Review Article

Molecular-Friendly Histopathology

Mehdi Nassiri[1], Mehrdad Nadji[1]

1.University of Miami, Jackson Memorial Hospital, Miami, Florida

ABSTRACT

The rapidly expanding fields of pharmacogenomics and pharmacodiagnostics have presented the pathology laboratories with many challenges and opportunities. As custodians of patient tissues, these laboratories are in the logical position to perform biomolecular testing for proper management of patients. In order to meet these challenges, the pathology laboratories of the twenty-first century should design and execute a biomarker-friendly, standardized tissue handling, including fixation and processing, to ensure uniform protection of macromolecules for clinically useful molecular assays. This important pre-analytic phase cannot be successful if tissues are handled in the traditional manner that includes the use of conventional fixation and processing. The recent progress in fixation and processing methods are rapidly replacing the time-honored routine formalin fixation and overnight processing. In this article we describe our experience with such system that not only produces good histomorphology but also preserves high quality RNA, DNA, and proteins in paraffin embedded material.

Key words: Molecular Histopathology, PCR, DNA, RNA

Why Molecular Histopathology?

Most pathology laboratories use fixation and processing technologies that are more than 100 years old. These traditional methods continue to be widely used because of their excellent qualities for routine morphological examination and their cost-effectiveness (1). However, at the molecular level their impact on biomolecule preservation is variable and unpredictable. Because of the rapidly advancing era of molecular medicine, it is not unreasonable to predict that molecular methods will eventually either replace existing morphologic approaches, or more likely will be used in conjunction with them. Most of today's diagnostic and therapeutic decisions are based on the evaluation of small tissue biopsies, and laboratories are expected to use the same precious small volume of tissues for histology and molecular tests. Pathology laboratories should therefore devise molecular-friendly tissue handling systems that allow for morphological diagnosis while rendering the same archival tissue suitable for advanced molecular testing (2).

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**Address communications to: M Nadji MD Holtz-2147 Department of Pathology1611 NW 12th Ave Miami, FL 33136 USA*
E-mail: mnadji@aol.com

What is a Complete Molecular-Friendly Histopathology Platform?

A complete molecular-friendly histopathology platform requires that specimens are properly handled from the moment they are removed from a patient to the time they are archived. With the introduction of fixatives and processing systems that protect molecular properties of tissue, two major steps toward this goal have been taken. It should be remembered however, that a molecular-friendly fixative alone or a simple formalin-free processing system by itself does not guarantee that the final product is suitable for advanced molecular studies. Molecularly compatible fixatives and tissue processing are merely two components of a complete molecular histopathology platform. No matter how efficient the fixation and processing systems, if the time interval between surgical excision and fixation is long, there will be considerable degradation of biomolecules. Therefore, an overall "molecularly conscious" laboratory philosophy with provisions for proper pre-fixation and post-processing handling of tissue is sometimes more important than the fixative or the processor alone.

Fixation

It has been suggested that the best fixative for preservation of tissue macromolecules is "no" fixative. This is because past experiences have shown that all chemical fixatives, in one way or another, modify, degrade or destroy nucleic acids and proteins. For this reason fresh, or fresh-frozen tissues have been used for most molecular studies. But these have limited value for assessment of histomorphology or for the performance of routine ancillary tests such as histochemistry (HC) and immunohistochemistry (IHC). In addition, transportation and long term storage of frozen tissue creates logistical problems that render it impractical for routine use in most diagnostic histopathology laboratories.

Formalin-fixed, paraffin-embedded histologic sections, however, are quite suitable for most HC and IHC tests, although they yield degraded nucleic acids suitable only for slide-based in situ hybridization as well as PCR amplification of short amplicons. An example is less than 500 bp for DNA and less than 100 for RNA. The material therefore is of limited value for performing molecular assays that require intact molecules, such as high molecular weight RNA and biologically viable proteins. For this reason a search for alternative non-formaldehyde molecular fixatives has been going on for the past several years (3,4).

Chemical solutions that can potentially protect macromolecules in tissue can be grouped into two general classes. One class protects nucleic acids well but the same tissue is unsuitable for histomorphologic evaluation. These "molecular preservatives" are therefore excellent alternatives to freezing the tissue, but of limited value as practical histology fixatives. In other words they must be used in addition to formalin, and that limits their utilization for small biopsies.

The second class could be regarded as true "molecular fixatives", because they not only protect macromolecules but preserve acceptable histomorphology. They can therefore be used as a single universal reagent to fix tissue and to preserve its molecular properties at the same time. Such complete molecular fixatives have not been available until recently.

In addition to protecting intact biomolecules and preserving microanatomy, an ideal molecular fixative should preferably be non-toxic, non-volatile, active at ambient temperatures, economically priced and cost-effective. A molecular fixative recently introduced by Vincek et al, for the most part meets these requirements (5). That fixative - a mixture of methanol and polyethylene glycol - is non-volatile and active at room temperature. It protects tissue DNA, RNA and proteins along with histomorphology. In addition, long exposure of tissue to the fixative,

up to six months at room temperature, does not significantly alter its molecular properties or histomorphology.

Processing

The value of harvesting microwave energy for histopathology in general and for tissue processing in particular, has been well established (6,7). In most published works conventional microwave ovens are used, including some that are adapted for histology purposes. In 2002, Morales and coworkers reported the development of a microwave assisted, continuous-specimen-flow, one-hour tissue processing method (8). This manual rapid tissue processing (RTP) system utilized microwave energy along with vacuum and a combination of common histologic reagents –minus formalin and xylene. It permitted preparation of paraffin blocks from either fresh or prefixed tissue in about one hour. The system utilized a specially designed cylindrical, low energy microwave that distributed energy uniformly throughout the chamber, thus avoiding the creation of hot-cold spots commonly observed in conventional microwave processing. The manual procedure is now automated into a rapid tissue processing system, complete with robotics, internal reagent containers, and user-friendly operating software. As a corollary, it has been observed that tissue samples processed by this methodology show improved RNA preservation, particularly when they are not prefixed in formalin. This unexpected but highly desirable "molecular-friendliness" led to a search for a fixative that could similarly protect tissue biomolecules. The result was development of the molecular fixative by Vincek et al, referred to above.

