed to the methylene bridge formed between the monomethylol bases of RNA and the amino bases of the proteins in the cytoplasm. Alternatively, the amino groups may be kept nonreactive because of the frequent secondary structure formation in the single stranded RNA.[44]

Effect of Formaldehyde Concentration, pH, and Temperature on Tissue Fixation

The overall rate of formalin-induced modification of DNA is dependent on the concentration, temperature, and pH of the fixative. It has been shown that when reacted at 5°C below the melting temperature, the denaturation rate of DNA increases with increasing concentration of formaldehyde.[38]

Formaldehyde fixation at room temperature results in poor preservation of high-molecular weight DNA, the size of the extracted DNA being directly related to the fixation temperature. Up to 30% of nucleic acids may be lost during fixation.[30,46] Multiple studies suggest that tissues fixed in formalin at 4°C exhibit the least amount of degradation of the nucleic acids.[35,47,48]

Prolonged tissue hypoxia reduces pH in tissues locally decreasing the yield of quantifiable nucleic acids.[11] So also, formaldehyde fixation at acidic pH or in the presence of formic acid causes degradation of tissue nucleic acids. As compared to neutral buffered formalin, tissues fixed in formaldehyde solution at pH 3.0 had a greater number of artificial mutations.[36] It has been suggested that a higher rate of depurination at lower pH can favor misincorporation of noncomplementary nucleotides (cytosine in place of thymine) at apurine and apyrimidine sites.[36] Although there have been several reports of successful amplification of DNA from neutral buffered formalin-fixed tissues, the PCR products are generally 500 bp or less.[35,47] The low-molecular weight DNA may be the result of scission of phosphodiester backbone of DNA.[36] Interestingly, secondary fixation in paraformaldehyde at high pH has been shown to drastically improve the signal of *in situ* hybridization of both low abundant (zinc finger transcription factor) and high abundant (actin) mRNA in human mammary gland tissue.[49]

Effect of Tissue Nucleases

DNase in tissue has been considered as one of the factors contributing to DNA degradation during fixation.[48] Formaldehyde solution containing DNase-neutralizing ethylenediaminetetraacetic acid is a better fixative for preserving the tissue DNA.[35] Tissue RNases contribute to the low yield of RNA *ex vivo*. Under conditions of relatively higher humidity (80%), formaldehyde induces aggregation of proteins including RNases. The accumulated RNase may compromise the yield of RNA from tissues stored for a period of time.

Tissue Penetration

Fixation depends on the coefficient of diffusibility of the fixative and the rate at which it reacts with the tissue components. The coefficient of diffusibility in 1 hour is the distance in millimeters that the fixative has diffused into the tissue and is inversely related to the square root of time.[13] In general, the higher the coefficient of diffusibility the better the fixative. Four percent formaldehyde has a coefficient of diffusibility of 0.78. On contact with water, formaldehyde rapidly becomes hydrated to form methylene glycol. When tissues are immersed in formaldehyde, they are rapidly penetrated by the methylene glycol. The actual covalent chemical fixation depends on the fraction of the formaldehyde forming bonds with the tissue components and dissociation of formaldehyde from methylene glycol. The equilibrium of this reaction strongly favors methylene glycol. Hence, formaldehyde penetrates tissues rapidly as methylene glycol but fixes slowly as carbonyl formaldehyde. To facilitate more uniform penetration of the fixative, it is imperative to fix small volumes of tissues (5 mm to1 cm). The volume of fixative should be in excess of 20 times the volume of the tissue.[31]

