Cold-Temperature Plastic Resin Embedding of Liver for DNA- and RNA-Based Genotyping

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The standard practice of tissue fixation in 10% formalin followed by embedding in paraffin wax preserves cellular morphology at the expense of availability and quality of DNA and RNA. The negative effect on cellular constituents results from a combination of extensive cross-linking and strand scission of DNA, RNA, and proteins induced by formaldehyde as well as RNA loss secondary to ubiquitous RNase activity and negative effects of high temperature exposure during paraffin melting, microscopic section collection, and tissue adherence to glass slides. An effective strategy to correlate cellular phenotype with molecular genotype involves microdissection of tissue sections based on specific histopathological features followed by genotyping of minute representative samples for specific underlying molecular alterations. Currently, this approach is limited to short-length polymerase chain reaction amplification (<250 bp) of DNA, due to the negative effects of standard tissue fixation and processing. To overcome this obstacle and permit both cellular morphology and nucleic acid content to be preserved to the fullest extent, we instituted a system of cold-temperature plastic resin embedding based on the use of the water-miscible methyl methacrylate polymer known as Immunobed (Polysciences, Warrminster, PA). The system is simple, easy to adapt to clinical practice, and cost-effective. Immunobed tissue sections demonstrate a cellular appearance equivalent or even superior to that of standard tissue sections. Moreover, thin sectioning (0.5–1.0 μ m thickness) renders ultrastructural evaluation feasible on plastic-embedded blocks. Tissue microdissection is readily performed, yielding high levels of long DNA and RNA for genomic and transcription-based correlative molecular analysis. We recommend the use of Immunobed or similar products for use in molecular anatomical pathology. (*J Mol Diag* 1999, 1:17–22)

The explosion of genetic information, coupled with development of high-throughput genotyping technologies, has set the stage for molecular approaches to disease diagnosis and treatment planning. Solid tissues present a major challenge in this regard due to intrinsic cellular heterogeneity, which can confound the results of testing if not carefully taken into account. The ideal approach, exemplified by microscopic slide staining techniques such as immunohistochemistry and *in situ* hybridization, allows each cell in a tissue specimen to be visually inspected for specific genotypic changes. Unfortunately, slide format approaches are limited in the extent to which genetic analytical techniques can be applied directly to them for detection and characterization by microscopic examination.

With the exception of a small number of genes, DNA mutational analysis cannot be carried out by means of a histological stain. Protein expression analysis using well established procedures for immunohistochemical staining is very often constrained by the lack of availability of specific and sensitive antibodies for the expanding repertoire of human cancer-related gene products. In addition, many available antibodies prove to be ineffective on formalin-fixed, paraffin-embedded tissue sections due to irreversible alterations to native proteins resulting from standard practices of fixation and tissue processing. The molecular analysis of RNA, in particular, is severely hampered as a result of RNA loss secondary to the combined actions of tissue RNases and high temperatures commonly used in specimen processing.

An alternative approach, permitting integration of cellular characteristics with underlying molecular alterations, is based on the use of tissue microdissection.^{1,2} Cellular areas in histological sections are first delineated under light microscopic and stereomicroscopic observation. Then, selected sites, chosen for their representativeness of biological processes operative in a tissue specimen, are removed with precision so as to minimize the inclusion of unwanted cellular elements not intended for genotyping. This can be accomplished simply and rapidly by manual methods³ or through the use of complex equipment specially designed for this purpose.⁴ Two commercially available units have recently been described.

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These are the PixCell II laser capture microdissection apparatus (Arcturus, Mountain View, CA)⁵ and the Robot-Micro-Beam (PALM) laser microdissector (Wolfratshausen, Germany).⁶ Both use focused laser energy to effect discrete removal of cellular material from microscopic slides, giving the operator the freedom to select topographic tissue targets. Using nucleic acid amplification, samples as small as a single cell can be analyzed for a broad array of gene alterations.⁷

The full potential of microdissection-based, polymerase chain reaction (PCR) format genotyping has not yet been realized due to inherent limitations related to tissue fixation and processing.⁸ Prolonged exposure of tissues to the effects of formaldehyde results in irreversible cross-linking of protein and nucleic acid, usually limiting maximum PCR amplicon size to 200 to 300 bp.⁸ In addition, tissues excessively fixed in formaldehyde suffer nucleic acid strand scission, diminishing the efficiency of nucleic acid amplification and further reducing maximum amplicon size.⁸ Although DNA survives fixation and embedding reasonably well, RNA content is seriously decreased due to a combination of RNase activity present in virtually all tissue specimens and the use of excessive heating during tissue processing. The latter occurs at many stages of tissue handling, eg, during melting of paraffin at the embedding step and during oven baking of tissue sections to maximize adherence to glass slides.