Validation

Molecular fixative and the RTP system must be used together to preserve macromolecules. Use of molecular fixative with conventional processing, or the use of RTP without a molecular fixative, will result in degradation of tissue nucleic acids and proteins. Since the complete system was intended for use as a molecular-friendly alternative to formalin fixation and conventional processing, a detail morphologic, immunohistochemical and molecular biologic evaluation of processed tissue was carried out as summarized in the following:

The Processing System

The histologic quality of the RTP system for formalin-fixed tissues was validated through comprehensive parallel studies, including a blinded review of slides by an expert external panel (9). In addition to integrity of histomorphology, quality of histochemical and immunohistochemical properties of processed tissues was established through extensive testing and reviewing. Altogether results confirmed that when formalin-fixed tissues were processed in the RTP system, no modification of protocols was necessary for Hematoxylin and Eosin (H&E) staining, histochemistry, immunohistochemistry, and in situ hybridization.

Combined Molecular Fixation and Processing Platform (MP)

When molecular fixative was used as an alternative to formalin, the RTP-processed tissues were subjected to new sets of validation studies. These experiments used parallel slices from surgically removed specimens. One slice was fixed in formalin and the other in molecular fixative; they both were then processed by the RTP system. The results are summarized below.

Histomorphology

As safe alternatives to formalin, alcohol-containing fixatives have been in use for many years and most pathologists are familiar with their histologic properties. As with other alcohol-based fixatives, the molecular fixative produces a histomorphology that is similar, but not identical

to formalin. For example, the molecular fixative-exposed tissues show an overall brighter, shiny appearance in hematoxylin and eosin-stained slides. The minor morphologic differences however in no way interfere with establishing the correct diagnosis.

Immunohistochemistry

IHC of MP specimens is different from that of formalin-exposed tissue, and therefore modification of staining protocols may be necessary. A comparative study showed that for most routinely used antibodies the sensitivity of immunohistochemistry performed on molecular-fixed tissue is either comparable or superior to formalin-fixed specimens. This is not surprising, because the superiority of alcohol-containing fixative for preservation of certain tissue antigens in general and intermediate filaments in particular has been observed before. A word of caution, however: since a number of stand-alone immunohistochemical tests are designed solely for the use on formalin-fixed tissue, one must adjust the antibody concentration or remove the antigen retrieval step to achieve comparable sensitivity in MP specimens.

Molecular Properties

As a general rule, any molecular test that can be performed on formalin-fixed tissue could be done on specimens processed in the molecular system. This includes PCR amplification of small segments of DNA and RNA, as well as in situ hybridization. The latter requires shorter predigestion time, usually about one-third of the time required to digest formalin-fixed tissues. This is because the formalin-induced cross-linking of tissue proteins is not a problem in a formalin-free system. Tests that require intact biomolecules cannot be performed on formalin-fixed paraffin-embedded specimens. The same tests however, are feasible on archival tissue prepared by the molecular platform.

The following summarizes validation of some of these tests as they apply to the preservation of intact RNA and proteins.

RNA Preservation

Tissues processed by the molecular platform yield an intact RNA comparable to that of fresh tissue, whereas in formalin-fixed specimens, RNA is significantly degraded as evidenced by the absence of 28S and 18S ribosomal bands. Similarly, in quantitative real-time PCR, the copy number of templates of molecular system is similar to fresh tissue, and significantly higher than that of formalin-fixed samples. In addition, extracted RNA from molecular and fresh tissue yields similar cDNA microarray profiles. Finally, it has been demonstrated that high molecular weight RNA can successfully be extracted by laser capture microdissection from H&E sections of paraffin blocks processed by the molecular platform (10). It should be remembered however, that validation of tissue RNA integrity has to be carried out under well-controlled RNase-free conditions. This includes maintenance of an RNase-free laboratory environment, meaning RNase-free instruments, glassware and reagents. Even more important is to ensure that endogenous RNase activity of tissue is immediately and efficiently blocked. To that end one has to establish strict operating room protocols that require immediate fixation of small biopsies, and timely delivery of larger specimens to the laboratory for proper handling.

Protein Preservation

Protein extracts from MP blocks show distinct spot patterns on 2D-gel electrophoresis similar to that of fresh tissue. This is in contrast to formalin-fixed specimens that produce small number of distinguishable spots. On Western blots, MP samples also reveal distinct bands with most antibodies tested, including some antibodies against phosphorylated proteins. Only a few

antibodies react with protein extracts of formalin-fixed tissues, and the intensity of bands is usually weaker and less distinct. Recent studies have also showed that proteins isolated from MP blocks are suitable for surface enhanced laser desorption and ionization spectrometry (SELDI-TOF), yielding high resolution protein profile patterns. No such pattern is observed with formalin-fixed specimens.

Conclusion

At this writing, several molecular preservative/ fixatives and formalin-free, tissue processing systems are available. While an all-inclusive, combined system similar to MP has yet to be validated; such systems will hopefully one day be commonplace. The most formidable obstacle to this might be existing mind-sets, but the benefits of establishing a complete molecular histopathology laboratory as a key component of "personalized" diagnosis and treatment outweigh all challenges.

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