Duration of Fixation

The speed of fixation depends on the rate of diffusion of fixative into the tissue and the rate of chemical reactions with various components.[50] In practice, it is assumed that these processes require at least 1 hour per mm of tissue thickness, but routinely the tissues are fixed for 24 to 48 hours. Although the relatively broad time range presumably has no effect on histopathology, the longer duration of fixation adversely affects the quality of tissue DNA.[26] As stated above, the rate-limiting step in formaldehyde fixation is the binding of carbonyl formaldehyde to the tissues.[51] All cells enter a period when they are exposed to an increasing gradient of fixative as it diffuses in. At very low concentration, the cells may be able to metabolize some fixatives, eg, formaldehyde by aldehyde dehydrogenase. As the concentration of the fixative increases the chemical kinetics will be altered and the fixative is in excess of the proteins. At the same time, metabolic pathways will be cutoff at different times, exerting various effects on organelles. In addition, the fixed tissue also acts as a barrier to subsequent inward diffusion of fixatives. Immediate microwave irradiation of tissues for 1 to 2 minutes to ~60°C has been shown to better preserve nucleic acids presumably because of reduced enzymatic degradation and enhanced fixation.[23] The average size of DNA extracted from tissues fixed in buffered formalin decreases with increasing fixation time. Tissues fixed in buffered formalin for 3 to 6 hours yield greater amounts of high-molecular weight DNA.[36]

In conclusion, criteria recommended in the literature for the use of formaldehyde as a tissue nucleic acid fixative are: 1) minimal prefixation time lag, < 2 hours; 2) use of cold 10% neutral formalin; 3) low salt concentration; 4) cold fixation (at 4°C); 5) duration of fixation (3 to 6 hours); 6) ethylenediaminetetraacetic acid (20 mmol/L to

50 mmol/L) as an additive; 7) absolutely avoid low pH environment.

Alternative Fixatives

A method to overcome the problems of formaldehyde is to use an alternative fixative that is better suited for the preservation of nucleic acids and proteins. Some of the alternative fixatives are discussed below.

Gluteraldehyde

Like formaldehyde, glutaraldehyde is another cross-linking agent for tissue fixation.[23] An aqueous solution of glutaraldehyde (glutaric dialdehyde) is a complex mixture consisting of ~4% free aldehyde, 16% monohydrate, 9% dihydrate, and 70% hemiacetal. Using its aldehyde functional groups, glutaraldehyde reacts primarily with the e-amino groups of amino acids and nucleic acids. The coefficient of diffusibility of 4% glutaraldehyde at 4°C is approximately half that of 4% formaldehyde solution.[23,31] Although widely used as a fixative for standard electron microscopy, the slow penetration and the need for periodic purification to maintain the functional aldehyde levels have greatly limited its use as a biological fixative. However, it has been shown that 1% glutaraldehyde at pH 7.0 better preserves high-molecular weight DNA as compared to 10% formalin.[36]

Genipin

Recently, a naturally occurring cross-linking agent, genipin, has been shown to react spontaneously with amino acids to form a dark blue pigment. After fixation with genipin, the resistance of the tissue against collagenase degradation increased significantly. It has been suggested that genipin may form intramolecular and intermolecular crosslinks with cyclic structure within collagen in biological tissues. Although no apparent cytotoxicity was observed, the effect of genipin fixation on tissue nucleic acids is not known.[52]

Organic Solvents or Alcoholic Fixatives

Ethanol and Methanol

Multiple studies have indicated that noncross linking alcoholic reagents always yielded superior results as nucleic acids fixatives than aldehydes.[23,31,47,53] Ethanol and methanol preserve nucleic acids better because they bring about little chemical change. Physical chemical measurements have shown that DNA is largely collapsed in ethanol (65% v/v) and methanol. When the denatured DNA is rehydrated there is substantial reversion to the original form.[31] The presence of salts is essential for maximum precipitation of nucleic acids from alcohol. This does not interfere with the subsequent use of nucleases, but there may be shrinkage and hence not suited for morphological studies.[23] One hundred percent ethanol and methanol are known to be excellent fixatives for preserving both high-molecular weight DNA and RNA. The low molecular weight and rapid tissue penetration of the alcohol is thought to contribute to the uniform tissue fixation and minimal loss of tissue components.[47,53]

Mixtures of Fixatives

Because no single fixative is ideal for the preservation of all tissue components, mixtures of fixatives have been tried in an attempt to compensate the shortcomings of one by another.