Standard practices of tissue fixation and embedding, therefore, impose a significant limitation on the application of molecular methods in everyday clinical practice. Alternative techniques to preserve nucleic acids, such as immediate freezing and cryostat sectioning, are not generally desirable in a clinical context due to the frozen section artifact impairment of cellular morphology that accompanies such an approach. Given the empirical foundation of microscopic analysis for current methods of tissue diagnosis and prognosis, the transfer of molecular methods into pathology practice has been greatly hindered.

The logical solution to this problem is a method for tissue fixation and processing that maintains cellular integrity while quantitatively and qualitatively preserving nucleic acids. Protocols designed to protect nucleic acids are also likely to influence favorably the availability of other cellular constituents including proteins, carbohydrates, and lipids. In this report, we present such a system, which is both easy and cost-effective to apply. The system is based on an established process of cold-temperature embedding of tissue specimens in an inert plastic resin with water-miscible characteristics (Immunobed Kit, Polysciences, Warminster, PA). The technique, termed the Immunobed method, has been used successfully for ultrastructural immunocytochemistry. We report here that the system, which allows tissue sections to be cut as thin as 0.5 μm , confers excellent retention of long DNA and RNA lengths suitable for detailed genetic analysis. Through the use of microdissection techniques, this system permits the positive attributes of both microscopic and molecular analysis to be realized for molecular pathology research purposes as well as for clinical diagnosis and treatment.

Materials and Methods

Molecular Fixation

Portions of liver biopsies were obtained from patients with a variety of hepatic diseases attending the Digestive Disease Clinic at the University of Pittsburgh Medical Center. Utilization of tissue was carried out in accordance with guidelines set forth by the institution's internal review board. Only after securing adequate tissue for routine diagnostic pathology purposes, based on formalin fixation and paraffin embedding, portions of liver tissue measuring 3–5 mm $\times$ 1 mm $\times$ 1 mm were fixed in 2% paraformaldehyde for 1 to 2 hours. Water for all solutions was treated with 0.1% diethyl pyrocarbonate to remove intrinsic RNase activity. Specimens were then rinsed and stored in cold 70% ethanol at -80°C until ready for embedding in plastic resin.

Cold-Temperature Embedding in Plastic Resin

Tissues were processed for plastic resin embedding according to the Immunobed protocol provided by manufacturer. Tissues underwent three 1-hour incubations in increasing grades of ethanol to 100% ethanol at 4°C . This was followed by immersion in 50%/50% ethanol/Immunobed solution for 2 hours at 4°C . Catalyst was added to the plastic resin and the tissue embedded overnight in catalyzed resin at 4°C using standard beam capsules as tissue supports. Blocks were stored at 4°C until the time of microtomy.

Preparation of Tissue Sections

Immunobed blocks were cut on a Reichert ultracut S microtome at thicknesses from 0.5 to 4 μm . Tissue sections were floated in a DEPC-treated water bath. Sections were picked up on clean glass slides and allowed to air dry at 4°C , after which they were stored at -20°C until ready for staining and microdissection. Immediately before microdissection, tissue sections were stained with hematoxylin-eosin and examined without coverslipping. To confirm accuracy of tissue microdissection, postmicrodissected stained histological sections were dehydrated through graded alcohols and xylene. Routinely processed tissue sections received a nonaqueous mount and plastic-embedded sections were covered with Crystalmount (Biomedica Corp., Foster City, CA).

