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Assessment of fixatives, fixation, and tissue processing on morphology and RNA integrity

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Abstract

Molecular characterization of morphologic change requires exquisite tissue morphology and RNA preservation; however, traditional fixatives usually result in fragmented RNA. To optimize molecular analyses on fixed tissues, we assessed morphologic and RNA integrity in rat liver when sections were fixed in 70% neutral-buffered formalin, modified Davidson's II, 70% ethanol, UMFIX, modified Carnoy's, modified methacarn, Bouin's, phosphate-buffered saline, or 30% sucrose. Each sample was subjected to standard or microwave fixation and standard or microwave processing, and sections were evaluated microscopically. RNA was extracted and assessed for preservation of quality and quantity. Modified methacarn, 70% ethanol, and modified Carnoy's solution each resulted in tissue morphology representing a reasonable alternative to formalin. Modified methacarn and UMFIX best preserved RNA quality. Neither microwave fixation nor processing affected RNA integrity relative to standard methods, although morphology was modestly improved. We conclude that modified methacarn, 70% ethanol, and modified Carnoy's solution provided acceptable preservation of tissue morphology and RNA quality using both standard and microwave fixation and processing methods. Of these three fixatives, modified methacarn provided the best results and can be considered a fixative of choice where tissue morphology and RNA integrity are being assessed in the same specimens.

Keywords: Fixation; Methacarn; Microwave; Morphology; Pathology; Processing; RNA; LCM; Histology; Molecular

Most methods currently used to manipulate tissues for microscopic examination were developed in the early 1900s. These methods evolved largely after the establishment of formalin as a fixative that could rapidly and permanently preserve fairly large tissue specimens. For this reason, histotechnology arguably can be viewed as a discipline devoted to optimizing the microscopic morphology of formalin-fixed tissues. Unfortunately, for molecular biology techniques, formalin fixation may be too successful in its ability "to prevent autolysis (the degradation of proteins into amino acids) and to coagulate cell contents into insoluble substances" (Stoddard, 1989).

Formalin preserves tissues by cross-linking proteins; however, RNA is fragmented, chemically altered, and difficult to isolate in quantity from formalin-fixed, paraffin-embedded (FFPE) samples (Goldsworthy et al., 1999; Masuda et al., 1999; Srinivasan et al., 2002). Isolation of intact RNA from tissues with pristine morphology has become more important with the advent of laser capture microdissection (LCM) and robust methods for RNA amplification because technology now exists to analyze mRNA from single populations of cells obtained from heterogeneous solid tissue samples (Mizuarai et al., 2005).

In general, investigations to harvest intact RNA from tissues examined microscopically have taken one of three approaches. The first concedes that RNA in fixed tissues is fragmented and instead uses snap-frozen tissues that are embedded in Optimal Cutting Temperature (OCT) media (TissueTek, Sakura Finetek, Torrance, CA). This method provides good quality RNA (Guo and Catchpoole, 2003); however, tissues need to be maintained at −70°C making the routine storage of large numbers of

Abbreviations: FFPE, formalin fixed paraffin embedded; H&E, hematoxylin and eosin; LCM, laser capture microdissection; NBF, neutral-buffered formalin; OCT, optimal cutting temperature; PBS, phosphate-buffered saline.

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Table 1
Fixatives used in this study

Fixative	Supplier	Fixative type	Notes
10% Neutral-buffered formalin (NBF)	Mallinckrodt Chemicals (Phillipsburg, NJ)	Aldehyde	Used as purchased
Modified Davidson's solution II	Rowley Biochemical (Danvers, MA)	Aldehyde	Used as purchased
70% Ethanol	PolyScientific (Bayshore, NY)	Alcohol	Used as purchased
UMFIX	Sakura Finetek (Torrance, CA)	Alcohol	Used as purchased
Modified Carnoy's solution	Freshly prepared	Alcohol	3 parts ethanol 1 part glacial acetic acid
Modified methacarn	Freshly prepared	Alcohol	8 parts methanol 1 part glacial acetic acid
Bouin's solution	Newcomer Supply (Middleton, WI)	Picrate	Used as purchased
Phosphate-buffered saline (PBS)	Ambion (Austin, TX)	Holding solution	Nuclease-free; pH 7.4
30% Sucrose	Freshly prepared	Holding solution	Diethylpyrocarbonate-treated water

samples expensive and often impractical. In addition, sectioning samples on a cryostat is time consuming, difficult, and provides morphology that is so compromised that it is often difficult to identify individual cell types.

The second approach recognizes that formalin is the fixative upon which histotechnology and diagnostic histopathology are based and tries to develop and/or improve methods to optimize RNA isolation from FFPE samples. Although morphology is maintained by using formalin, and this approach allows for the use of standard fixed and processed archival samples, RNA quality and quantity are compromised. These methods yield 50% to 99% less RNA than fresh tissue, and amplicons generated from this RNA are generally less than 300 bases (Lewis et al., 2001; Abrahamsen et al., 2003; Cronin et al., 2004).

The third approach tries to find a middle ground between tissue morphology and RNA integrity by using fixatives and different processing methods that offer morphology essentially equivalent to formalin-fixed tissues while being less damaging to RNA (Gloghini et al., 2004; Benchekroun et al., 2004; Parlato et al., 2002; Perlmutter et al., 2004; Shibutani and Uneyama, 2002; Shibutani et al., 2000; Vincek et al., 2003).

To define a reasonable compromise between optimal morphology and RNA quantity and quality, we evaluated tissue morphology and extractable RNA quantity and quality from rat liver prepared using nine different fixatives, standard and microwave fixation, and standard and microwave processing. RNA analysis was performed on both microtome and cryostat sections as well as on samples obtained by LCM.