Carnoy's

Ethanol, 60%; Chloroform, 30%; and Glacial Acetic Acid, 10%: Isolated studies suggest Carnoy's fixative is best for optimal preservation of nucleic acids in tissues. When compared to neutral buffered formalin (NBF) fixation, RNA was found to be easily extractable from Carnoy's-fixed mouse liver and well preserved with only a slight degradation of high-molecular weight RNA.[26] Formaldehyde vapor postfixation reduces the loss of cellular RNA from the Carnoy's-fixed tissue that may occur during treatments before *in situ* hybridization.[54] It has been suggested that at centers without access to frozen-tissue banking, fixing tissues in alcohol-based fixatives should be considered as an alternative for preserving the tissue molecular integrity, at least for those limited tissues on which subsequent RNA/DNA analysis may be of interest.[53]

Methacarn (Methanol, 60%; Chloroform, 30%; and Glacial Acetic Acid, 10%)

Substitution of methanol for ethanol in Carnoy's fixative gives methacarn, which has been shown to be an excellent fixative for preserving tissue RNA.[55] Using cultured cell lines and rodent tissues, Shibutani and colleagues[55] have shown that not only the extraction efficiency but also the integrity of the extracted total RNA from methacarn-fixed tissues are comparable to that from unfixed frozen cells. Furthermore, the concentration of contaminating genomic DNA is very low in methacarn-fixed tissues. In contrast to formalin-fixed tissues, 300- to 700-bp fragments of both abundant mRNA and low copy number RNA species could be amplified from methacarn-fixed tissues.[55] It has been hypothesized that the methacarn fixation causes precipitation of ribosomal proteins in the cells inactivating endogenous RNases and/or masking the mRNA from RNase action. Methacarn fixation is superior to NBF in retaining antigen immunoreactivity and does not require antigen retrieval. It has been recommended for prospective immunohistochemical studies assessing mitotic indices in tissues.[2,56] Using methacarn-fixed, paraffin sections from fetal and infant breast, immunostained with a panel of antibodies to cytoskeletal proteins and kappa-casein, it has been possible to define in detail the chronological evolution of the major cell types in the human breast from 16 weeks of intrauterine life to 2 years of age, in both sexes.

Acetone (AMeX)

Acetone has been used as a fixative in the acetone-methylbenzoate-xylene (AMeX) technique.[57] The method involves overnight fixation of tissues in acetone at −20°C, then clearing in methylbenzoate and xylene before paraffin embedding. The product has been shown to preserve good morphology while retaining the immunoreactivity for labile proteins such as lymphocyte membrane antigens. Significantly, AMeX-processed tissues yielded good quality spooled high-molecular weight DNA suitable for molecular analysis. In addition, specific signals (c-*myc* mRNA and albumin mRNA) comparable to RNA from fresh-frozen tissues could be detected by dot-blot hybridization using RNA isolated from AMeX-fixed tissues.[58–60]

HOPE Fixation

Recently a novel method of fixation has been reported that is compatible for complete pathological analysis including retaining relevant positive features for both immunohistochemistry and molecular pathology.[61] The HOPE technique (Hepes-glutamic acid buffer-mediated organic solvent protection effect) consists of incubation of fresh tissues in a protecting solution comprising of a mixture of amino acids at pH 5.8 to 6.4. The protection solution is thought to penetrate the tissues by diffusion. This is followed by incubation in acetone at 0 to 4°C for dehydration. It is suggested that the protective solution also helps in keeping the capillaries open for the infusion of acetone, thus accelerating dehydration. Finally the specimens are directly transferred into low melting paraffin and subsequently embedded in paraffin. Essentially, HOPE-fixed sections have been shown to exhibit formalin-like morphology and provide an excellent preservation of proteins and antigenic structures for differential analysis by immunohistochemical and/or enzyme histochemical techniques. More importantly, HOPE-fixed specimens yielded sufficient amounts of good quality DNA and RNA even after a period of 5 years.[61] The DNA and RNA were suitable for further molecular analysis by PCR, RT-PCR, and *in situ* hybridization. Absence of cross-linking and greater yield of extractable nucleic acids suggests that HOPE fixation offers a distinct alternative fixation method for projects that aim at establishing tissue banks.

