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Review

Excavation of a buried treasure – DNA, mRNA, miRNA and protein analysis in formalin fixed, paraffin embedded tissues

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Summary. Fresh or frozen tissue samples will always be the best tissue source for the analysis of nucleic acids and proteins from tissues. However, their long-term storage is expensive and laborious. Much interest has therefore been focused on the question whether the almost infinite resources of formalin fixed and paraffin embedded tissue samples in the archives of pathology and histology departments can be used for research on biomarkers and molecular mechanisms of disease. In recent years the methods and protocols for the extraction of DNA, mRNA, miRNA and proteins from formalin-fixed and paraffin-embedded tissue samples have improved enormously. Especially, the possibilities of analysing DNA and miRNA in FFPE have reached a level that allows their application as a first line approach in the search for biomarkers. In contrast, many questions remain in terms of quantification of mRNA and protein expression levels in formalin-fixed and paraffin-embedded tissue samples. This review gives an overview on current potentials and limitations of the quantification of DNA, miRNA, mRNA and the proteome in FFPE tissue samples. The chemical events during formalin fixation and paraffin embedding and alternatives to formalin fixation are described. In addition, methods and general problems of DNA, miRNA, mRNA and protein extraction and the current knowledge on the feasibility and accuracy of quantitative gene expression analysis in FFPE tissues is summarized.

Key words: FFPE, miRNA, DNA, Microarray, Proteomics

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Introduction

Formalin fixation and paraffin embedding for the conservation of tissue samples has been applied for over 100 years in all areas of biomedical research and diagnostics. Hence, archives with formalin-fixed and paraffin-embedded (FFPE) diagnostic tissue samples with associated clinical information or samples from elaborate and costly scientific experiments of tremendous value have accumulated worldwide. These precious biologic samples have commonly been used to answer the scientific or diagnostic questions by application of histologic techniques to visualize morphologic, and in some instances immunohistochemical features associated with certain pathologic or physiologic conditions.

With the discovery of DNA, RNA and, recently, small interfering RNA like miRNA as well as associated amplification and visualization techniques the possibilities for characterizing diseases have expanded far beyond the level of morphology. Hence, bringing together both, the enormous FFPE tissue archives and the constantly growing number of methods for the molecular analysis of cells should be rewarding. However, nothing comes without a price and this is also true for the relatively cheap and simple storage of morphologically well preserved FFPE tissue samples, which in terms of molecular characterization are quite challenging due to the damaging effect of formalin on nucleic acids.

This review summarizes the current knowledge on potentials and limits of DNA, miRNA, mRNA and proteome analysis in FFPE tissue samples. A focus will be laid on chemical events during formalin fixation and paraffin embedding, alternatives to formalin fixation and general problems of DNA, miRNA, mRNA and protein extraction and the current knowledge on the feasibility

and accuracy of quantitative gene expression analysis in FFPE tissues.

Effect of formalin fixation on proteins and nucleic acids

Effect on proteins and tissue morphology

Formalin, the aqueous solution of formaldehyde, is the most commonly used fixative to prevent biological tissues from decaying. The dominance of formalin as a fixative is mostly driven by its low cost and its serviceability in terms of biosecurity and preservation of morphologic details. The principle of its action is the reversible cross-linking of proteins by the formation of reversible methylol derivatives, Schiff-bases and stable methylene bridges, mostly between primary amino groups of the basic amino acid lysine but also to other proximal nitrogen molecules (Kunkel et al., 1981; Metz et al., 2004). These cross-links stabilize the morphological details of the tissues and allow their storage for years (Fox et al., 1985). In addition, the protein denaturation not only directly preserves the tissue morphology by fixation of structural proteins and in situ fixation of soluble proteins to cytoskeletal proteins, but also by the inhibition of intrinsic proteinolytic or nucleic acid degrading enzymes and so prevents autolysis. Due to its microbicidal properties, it also inhibits the activity of bacterial enzymes on cellular proteins and nucleic acids, preventing heterolysis, and reverses highly infectious tissues into biologically unobjectionable material.

These positive effects of formalin fixation on structural integrity and prevention of enzymatic degradation are, however, accompanied by its negative effects on the quality of nucleic acids and proteins (Lehmann and Kreipe, 2001; Dedhia et al., 2007).