We found that the fixative is the most important factor in the preservation of RNA quality, and while microwave protocols can modestly improve morphology, they have no significant impact on RNA preservation. Modified methacarn was the best fixative for the combined analysis of morphology, RNA quality, and efficiency of amplification from LCM samples.

Methods

Fixatives

Nine fixatives were used in this study (Table 1). Phosphate-buffered saline (PBS) and 30% sucrose are more accurately termed holding solutions, but for the sake of brevity, they will be grouped with the fixatives in this study. PBS and sucrose were chosen because these are often used to transiently prevent desiccation of tissues that will be subjected to LCM (Parlato et al., 2002).

Tissue preparation

Livers from four adult Sprague–Dawley rats were collected as per Institutional Animal Care and Use Committee (IACUC) approved procedures. Representative tissue samples were trimmed to approximately 15 mm × 8 mm × 3 mm. Two samples were embedded separately in cryomolds containing OCT, frozen on dry ice, and stored at −80°C until sectioning. Additional samples were immediately placed into one of nine fixatives at room temperature. One sample from each fixative was subjected to either (1) standard fixation and standard processing, (2) standard fixation and microwave processing, (3) microwave fixation and standard processing, or (4) microwave fixation and microwave processing.

Samples for standard fixation were immersed in the appropriate fixative for 48 to 72 h prior to processing. Overnight processing (including dehydration, clearing, and paraffin embedding) used fresh solutions other than paraffin and was performed using a Tissue Tek VIP5 automated processor (Sakura Finetek, Torrance, CA).

Microwave-assisted fixation and processing were performed using a Shandon Histowave (ThermoElectron, Pittsburgh, PA). For fixation, we used setting 2 at 70% power for 30 min. The protocol for microwave-assisted tissue processing was previously developed in-house for formalin fixation (Table 2). Microwave-assisted fixation was not used for Bouin's or modified Carnoy's solutions to avoid exposure to hazardous fumes. After embedding, 3-μm paraffin sections from a block prepared from each fixative and fixation/processing method were stained with hematoxylin and eosin (H&E). The morphology of each fixative and fixation/processing combination was evaluated based on a 1–4 grading system.

RNA extraction

Under conditions minimizing exposure to RNases, six 10-μm sections of tissue from each fixative, fixation, and processing method were placed in nuclease-free microcentrifuge tubes. To avoid contamination, a new microtome blade was used for each block. Six 10-μm sections of OCT-embedded tissue were similarly prepared using a Microm 560 cryostat (Richard-Allan Scientific, Kalamazoo, MI) and were stored at −80°C.

Table 2
Schedule used for microwave tissue processing (Shandon Histowave, ThermoElectron, Pittsburgh, PA)

Solution	Time (minutes)	Temperature (°C)	Wattage * (setting #)
100% Ethanol	15	55	5
100% Ethanol	15	65	6
Isopropanol	15	65	5
Isopropanol	15	65	6
Paraffin	20	70	6

* Power at 100%.

RNA isolation from all samples used a modified TRIzol® (Invitrogen, Carlsbad, CA) extraction method. Briefly, 800 μl of TRIzol® and 100 μl Proteinase K (20 mg/ml) were added to the 60-μm tissue sample, vortexed, and incubated at 55°C for 1 h. The temperature was increased to 70°C for 10 min to inactivate the Proteinase K, and 200 μl chloroform was added. After gentle vortexing, samples were centrifuged at 16,000 *g* for 15 min at 4°C. Owing to the change in temperature, after phase separation, the paraffin in the solution solidified between the two phases, allowing easy removal of the aqueous phase without contamination. Isopropanol (500 μl) was added, samples vortexed, and stored at −80°C. Following precipitation, total RNA samples were rehydrated in 40 μl RNA Storage Solution (sodium citrate, Ambion, Austin, TX). Samples were further purified using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). DNA was removed using the Ambion TURBO DNA-free kit.

RNA quantity was measured using the Nanodrop ND-1000 UV Spectrophotometer (Nanodrop Technologies, Wilmington, DE). A minimum of five replicates were extracted per sample type, and the Grubb's test was performed to detect and eliminate outlier values. Mean concentration and standard deviation were calculated, and a one-way test of variance was applied to define differences