Other Fixatives

The use of additional fixatives that incorporate picric acid (Bouin's solution), or mercuric chloride (B5, Zenker's, Helly's, and Ridley's solutions) or tannic acid to increase tissue penetration, or that contain phenol or heavy metal solutions such as zinc sulfate to improve protein precipitation have been validated in immunohistochemical studies, but there is paucity of data in their use as nucleic acid preserving agents.[54] Several studies have shown that B5 is a poor fixative for nucleic acid preservation.[23,33] Table 2 presents a compilation of the effect of different fixatives on tissue nucleic acids.

Microwave (MW) Irradiation

Electromagnetic waves with a frequency between 300 MHz and 300 GHz are classified as microwaves.[62] MW irradiation for tissue fixation was first introduced by Mayers,[63] who reported that direct exposure to MWs produced satisfactory fixation in both mouse and human postmortem tissues. MW fixation depends on the chemical environment around the specimen during irradiation, duration of microwave exposure, and sequence of microwave irradiation and other chemical or physical fixation methods. Thus, five MW fixation methods have been defined. 1) Stabilization wherein specimens are subjected to MW irradiation *in situ* or when immersed in a physiological salt solution in an attempt to preserve structures without the superimposed effects of a chemical fixative. 2) Fast or ultrafast primary MW chemical fixation in which specimens are irradiated by MW energy in a chemical environment for a short period of time ranging from milliseconds to seconds. 3) MW irradiation followed by chemical fixation involves continued immersion of specimens in a chemical fixative such as formaldehyde for minutes to hours after MW irradiation to improve uniformity of fixation. 4) Primary chemical fixation followed by MW irradiation to facilitate crosslinking of fixatives within the specimen. 5) MW irradiations in combination with freeze fixation limits freezing artifacts. In general, irradiation times less than 60 seconds, final irradiation temperature between 50 to 55°C and solution volume less than 50 ml in containers with at least one dimension that is ~1 cm are recommended for good MW fixation.[64]

The great advantage of microwave stabilization is that there are no chemicals involved that would extract important components from the tissue. However, significant disadvantages in this method include shrinkage, sponginess of tissue, and breakdown of red blood cells.[65] Fast primary MW chemical fixation can be used to enhance diffusion of fixation reagents into the tissue, and to accelerate the chemical process by which the fixative cross-links with the protein of the tissue. The reduced exposure to cross-linking chemicals makes it an attractive alternative method of fixation for ultrastructural and genetic studies. However, simply microwaving tissue in formalin often produces disappointing results, because the outside of the tissue fixes so rapidly and well that it effectively prevents further diffusion of fixative into the central part of the biopsy. Hence, a hybrid procedure consisting of first soaking tissue blocks in formalin for a few hours at room temperature followed by microwave irradiation for 1 to 2 minutes at 55°C has been recommended.[51] Excellent immunostaining has been achieved using this hybrid method of fixation.[66] The speed with which microwaves can accomplish fixation of both large and small biopsy specimens is a major asset. The MW fixation method considerably reduces the processing time with one-step dehydration and one-step clearing before paraffin infiltration.[65,67]

Household MW ovens provide an economical method for accelerated sample preparation. However, they have severe limitations in terms of safety and reproducibility. The magnetrons with variable warm-up time causes in-