Effect on DNA

DNA Fragmentation due to the low pH of unbuffered formaldehyde solutions that are oxidized to formic acid is a main cause of poor nucleic acid quality in FFPE (Bonin et al., 2003). DNA is fairly stable in a faintly acidic environment, but below pH 6 the glycosidic bonds in the purine bases are hydrolysed (McGhee and von Hippel, 1977b; Voet and Pratt, 1999; Bonin et al., 2003). The acidic environment induces protonation of the purine bases guanine and adenine, which, in this form, are easy targets for cleavage by hydrolysis. The resulting depurinated DNA is then susceptible to cleavage by hydroxyl ions and thus single strand breaks (Akalu and Reichardt, 1999; Voet and Pratt, 1999).

Another major problem of formalin fixation is the extensive cross-linking of proteins with nucleic acids, which leads to fragmentation of DNA and RNA and their unavailability with extraction protocols used for fresh frozen tissues (Lehmann et al., 2001). The mentioned effects lead to a failure of amplification of DNA fragments longer than 200 bp, especially in non-buffered formaldehyde solutions (Ninet et al., 1999; Lehmann et al, 2001). However, amplification of DNA fragments of up to 600 bp in length have been reported (Weiss et al., 2010).

Effect on mRNA

Similar to DNA, RNA also becomes modified and degraded during formalin fixation. Especially, the covalent bondage of monomethylol groups to purine bases, mostly adenines, hinders extraction of high quality RNA and disturbs accurate quantification (Feldman, 1973; Masuda et al., 1999; Doleshal et al., 2008). An important effect of formalin fixation on mRNA is the modification or complete loss of the poly A tail that subsequently inhibits the annealing of oligo (dT) primers during d the reverse transcription reaction (McGhee et al., 1977a; Srinivasan et al., 2002). Consequently, the resulting cDNA pool that is measured with RT-PCR or other methods does not reflect the true proportion of mRNA, but is highly influenced by the intactness and modification status of the specific mRNA type.

Effect on miRNA

In contrast, the quality of microRNAs (miRNA) seems to be less affected by formalin fixation. miRNA is a newly discovered very short RNA, on average only 22 nucleotides long, which in contrast to mRNA types are not translated into proteins (Ambros, 2004). They post-transcriptionally repress mRNA translation by binding to their 3' UTRs and consecutive silencing or degradation of mRNA (Bartel, 2004). miRNA are thought to be a newly discovered but very old way of genetic regulation, which besides the well known genetic or epigenetic transcriptional regulation mechanisms, dramatically influences the function and metabolism of cells under physiologic and pathologic conditions. Their constitutional shortness seems to significantly weaken the degrading effect of formalin on miRNA and may be one of the reasons why several recent studies found that miRNAs apparently are relatively unaffected and well preserved in FFPE tissues (Li et al., 2007; Xi et al., 2007; Szafranska et al., 2008; Liu et al., 2009). In addition, their uniform structure may also cause a similar and proportional degrading effect on the different miRNA types and thus keep their relative amounts to some extent equal.

Effect of paraffin embedding, storage and prefixation treatment on nucleic acids and proteins

There are few studies on the effect of paraffin embedding on nucleic acid integrity and quality. Several

types of paraffins are used for embedding in the different laboratories. Especially low quality paraffins that contain beeswax may contain contaminants and therefore interfere with the extraction of biomolecules (Fergenbaum et al., 2004). In addition, the incubation of tissues in 60°C or warmer paraffin may enforce protein cross-linking and thereby increase DNA damage to some extent (Fergenbaum et al., 2004).

Contradictory information is available on the effect of storage duration on nucleic acid and protein yield and quality in paraffin blocks. One study found no loss of sensitivity for detection of Hepatitis C virus RNA in paraffin blocks of formalin-fixed liver tissues stored for more than four years (Guerrero et al., 1997). In contrast, a progressive decrease in signal intensity of type III collagen mRNA during storage of non-deparaffinized histological sections has been shown, although this decrease may also be caused by oxidation of mRNA (Lisowski et al., 2001).

Another very important parameter that affects quality and quantity of extractable nucleic acids from FFPE that is not directly associated with the fixation process is pre-fixation treatment of tissues to be fixed. Ideally, tissues are fixed immediately after surgical removal or necropsy. In an experimental setting, in vivo perfusion of animals is the best way to do so. However, in the routine diagnostics on human or veterinary excisional biopsies the duration between excision and complete fixation is mostly unknown but tremendously influences nucleic acid quality and quantity in the biopsies (Hipfel et al., 1998; Labat-Moleur et al., 1998; Srinivasan et al., 2002). For instance, pre-fixation anoxia of less than 10 minutes may already lead to substantial changes in the protein and mRNA composition of the cell (Kingsbury et al., 1995). After 30 min the mitotic figures, a morphologic correlate of the cell cycle status and the metabolic status of dividing cells, are reduced to 50% of the original number (Cross et al., 1990).