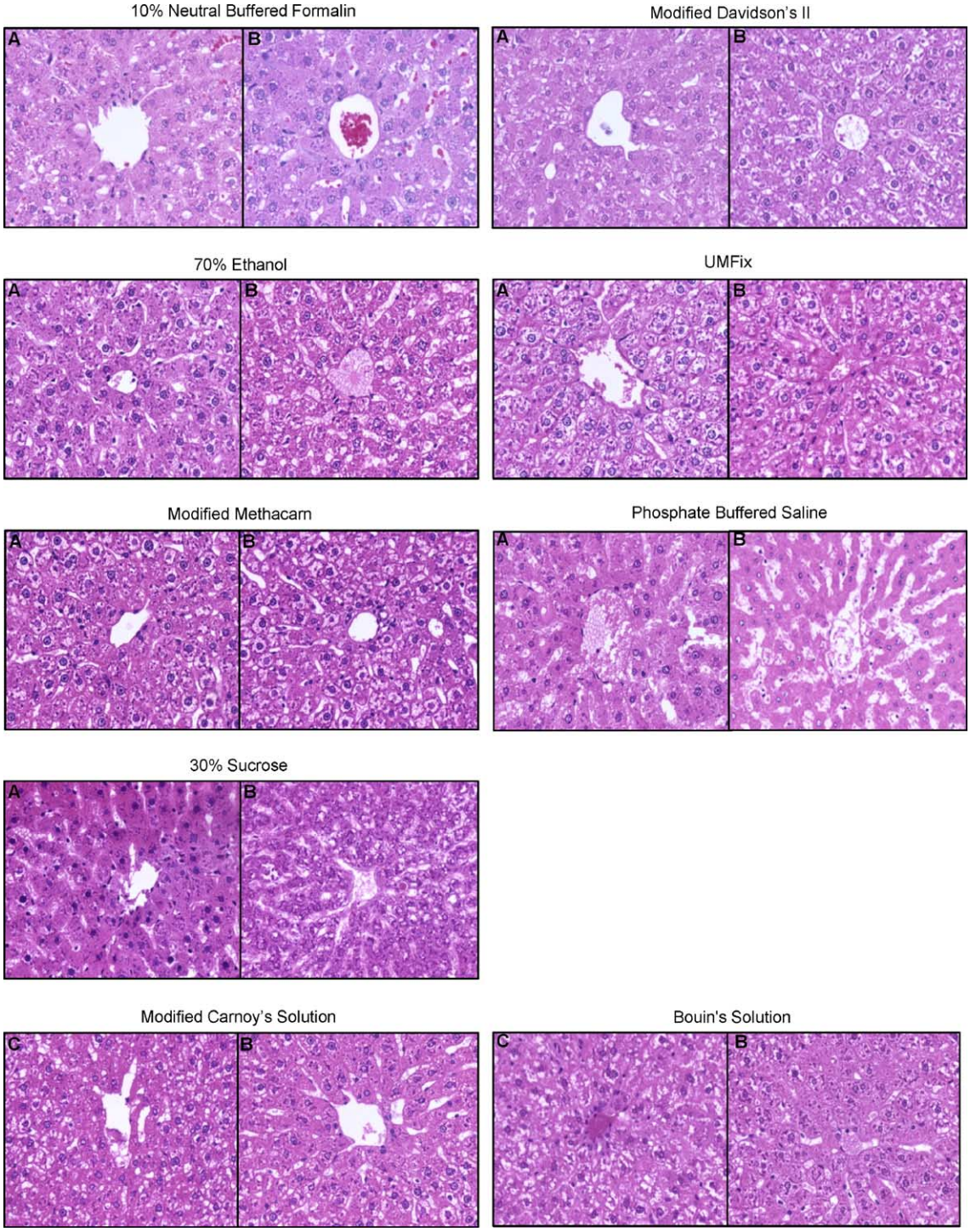

Fig. 1. Photomicrographs of liver centrilobular regions for all fixatives with two methods of fixation/processing (40× magnification; hematoxylin and eosin staining). (A) Microwave fixation and microwave processing, (B) standard fixation and standard processing, (C) standard fixation and microwave processing.

among the nine fixatives and the four combinations of fixation and processing methods per fixative. RNA quality was classified based on RNA fragment size using the Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent, Palo Alto, CA) with the RNA 6000 ladder (Ambion) as standard. Control rat liver total RNA was purchased from Ambion for comparison of RNA quality.

Laser capture microdissection and Taqman qRT-PCR

Four microwave-fixed, standard processed samples were chosen for LCM: 70% ethanol, modified methacarn, UMFIX, and 10% NBF. Each sample was sectioned to a thickness of 6 μm, and the sections were exposed to three washes in xylene of 2 min each for deparaffinization. This was followed by 30 s washes in ascending alcohols, another xylene wash, and drying at room temperature for 5 min. LCM was performed with an Arcturus Pixcell IIe (Arcturus, Mountain View, CA) and 1500 pulses of 7.5 μm each were collected from each sample in duplicate. RNA isolation followed using the RNAqueous-Micro kit (Ambion) including DNase treatment, according to manufacturer's instructions. Samples were quantified by UV spectrophotometry, and 5 ng of each was reverse transcribed using the SuperScript™ III Platinum® Two-step qRT-PCR Kit (Invitrogen) following manufacturer's instructions. The same kit was used for PCR amplification with the reaction size scaled to 10 μl, including 5 ng cDNA for each sample. Three Applied Biosystems (Foster City, CA) predesigned Taqman primer/probe sets were assayed: eukaryotic 18S rRNA (GenBank accession number X03205, catalogue number Hs99999901_st), PPIA (peptidylprolyl isomerase A, also known as cyclophilin A, GenBank accession number NM_017101, catalogue number Rn00690933_m1), and HPRT (hypoxanthine guanine phosphoribosyl transferase, GenBank accession number NM_012583, catalogue number Rn01527838).

RNA from each LCM sample from selected fixatives was run in triplicate on 2 different days. Triplicate determinations were averaged and mean Ct calculated from the two replicates from one plate. Finally, a mean Ct was calculated from reactions performed on different days. Failed reactions were not included. The final control mean Ct was subtracted from each final fixative mean Ct. This set the control at zero and allowed a comparison of the relative amplification efficiencies of the RNA derived from each fixative by LCM isolation.

Results

Morphology

Examples of typical morphology for each of the fixatives are shown in Fig. 1. Grading was on a four-point scale, with 1 being poor, and 4 being excellent (Table 3). Tissue was adequate for histologic examinations with all fixatives except Bouin's, PBS, and 30% sucrose, while modified methacarn had the best morphology. In comparison to standard methods, microwave fixation/microwave processing protocols resulted in modest improvement in morphology for all fixatives without any significant impact on the reviewer's ability to conduct microscopic interpretations. This was especially evident with 30% sucrose and PBS and to some extent with UMFIX. In addition, bacteria were noted in the tissues held in PBS for standard fixation.