Tissue Microdissection

Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated by passage through graded alcohols, and air-dried. Plastic resin-embedded tissue sections were simply air-dried in preparation for microdissection. Microdissection was carried out under stereomicroscopic observation using a Zeiss stereomicroscope. Selected areas of liver tissue were removed using a no. 11 scalpel and placed directly into 500-ml tubes for molecular analysis.

Detection and Genotyping of Hepatitis C Virus (HCV) and Internal Nucleic Acid Controls

Pairs of oligonucleotide primers were used to amplify by PCR and to DNA sequence two separate regions of HCV.^{9,10} A short (153 bp) amplicon was generated from the 5' nontranslated region⁹ and a longer (364 bp) PCR product was created from the NS5 region.¹⁰ In both instances, the upstream primers were used to sequence the amplified material according to methods described below. Positive HCV controls consisted of fresh-frozen blood and/or tissue known to contain the virus. Amplification and sequencing of housekeeping genes in each tissue specimen were used as internal positive controls for DNA and RNA content. DNA amplification reactions were targeted to a 335-bp fragment derived from the 3' nontranslated region of the epidermal growth factor receptor gene.¹¹ In a similar fashion, a 264-bp amplicon was generated from mRNA of the β -actin gene.¹²

Microdissected tissue for RNA genotyping (HCV and β -actin) was treated with guanidinium isothiocyanate for isolation of RNA.¹³ The extracted RNA was reverse transcribed with avian myeloleukemia virus, and the resulting cDNA was split into two parts; one portion was used for HCV genotyping and the remaining portion was destined for housekeeping gene amplification. Microdissected tissue for DNA genotyping was treated with Proteinase K (10 mg/ml) for 1 hour. A one-fourth portion of the postdigested material was placed directly in a microcentrifuge tube for PCR amplification using the GeneAmp kit (Perkin-Elmer, Norwalk, CT).

The products of PCR amplification were electrophoresed in 2% agarose and amplicon bands, visualized with ethidium bromide, and excised on a transilluminator. PCR-generated DNA was isolated with glassmilk (GeneClean, Bio 101, La Jolla, CA) and manually sequenced according to manufacturer's instructions (Sequenase, United States Biochemical Corp, Cleveland, OH). The DNA sequence was read from autoradiograms of 6% polyacrylamide gels exposed overnight.

Results

The microscopic appearance of liver biopsy specimens, fixed for 1 to 2 hours in 2% paraformaldehyde and processed by cold embedding in plastic resin, was compared to that of the same specimens routinely processed in 10% neutral buffered formalin and paraffin-embedded (Figure 1). The quality of cellular morphology in 4- μ m-thick sections was judged to be equivalent between the two methods of tissue handling.

Tissue microdissection was performed by hand under stereoscopic observation. Microdissection targets for plastic resin-embedded specimens varied in size from approximately 20 to several hundred cells. Targets of similar size were selected from routinely processed specimens. In addition, larger targets from formalin-fixed, paraffin-embedded sections were collected, amounting to one to three whole biopsy sections. Unlike paraffin-embedded sections, plastic resin-embedded sections re-

Figure 1. Histological appearance of liver biopsy specimens subject to routine (**left**, 10% neutral buffered formalin/paraffin embedding) and molecular (**right**, 2% paraformaldehyde/plastic resin embedding) processing. Liver cells fixed and processed routinely manifest homogeneous velvety cytoplasm with round vesicular nucleus including prominent nucleolus. Liver tissue subject to molecular fixation is indistinguishable in histological appearance. Hematoxylin-eosin; original magnifications, $\times 250$ and $\times 400$.

quired no preparation for manual microdissection. Minute targets could be lifted very easily and quickly using the point of a scalpel tip, whereas routinely processed tissue required careful wetting to gather the sample properly using the end of the scalpel. Flaking of the histological section with scattering of minute dried tissue pieces was a problem with microdissection of deparaffinized sections. In general, microdissection of plastic-embedded tissue was easier and quicker to perform and was also found to be more precise and reliable than that of paraffin-embedded sections.