Table 2. Review of the Effect of Fixatives on Nucleic Acid Analysis

Serial number	Fixative	Temperature of fixative	Duration of fixative	Temperature of paraffin block	Duration of storage	Tissue
1	10% NBF	4 C	1 hour	NA	NA	Rat liver
2	10% NBF	RT	2,7,24 hours	RT	1–4 weeks	Mouse spleen
3	Routine fixation (10% NBF)	NA	24 hours	RT in a light resistant closed box	1,4,9,14,20,27,35 days	Rat skin wounds, myocardial infarcts
4	Routine fixation (10% NBF)					Human
5	10% NBF	RT?	18 hours	NA	NA	Human skin and lung
6	10% NBF	RT?	6,24,48,72 hours, 1 week	NA	NA	Human breast (adenoma/cyst)
7	10% NBF	RT?	NA	NA	NA	Human lymphoid, parotid, gut, liver, and kidney
8	Ethanol (100%)	RT	6,24,48,72 hours, 1 wk	NA	NA	Human breast adenoma
9	Methanol (100%)	RT	12 hours	NA	NA	Human cancerous tissues
10	Alcoholic formalin			RT	5 years	Human skin
11	Omnifix		6,24,48,72 hours, 1 wk			Human breast adenoma
12	Carnoy's fixative	NA	2,8,48 hours	RT	1–4 weeks	Mouse spleen
13	Carnoy's fixative	RT	Overnight	RT	2 years (maximum)	Mouse liver
14	Methacorn	4 C	1 hour			Rat liver lung, and kidney
15	Acetone	4 C	18 hours	NA	NA	Human skin, lung
16	Bouin's	4 C	18 hours	NA	NA	Human skin, lung
17	AMeX	−20 C	Overnight	NA	NA	Mouse liver and human lymphoma
18	HOPE	0–4 C	Overnight	NA	1–5 years	Mouse liver
19	PBS	Microwave for 10–20 seconds		NA	NA	Human liver

NA = not available, RT = room temperature, RT? = authors believe room temperature was implied.

consistent production of MW power resulting in nonuniform MW fields. In addition, the MWs have restricted penetration ability that is further limited by the poor heat-conducting properties of the tissues.[68] In an attempt to overcome these limitations, experimental tools have been developed to standardize and calibrate MW ovens for tissue fixation. The neon bulb calibration tool maps regions of high and low power in a MW oven. Agar-saline-Giemsa tissue phantoms help determine the MW irradiation conditions that will uniformly heat a tissue.[64] Using hepatitis B virus DNA sequences and the type I human procollagen gene as markers and liver tissue as a target, Hsu and colleagues[69] have shown that microwave fixation in phosphate-buffered saline is superior to routine formalin for the preservation of excellent quality of genomic and viral DNAs for nucleic acid hybridization analysis. However, the morphological preservation of various tissues depends on generation of an optimal temperature for each tissue, ranging between 70°C and 85°C. Heating above or below these temperatures creates various artifacts such as vacuolation and changes in chromatin pattern.[69]

Postfixation Parameters

Storage

Patient tissues obtained for diagnosis are routinely stored in pathology archives. The stored paraffin blocks constitute a valuable resource that allow investigators to apply modern technology to verify plausible hypotheses in diverse populations than would be possible with fresh or frozen tissues. However, there are relatively few studies addressing the concerns regarding the specific quality control and quality assurance practices for appropriate storage of the paraffin blocks. The activities of glucokinase and phosphofructokinase decrease with increasing periods of storage at −80°C as a result of conformational changes or because of the effect of proteinases or deamidation of amide group of proteins. In contrast the activities of mitochondrial enzymes and isoenzymes and xanthine oxidase increase with periods of storage at −80°C presumably because of disintegration of tissue caused by freezing and thawing. Although Guerrero and colleagues[70] found no loss of sensitivity of