RNA

RNA quantity was assessed by UV spectrophotometry, and the concentration is reported in micrograms (Fig. 2). The aldehyde-based fixatives and Bouin's solution had extremely low yields; the highest average yield among these (10% NBF with microwave fixation and standard processing) did not exceed 2.2 μg. The RNA yield from OCT-embedded tissue was the next highest. While the average yield from Carnoy's solution was approximately 20 μg, all of the other alcohol-based fixatives had average yields close to 35 μg. Microwave-fixed

Table 3
Histologic assessment of morphologic quality of liver sections examined with H&E staining using microwave (MW) fixation and processing and standard fixation and processing

Fixative/Processing method	Nuclear detail	Cytoplasmic detail	Cell membrane detail	Total
10% NBF				
MW fixation/MW processing	4	3	2	9
Standard fixation/ processing	3	3	3	9
Modified Davidson's II				
MW fixation/MW processing	3	2	3	8
Standard fixation/ processing	3	3	3	9
70% Ethanol				
MW fixation/MW processing	4	4	3	11
Standard fixation/ processing	4	3	3	10
UMFIX				
MW fixation/MW processing	3	2	3	8
Standard fixation/ processing	2	2	3	7
Modified Carnoy's				
Standard fixation/MW processing	4	2	3	9
Standard fixation/ processing	4	3	3	10
Modified methacarn				
MW fixation/MW processing	4	4	4	12
Standard fixation/ processing	4	3	4	11
Bouin's				
Standard fixation/MW processing	2	2	2	6
Standard fixation/ processing	3	3	3	9
PBS				
MW fixation/MW processing	2	4	1	7
Standard fixation/ processing[a]	1	1	1	3
30% Sucrose				
MW fixation/MW processing[b]	2	4	2	8
Standard fixation/ processing	1	3	1	5

Grading was 1 (poor) to 4 (excellent).
[a] Bacteria were noted in this sample.
[b] This sample was overmicrowaved.

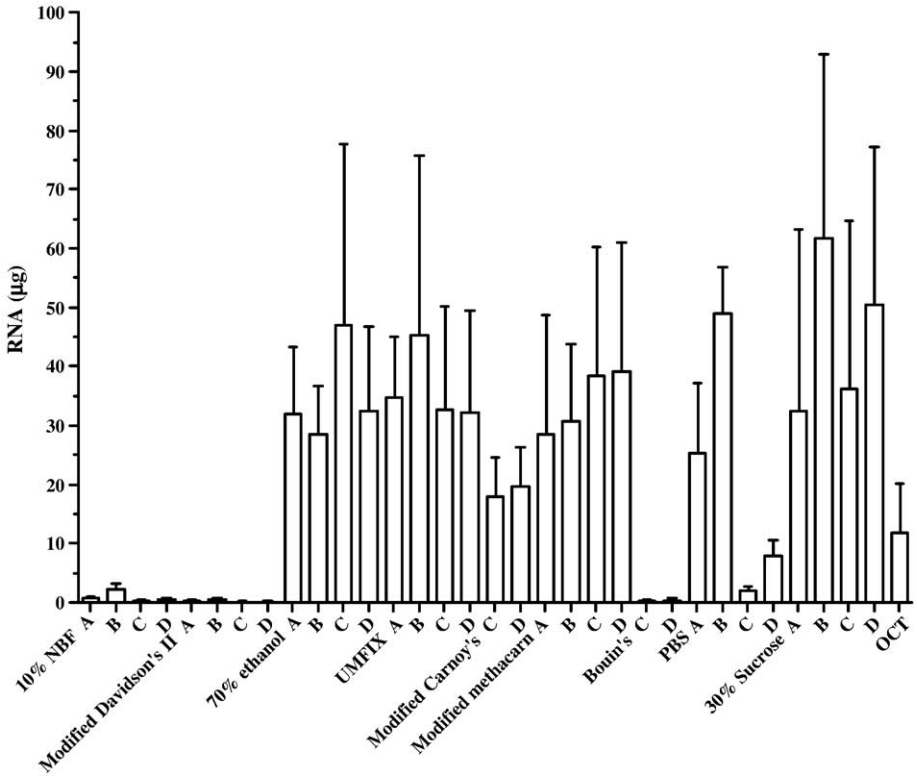

Fig. 2. Amount of RNA isolated (µg) based on different fixation and processing methods. (A) Microwave fixation and microwave processing; (B) microwave fixation and standard processing; (C) standard fixation and microwave processing; (D) microwave fixation and microwave processing.

PBS samples had yields comparable to the alcohols, although standard fixation resulted in much lower RNA yields. The solution with the highest average yield of RNA was 30% sucrose.

RNA quality was assessed by capillary electrophoresis (Agilent RNA Bioanalyzer, Fig. 3). Small fragments are observed earlier, thus RNA transcripts increase in size from left to right in Fig. 3. Ambion rat liver total RNA was used for comparison with 28S:18S rRNA peaks in an approximate 2:1 ratio at 49 and 43 s, respectively. RNA from OCT-embedded experimental rat tissue (Category 1) had distinct 28S and 18S peaks, although the ratio was approximately 1:2 28S:18S, and the baseline was elevated between and to the left of these peaks indicating partially degraded RNA. Some RNA fragments were up to and above 6000 bases, and the majority of RNA fragment sizes were still relatively large. Peaks for 28S and 18S were still apparent in the modified methacarn and UMFIX (Category 2) but were considerably reduced from those found in the OCT-derived RNA. Some fragments were up to 6000 bases, but the majority of fluorescence was observed between 400 and 3000 bases. Seventy percent ethanol, modified Carnoy's and PBS (microwave fixation conditions), fell into Category 3, in which the 28S peak was missing, but a small 18S peak remained. These RNAs were up to 4000 bases, with the majority between 25 and 2000 bases. 30% Sucrose had lower, broad spectra, without any rRNA peaks, indicating that some of the fragments had sizes of 1000 bases, but the majority were between 25 and 500 bases. RNA isolated from 10% NBF, modified Davidson's II, and PBS (standard fixation conditions) had extremely fragmented RNA, with the largest fragments approximately 500 bases, but the majority were 25–200 bases.