PCR amplification of DNA gene targets <250 bp in length occurred in a reproducible fashion in both routine and molecular processed tissue (Figures 2 and 3). Longer DNA amplicons could not be achieved with routinely fixed tissue, in contrast to plastic resin-embedded

Figure 2. Robust amplification of β -actin RNA by RT/PCR (**leftmost 8 samples**) and epidermal growth factor receptor-2 DNA (**rightmost 8 samples**) from minute cold-temperature plastic-embedded tissue samples. Microdissected tissue samples consisting of as few as 20 cells from 4- μ m-thick histological sections demonstrated strong positive amplification of RNA and DNA gene targets. P, primers only PCR, negative control.

Figure 3. RT-PCR amplification of hepatitis C virus for amplicons that are short (**left**, 5' nontranslated region, 153 bp) and long (**right**, NS5 region, 364 bp). **Left:** All samples subject to molecular fixation and processing yielded strong positive amplification without the requirement for nested primer amplification. All samples routinely fixed failed to effectively amplify consistent with total or nearly total loss of viral RNA. **Right:** A series of HCV-positive and -negative (N) control samples were subject to RT-PCR for specific detection targeting a 364-bp segment of the NS5 region. P, primers only negative control.

specimens, which gave a strong PCR product band (Figure 3). Only short RNA reverse transcription PCR (RT-PCR) amplification products were generated from routinely processed tissue; however, this required nested PCR, which was more tedious to perform and which carried a significant risk of cross-contamination. Furthermore, the product bands in these cases tended to be inconsistent and relatively weak in intensity. In contrast, RNA extracted from microdissected plastic resin-embedded tissue sections generated in a robust manner both short and long RNA amplification products (Figure 3). Even microdissection targets as small as 20 cells from plastic resin sections yielded discernable ethidium bromide-stained bands on agarose gel electrophoresis (Figures 2 and 3). Both short and long RNA products could be detected and consistently genotyped from plastic resin tissue sections (Figure 4).

Figure 4. HCV genotyping from plastic resin-embedded tissue section. Attainment of abundant PCR product was found to be the critical factor permitting clear and strong DNA sequencing of tissue HCV. **Left:** Genotyping based on the 5' nontranslated region reveals type 1 strains in two samples. **Right:** NS5 region genotyping was preferred due to greater interstrain sequence variation, which in turn permitted detection and characterization of HCV quasiespecies.

Discussion

Anatomical pathology must meet the challenge of providing the means whereby insights gained through efforts such as the Human Genome Project¹⁴ may be translated into practical, cost-effective clinical testing of tissue specimens. The goal, therefore, should be to establish operational conditions whereby traditional histopathological analysis (emphasizing cellular morphology) and molecular biological techniques (providing detailed information on DNA structure, RNA expression, and protein composition) can complement each other. At present, the two fields of diagnostic histopathology and molecular biology are often in conflict, competing for the same precious tissue specimens on which to perform specific analyses. Given the increasing reliance in recent years on biopsy procedures to obtain diagnostic material, efforts must be made to harmonize the two disciplines so that the insights from each can be integrated for more effective diagnosis and treatment planning.

Appreciation of cellular heterogeneity is fundamental to any effective genotyping strategy for solid tissue specimens. Failure to take this essential factor into account invariably leads to averaging of genetic information between divergent cell types. For example, the presence of a highly proliferative cell type, such as reactive fibroblasts, in a tissue sample may contribute to a potentially false impression that a rapidly growing tumor cell population exists in the sample under study. In this way, a benign neoplastic process that evokes an exuberant reactive cellular response may be mistaken for malignancy. Molecular analysis must be correlated on a cell-by-cell basis with the cell population of the sample being studied. Until this basic fact is completely understood, advanced techniques such as DNA sequencing and RNA expression profiling will invariably fail to have the expected impact in the practice of clinical medicine.