Table 2. *Continued*

Molecular analysis	Results (positive isolation)	Reference
Total RNA	19% successful	Shibutani et al.[55]
HPRT mRNA, GAPDH mRNA	ND	Foss et al.[26]
Type II collagen (protein) matrix metalloproteinase-2 (protein)	Good by ISH only in tissues stored for 1 and 4 days 35–50% reduced in slides stored for 35 days	Lisowski et al.[71]
β-actin mRNA	Good with nested PCR	Stanta and Bonin[73]
GAPDH	Good	Tyrrell et al.[43]
Total RNA	Good yield up to 48 hours of fixation	Ben-Ezra et al.[74]
Aldolase A fragment (181 bp) porphobilinogen deaminase (127 bp)	Not consistent—good housekeeping gene	Finke et al.[42]
Total RNA	Good at all duration of fixation	Ben-Ezra et al.[74]
High molecular weight DNA, p53	Good for both PCR and RT-PCR	Noguchi et al.[47]
β-actin GAPDH	Moderate	Tyrrell et al.[43]
Total RNA	Good at all duration of fixation	Ben-Ezra et al., JHC 1991
HPRT (168 bp)	Detectable in 50% of tissues fixed for 8 and 24 hours	Foss et al.[26]
ApoSAA (600 bp)	Good (but degrades if not postfixed in PF)	Urieli et al.[54]
β-actin (540 bp) GAPDH (158 bp)	good good	
α2-globulin mRNA HGFR mRNA	Very good (as compared to frozen)	Shibutani et al.[55]
β-actin GAPDH	Very good	Tyrrell et al.[43]
β-actin GAPDH	Not detectable	Tyrrell et al.[43]
Total DNA, C-ras gene, total RNA	DNA quantity comparable to the fresh tissue RNA quantity and quality poorer than fresh tissue, but useful for PCR	Sato Y et al.[57,59]
Total RNA	Good quantity and quality	Olert J[61]
Hepatitis B DNA, type I human procollagen	Viral and genomic DNA yield comparable to that of fresh tissue	Hsu HC et al.[69]

hepatitis C virus RNA detection in paraffin blocks of formalin-fixed liver tissues stored at room temperature for more than 4 years, Lisowski and colleagues[71] showed progressive decrease in signal intensity of type III collagen mRNA with increasing periods of storage of nondeparaffinized histological sections. However, it is not known whether storage of paraffin blocks and/or the histological sections under different conditions of temperature could prevent the nucleic acid degradation. Interestingly, thawed muscle tissues incubated at room temperature and at 4°C over a range of time from 0 to 8 hours and from 0 to 96 hours, respectively, has been reported to show a time-dependant decrease in mitochondrial DNA as detected by quantitative Southern blot analysis.[72]

Oxidation of tissue sections may decrease antigen recognition when using antibody detection methods and it may also have a similar effect on nucleic acid recovery or recognition. It is well established that vacuum packing of food substances including meat, lengthens their effective shelf life by preventing slow degrading changes by oxidation. One of the fundamental principles of chemistry is that reactions increase in rate with increase in pressure and hence vacuum packing of tissues in fixative should enhance fixation. In the context of storage of surgical specimens for future research purposes a similar attempt to vacuum pack the tissues may be worth studying.

Conclusion

Undoubtedly, there exists a tremendous need for human tissues for research and development. Recently considerable effort is directed toward developing standardized methods of tissue procurement and processing for more accurate comparison of molecular data obtained by using tissues from multiple tissue repositories. Great strides have been made in the field of biotechnology that facilitate rapid unraveling of disease etiology, which in turn has led to the development of better therapeutic targets. Molecular profile libraries of specific diseases (eg, prostate cancer) are being constructed from archival tissues. However, caution must be exercised in adapting the information for diagnostic purposes and/or drug development because it is possible that alterations in gene expression profiles can occur either during and or after the resection of the tissue. This is a critical issue that needs to be addressed experimentally in the future by comparing the molecular profiles of fresh tissue with fresh-frozen (removal and immediate freezing) samples,

and samples of the same tissue fixed in different fixatives. Such an exercise will help in the identification of the best fixation procedure to develop an ideal tissue template for future molecular profiling studies.

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