Except for PBS, no statistically significant ($P < 0.05$) differences were found among mean quantities of RNA within the four combinations of fixation and processing used in this study. There were also no obvious RNA quality differences among combinations of fixation and processing methods. Only PBS with microwave fixation had better RNA quality and quantity than with standard fixation.

LCM and qRT-PCR were performed to assess the practical use of RNAs isolated from four of these fixatives. With all three genes assayed, there were clear and reproducible differences in the average Ct when compared to the control (Fig. 4). Modified methacarn had the smallest average Ct above the control of all of the fixatives, for all three genes assayed. UMFIX was next best, while 70% ethanol and 10% NBF demonstrated the highest Ct values measured (Table 4).

Discussion

The objective of this study was to identify a fixative and an appropriate fixation and processing method that collectively provide the best balance of preservation of tissue morphology and RNA quality/quantity. Using the rat liver as a model, we determined that the fixative is the most critical consideration in optimizing both tissue morphology and RNA integrity.

The morphology associated with each fixative and fixation/processing combination was evaluated based on a 1–4 grading system where nuclear, cytoplasmic, and cell membrane detail

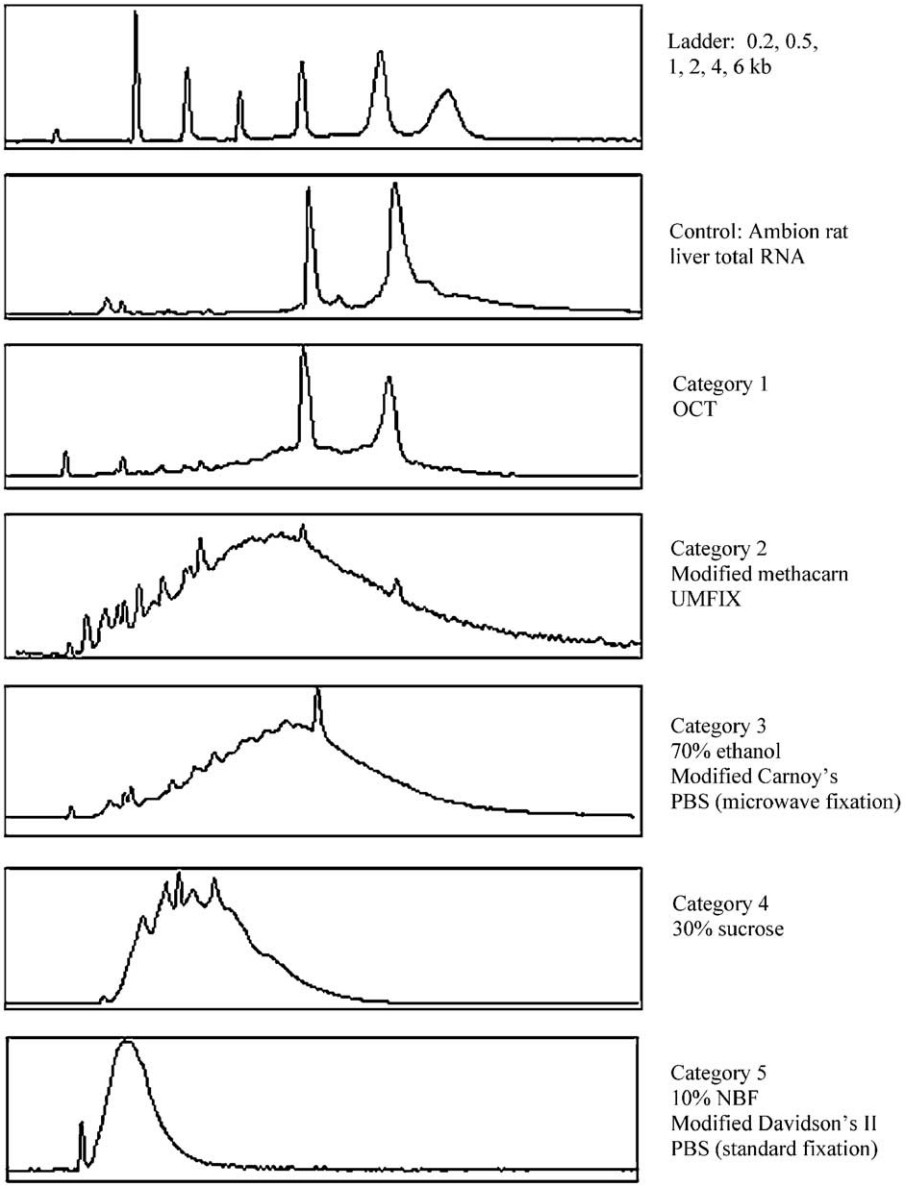

Fig. 3. Categories of RNA classification for samples run on the Agilent Bioanalyzer.

were given equal weight. Based on this scale, modified methacarn had the best morphology; however, there were no difficulties in making detailed morphologic assessment of tissues using 70% ethanol, 10% NBF, or modified Carnoy's. Since modified methacarn, 70% ethanol, and modified Carnoy's do not preserve tissues by cross-linking proteins, the morphology produced by these fixatives is not identical to formalin. This may not be preferable to anatomic pathologists skilled at the interpretation of cellular changes through formalin-induced artifacts. Nevertheless, the study does demonstrate that when preservation of RNA is an objective, very good morphology can be obtained with fixatives other than formalin.