The best approach might take the form of a microscope slide staining procedure capable of revealing molecular aberrations in neoplastic and supporting cells. Unfortunately this is not feasible, because detailed molecular analysis of multiple genes cannot be achieved by the application of any one staining technique. Rather, multigene analysis has become a reality through the use of non-slide-based high-throughput technologies that are effective with samples as small as a single cell.⁷ In response, tissue microdissection approaches have gained considerable interest as the best way to bridge the gap between cellular morphology and molecular diagnostics.¹⁻⁶

Tissue microdissection logically places genetic analysis after histopathological evaluation. This ensures that the full measure of information derived from microscopic evaluation encompassing concepts related to cellular pathogenesis can be channeled into obtaining the very best sample for molecular analysis. This process of sample selection is far less subjective than microscopic diagnosis, which often is imprecise in establishing a tissue diagnosis and even more inexact in predicting tumor behavior and treatment responsiveness. In an era of

staggering achievements in our understanding of the molecular basis of medicine, the role of the pathologist will inevitably grow to include the careful selection of representative tissue samples for genetic analysis. This should lead to a more objective means of detecting and classifying cancer, better prognostication of tumor behavior, and better rationalization of treatment.

At present, the major impediment to achieving this goal is the well recognized detrimental effect of routine tissue fixation and processing on nucleic acid and protein content and structure. Ten-percent neutral buffered formalin fixation followed by paraffin wax embedding produces histological sections of excellent quality, albeit at the expense of virtually total RNA loss and serious cross-linking and strand scission of DNA.⁸ The latter can be attributed to prolonged formaldehyde fixation beyond the time sufficient to achieve optimal cellular morphology. Although small loss of RNA may be unavoidable given the ubiquitous presence of RNases in tissues and environment, major degradation of RNA is preventable by simple measures such as the use of RNase-free water and cold temperature for tissue processing and embedding. This rationale served as the basis for a system of molecular processing designed to maintain cellular integrity while preserving tissue nucleic acid and proteins.

Cold-temperature plastic resin embedding was chosen as it already has proven to be an effective technique for ultrastructural analysis.¹⁵⁻¹⁷ Two properties of Immunobed made it especially attractive. First, Immunobed can be polymerized in the cold without the need for ultraviolet radiation, which may exert damaging effects on nucleic acids. Second, Immunobed is a water-miscible plastic resin that allows full access by water-soluble molecules to tissue constituents. Its most common use currently is in the context of ultrastructural immunocytochemistry. In addition to these properties, Immunobed can be thin-sectioned for both light and electron microscopic analysis. We have shown in this report that the microscopic appearance of standard 4- μ m-thick histology sections is equivalent to that seen with paraffin embedding. In fact, cellular morphology can be improved even further by virtue of Immunobed's thin sectioning properties.

Cold-temperature embedding as described in this study was primarily responsible for preserving long DNA and RNA lengths for PCR format genotyping. Exogenous (HCV) and endogenous (β -actin) RNA was easily amplified in a highly sensitive fashion (Figures 2 and 3). Samples as small as 20 cells microdissected by manual methods were sufficient to detect and sequence HCV RNA from liver biopsies (Figure 4). A similar beneficial effect of Immunobed can be expected on tissue histochemical and immunohistochemical staining due to the improved preservation of cellular constituents and antigen reactivity already demonstrated for this embedding medium.¹⁵⁻¹⁷ Thus, the methods used here to integrate genomic and RNA expression analysis with histopathology may also be expected to facilitate proteome analysis into standard tissue pathology.

Tissue fixation was modified by substituting 2% paraformaldehyde for 10% neutral buffered formalin. The water used to constitute the paraformaldehyde fixative was rendered free of RNases through the action of 0.1% diethyl pyrocarbonate. Fixation was limited to 2 hours to avoid excessive cross-linking of cellular constituents. These simple measures, together with cold-temperature plastic resin embedding, can easily be adapted to anatomical pathology practice without significant increase in cost or turnaround time. The effort involved in its setup is more than justified by the benefits resulting from the potential for full application of molecular analysis.

Tissue microdissection was performed manually on plastic resin-embedded sections. In contrast to deparaffinized sections, plastic-embedded sections did not require exact wetting conditions to effect tissue removal. The action of sample removal was found to be greatly facilitated due to the support properties of the plastic resin. The entire process was found to be much easier and more quickly carried out than mechanical microdissection systems. Continued experience with this plastic resin embedding, including modifications for its introduction and possible removal before microdissection, may be expected to facilitate the further integration of histopathology and molecular biology. Thus, we recommend cold-temperature plastic resin embedding as a molecular tissue processing approach well suited to molecular analysis in anatomical pathology.

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