In this study, the non-aldehyde-based fixatives with the best morphology were all alcohol based. The observation that tissues fixed in alcohol can offer superior morphology was also noted by Gillespie et al. (2002) who ranked 70% ethanol:100% methanol (3:1), 70% ethanol, and 95% ethanol as having superior morphology to 10% NBF based on nuclear morphology, cellular morphology, tissue architecture, and staining characteristics. Two of the solutions used in this study, 30% sucrose and PBS, are actually considered to be holding agents rather than fixatives because they do not chemically alter tissues. They were chosen because they are often used to transiently prevent desiccation of tissues that will be subjected to LCM (Parlato et al., 2002). In our experience, tissues that were maintained in these solutions, processed, and then paraffin embedded had poor morphology for adequate microscopic interpretations.

Most fixatives had slightly better morphology when microwave fixation/microwave processing protocols were followed. This was especially evident with 30% sucrose and PBS, most likely because microwave processing results in rapid infusion of paraffin and cessation of autolysis. One should note that both tissue trimming and fixation times need to be optimized for microwave methods (Hafajee and Leong, 2004).

In this study, we were rarely able to correlate RNA quality with quantity. The exceptions were the two extremes: 30% sucrose consistently provided the most RNA, but the RNA was degraded to fragments of 25 to 500 bases. In contrast, frozen sections obtained from OCT-embedded tissues provided less RNA, but it was of the highest quality with fragment sizes consistently near control distributions, through and above 6000 bases.

Ribosomal RNA is used as a standard for RNA quality because the size of these transcripts (eukaryotic 28S and 18S rRNAs are approximately 4800 and 2100 bases, respectively) reflect the average size range of mRNA transcripts present in total RNA. A 2:1 ratio of 28S rRNA:18S rRNA is optimal; the 28S rRNA is slightly more than twice as long as the 18S and will be detected twice as much in the same sample. A change from this ratio usually indicates that the larger 28S RNA is degrading. As shown in Fig. 3, the OCT preserved tissues had a 1:2 ratio of 18S to 28S, a decrease in quality compared to the control sample purchased from Ambion. We attribute this decrease in quality to the method used to freeze the samples in OCT medium in order to optimize morphology.

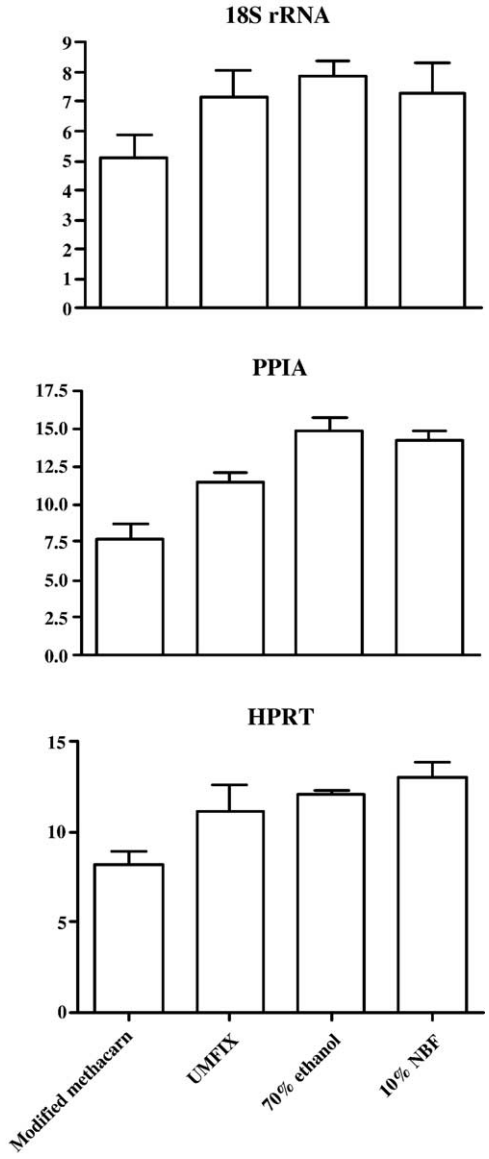

Fig. 4. Average Ct of each fixative-derived RNA above the mean for three specific transcripts: 18S rRNA, PPIA (peptidylprolyl isomerase A/cyclophilin A) and HPRT (hypoxanthine guanine phosphoribosyl transferase).

Table 4
Approximate size of RNAs extracted from rat liver preserved with various fixatives

Fixatives	Classification	Approximate RNA fragment size (nucleotides)
OCT	Category 1: 28S and 18S rRNA peaks	Through and above 6000; majority 1000–4700
Modified methacarn; UMFIX	Category 2: small 28S and 18S rRNA peaks	Up to 6000; majority 400–3000
70% Ethanol; modified Carnoy's; PBS microwave fixation	Category 3: 18S rRNA peak	Up to 4000; majority 25–2000
30% Sucrose	Category 4: no rRNA peaks; small RNA fragments	Up to 1000; majority 25–500
PBS standard fixation; NBF; Davidson's; Bouin's	Category 5: no rRNA peaks; extremely small RNA fragments	Up to 500; majority 25–200

Alcohol-based fixatives allow for the isolation of RNA with better integrity than formalin-based fixatives (Shibutani et al., 2000; Kim et al., 2003; Vincek et al., 2003; Perlmutter et al., 2004; Takagi et al., 2004). Our data support this observation, as modified methacarn and UMFIX had slightly higher yields but slightly less intact RNA than that obtained from frozen sections. The use of methacarn and UMFIX for gene expression studies has been previously documented and further confirms that additives to alcohols can protect RNA and better preserve morphology (Shibutani and Uneyama, 2002; Kim et al., 2003; Vincek et al., 2003, 2005; Takagi et al., 2004). It is reasonable to assume that chloroform (present in methacarn but not in the modified methacarn used herein) is not needed to preserve either morphology or RNA integrity. Modified methacarn can also be used with microwave fixation and microwave processors where volatilization of chloroform is a concern.

(Shibutani and Uneyama, 2002; Kim et al., 2003; Vincek et al., 2003, 2005; Takagi et al., 2004)The fixatives with the poorest quality RNA (fragments ≤ 500 bases) were PBS with standard fixation (because tissues autolyzed during the fixation period), Bouin's solution, Davidson's solution, and 10% NBF. Our results are consistent with a large body of literature (Goldsworthy et al., 1999; Masuda et al., 1999; Srinivasan et al., 2002; Abrahamsen et al., 2003; Cronin et al., 2004) that have indicated that aldehyde-fixed tissues invariably yield smaller fragments of RNA. This does not preclude their use in gene expression studies but until recently could limit the use of the RNA. Creation of cDNA libraries, Northern analysis, and to a lesser extent, nuclease protection assays required relatively intact RNA transcripts. Some reverse transcription and labeling

procedures also relied on the presence of the poly-A tail on transcripts. Although newer techniques such as quantitative RT-PCR and random primer extension labeling might mitigate some RNA hydrolysis, it is still probable that small transcripts could drop out of these protocols. An assay run with high quality RNA is always preferable.

New techniques in molecular biology allow for the use of small quantities of RNA in quantitative RT-PCR and microarray analysis. However, assigning expression profiles to individual cell populations is impossible if whole tissues are analyzed (Gjerdrum et al., 2004). LCM allows the isolation of individual cell populations from which RNA can be extracted, and gene expression levels can be compared among cells to identify changes that would be obscured in whole tissue analysis. This technique requires tissues with optimal morphology to distinguish between cell populations. Formalin-fixed paraffin-embedded tissues have been used successfully for LCM and RNA isolation when applied to the amplification of very short transcripts in quantitative RT-PCR (Specht et al., 2001), but product sizes and amplification efficiencies are higher in tissues preserved in ethanol or acetone-based fixatives (Koopmans et al., 1993; Goldsworthy et al., 1999; Takagi et al., 2004; Vincek et al., 2005).

Taqman quantitative RT-PCR reactions were performed to test the practical use of RNA derived from fixed tissues obtained by LCM. Starting with the same amount and concentration of RNA for all reactions, we observed clear and reproducible differences in amplification efficiency among the fixatives. Some samples yielded longer transcripts of total RNA than others, but we assumed that all samples contained essentially equivalent RNA information content. Thus, two possibilities may account for the differences observed in the amplification of each of the three genes assayed. The first is that the RNA with shorter fragments had more cleavage within the approximately 100-base Taqman target sequences. The second possibility is that less robust amplifications were due to modifications to the RNA and/or chemicals co-purified in the extraction process that inhibited efficient enzyme activity.

Microwave fixation and/or processing had no significant effect on RNA preservation. Prior to beginning this study, we evaluated the effects of microwave energy on RNA integrity. Control RNA was subjected to different times of microwave exposure and temperature gradations ranging from 5 min at 23°C to 60 min at 65°C. No degradation of RNA occurred at any of these settings when compared to RNA maintained at −80°C (data not shown). While the use of microwave-based methods may result in a shorter histology sample turn-around time and improved tissue morphology, our data demonstrate that these methods are irrelevant to the quantity or quality of isolated RNA.

Of the nine fixatives used in this study, modified methacarn, 70% ethanol, and modified Carnoy's solution had the best combination of morphology and RNA quality. Although UMFIX had RNA quality the equivalent of modified methacarn, the morphology was not acceptable. Other advantages to modified methacarn are that it is inexpensive and can be made in any laboratory. Like other alcohol-based fixatives, it is extremely gentle on tissue membranes compared to formalin, and immunohistochemistry can be performed with shorter incubations, higher dilutions of antibodies, and little need for antigen retrieval. In addition, methacarn allows for high-quality protein extracts and Western blots (Shibutani et al., 2000). These features support its use for LCM methods where preparation time and maintenance of tissue integrity are critical (Kim et al., 2003; Takagi et al., 2004).

In summary, the quandary between optimal morphology and RNA quality has no perfect solution; however, when the basis for RNA sampling is the microscopic examination of tissues, our study shows that modified methacarn is the fixative of choice.

References

Abrahamsen, H., Steiniche, T., Nexo, E., Hamilton-Dutoit, S.J., Sorensen, B.S., 2003. Towards quantitative mRNA analysis in paraffin-embedded tissues using real-time reverse transcriptase-polymerase chain reaction. J. Mol. Diagn. 5, 34–41.

Benchekroun, M., DeGraw, J., Gao, J., Sun, L., von Boguslawsky, K., Leminen, A., Andersson, L.C., Hieiskala, M., 2004. Impact of fixative on recovery of mRNA from paraffin-embedded tissue. Diagn. Mol. Pathol. 13, 116–125.

Cronin, M., Pho, M., Dutta, D., Stephans, J.C., Shak, S., Kiefer, M.C., Esteban, J.M., Baker, J.B., 2004. Measurement of gene expression in archival paraffin-embedded tissues. Am. J. Pathol. 164, 35–42.

Gillespie, J.W., Best, C.J.M., Bichsel, V.E., Cole, K.A., Greenhut, S.F., Hewitt, S.M., Ahram, M., Gathright, Y.B., Merino, M.J., Strausberg, R.L., Epstein, J.I., Hamilton, S.R., Gannot, G., Baibakova, G.V., Calvert, V.S., Flaig, M.J., Chuaqui, R.F., Herring, J.C., Pfeifer, J., Petricoin, E.F., Linehan, W.M., Duray, P.H., Bova, G.S., Emmert-Buck, M.R., 2002. Evaluation of non-formalin tissue fixation for molecular profiling studies. Am. J. Pathol. 160, 449–457.

Gjerdrum, L.M., Abrahamsen, H.N., Villegas, B., Sorensen, B.S., Schmidt, H., Hamilton-Dutoit, S.J., 2004. The influence of immunohistochemistry on mRNA recovery from microdissected frozen and formalin-fixed, paraffin-embedded sections. Diagn. Mol. Pathol. 13, 224–233.

Gloghini, A., Canal, B., Klein, U., dal Maso, L., Perin, T., Dalla-Favera, R., Carbone, A., 2004. RT-PCR analysis of RNA extracted from Bouin-fixed and paraffin-embedded lymphoid tissues. J. Mol. Diagn. 6, 290–296.

Goldsworthy, S.M., Stockton, P.S., Trempus, C.S., Foley, J.F., Maronpot, R.R., 1999. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol. Carcinogen. 25, 86–91.

Guo, D., Catchpoole, D.R., 2003. Isolation of intact RNA following cryosection of archived frozen tissue. BioTechniques 34, 48–50.

Hafajee, Z.A.M., Leong, A.S.-Y., 2004. Ultra-rapid microwave-stimulated tissue processing with a modified protocol incorporating microwave fixation. Pathology 36, 325–329.

Kim, J.-O., Kim, H.-N., Hwang, M.-H., Shin, H.-I., Kim, S.-Y., Park, R.-W., Park, E.-Y., Kim, I.-S., van Wijnen, A.J., Stein, J.L., Lian, J.B., Stein, G.S., Choi, J.-Y., 2003. Differential gene expression analysis using paraffin-embedded tissues after laser microdissection. J. Cell. Biochem. 90, 998–1006.

Koopmans, M., Monroe, S.S., Coffield, L.M., Zaki, S.R., 1993. Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. J. Virol. Methods 43, 189–204.

Lewis, F., Maughan, N.J., Smith, V., Hillan, K.P.Q., 2001. Unlocking the archive: gene expression in paraffin-embedded tissue. J. Pathol. 195, 66–71.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., Okubo, K., 1999. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 27, 4436–4443.

Mizuarai, S., Takahashi, K., Kobayashi, T., Kotani, H., 2005. Advances in isolation and characterization of homogenous cell populations using laser microdissection. Histol. Histopathol. 20, 139–146.

Parlato, R., Rosica, A., Cuccurullo, V., Mansi, L., Macchia, P., Owens, J.D., Mushinski, J.F., De Felice, M., Bonner, R.F., Di Lauro, R., 2002. A preservation method that allows recovery of intact RNA from tissues dissected by laser capture microdissection. Anal. Biochem. 300, 139–145.

Perlmutter, M.A., Best, C.J.M., Gillespie, J.W., Gathright, Y., Gonzalez, S., Velasco, A., Linehan, W.M., Emmert-Buck, M.R., Chuaqui, R.F., 2004. Comparison of snap freezing versus ethanol fixation for gene expression profiling of tissue specimens. J. Mol. Diagn. 6, 371–377.

Shibutani, M., Uneyama, C., 2002. Methacarn: a fixation tool for multipurpose genetic analysis from paraffin-embedded tissues. Methods Enzymol. 356, 114–125.

Shibutani, M., Chikako, U., Keiko, M., Toyoda, K., Hirose, M., 2000. Methacarn fixation: a novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. Lab. Invest. 80, 199–208.

Specht, K., Richter, T., Muller, U., Walch, A., Werner, M., Hofler, H., 2001. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am. J. Pathol. 158, 419–429.

Srinivasan, M., Sedmak, D., Jewell, S., 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am. J. Pathol. 161, 1961–1971.

Stoddard, R.W., 1989. Fixatives and preservatives: their effects on tissue. Conservation of Natural History Specimens: Spirit Collections. Manchester Museum and Department of Environmental Biology, The University of Manchester, pp. 1–25.

Takagi, H., Shibutani, M., Kato, N., Fujita, H., Lee, K.-Y., Takagami, S., Mitsumori, K., Hirose, M., 2004. Microdissected region-specific gene expression analysis with methacarn-fixed, paraffin-embedded tissues by real-time RT-PCR. J. Histochem. Cytochem. 52, 903–913.

Vincek, V., Nassiri, M., Nadji, M., Morales, A.R., 2003. A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. Lab. Invest. 83, 1427–1435.

Vincek, V., Nassiri, M., Block, N., Welsh, C.F., Nadji, M., Morales, A.R., 2005. Methodology for preservation of high molecular-weight RNA in paraffin-embedded tissue. Diagn. Mol. Pathol. 14, 127–133.