Taken together, to maintain a certain quality, above all for longer nucleic acid in a FFPE tissue sample, it is recommended to sufficiently fix the tissue for at least one day (avoiding autolysis) in neutrally buffered formaldehyde solution (avoiding complete DNA degradation) before paraffin wax embedding. However, in addition to the negative effect of formalin-fixation itself, a prolonged time between surgical removal of the tissues and fixation may also heavily influence nucleic acid quality of the FFPE tissue (Figs. 1-3).

Alternative fixation methods

The positive aspects of formalin fixation are undeniable: It is an easy and cheap method that reliably preserves the anatomical structure of the fixed tissue. Nevertheless, it is a hazardous material for both human health and harmful to the environment (NTP, 2005) and has detrimental effects on nucleic acid. Consequently, the demand for a less harmful fixative, which has nucleic acid degrading properties, but with similar structural preserving properties has led to several studies on other fixation methods.

Fresh frozen tissue samples – the gold standard

Obviously fresh frozen tissue samples, eventually stored in a cryo-protective embedding medium like OCT, TBS or Cryogel at temperatures below -80ºC are the gold standard and the best way to keep an almost physiologic nucleic acid integrity. Nevertheless, there are also disadvantages to this fixation procedure: Fresh frozen tissue sections have inferior morphological details then FFPE tissues, most infectious agents keep their biohazard potential and the technical equipment and the maintenance costs are high.

Glutaraldehyde

Glutaraldehyde is another aldehyde that is regularly used for fixation of tissue for consecutive ultrastructural analysis. Similar to formaldehyde, it causes cross linking and deformation of the alpha-helix structure of proteins, but due to its increased length and two aldehyde groups it leads to a more rigid fixation than formaldehyde. Furthermore, due to its larger size glutaraldehyde has a slower diffusion rate across intact cell membranes and therefore perfusion of voluminous tissue samples is a problem.

Glutaraldehyde causes similar DNA degradation to formaldehyde due to its similar structure and mechanism of fixation. Moreover, the few studies on DNA extraction from glutaraldehyde fixed tissues indicate that its stronger protein cross-linking has an even more severe negative effect on all nucleic acid types (Bramwell and Burns, 1988, O'Leary et al., 1994). However, a recent study found that a mixture of 4% paraformaldehyde/0.1% glutaraldehyde is a good compromise to combine well-preserved morphology with acceptable DNA extraction and in situ hybridization signalling (Falconi et al., 2007).

Alcohols

The most common alcoholic fixatives are ethanol, methanol and acetone. They also precipitate and denature protein molecules by breaking hydrophobic bonds thus disrupting their tertiary protein structure but not by cross linking of proteins with other proteins or nucleic acids. Alcohol fixation leads to massive shrinkage of the tissue. Acidic acid, which leads to a swelling of the tissue, is therefore occasionally used as an additive to attenuate shrinkage but causes severe degradation of nucleic acids. In daily practice, alcohols are nowadays commonly used to fix frozen sections and cytologic smears, but not for the routine fixation of larger tissue samples, mainly due to their higher price and their easy evaporation. Some studies analyzed

whether fixation with ethanol, methanol, acetone or a mixture of methanol and acetic acid (MAA) and Carnoy's (alcohol with acidic acid and chloroform) may be a better compromise between preservation of anatomical structure and nucleic acid integrity when compared to formalin fixation. In one of the first studies on the effect of alcohol fixation on the amount and quality of DNA extracted from snap frozen and formalin fixed tissue samples were compared with those from ethanol and MAA fixed tissues (Bramwell and Burns, 1988). MAA produced strong nucleic acid degradation that was, however, less severe than formalin fixation. Ethanol fixation for up to 120 hrs yielded high amounts of well preserved nucleic acids similar to fresh frozen tissue sample. Ethanol fixation was therefore recommended as a good alternative to formalin and snap frozen tissue samples in cases weretransport or storage conditions limit the availability of cooling equipment (Bramwell and Burns, 1988) Similar studies on the effect of ethanol fixation are not available for miRNA but an analogue effect can most probably be anticipated. Several other studies confirmed that short term ethanol fixation or storage (days to a few weeks) may result in mRNA and DNA yields and quality almost indistinguishable from snap freezing (Smith et al., 1987; Jackson et al., 1990; Kilpatrick, 2002; Soukup et al., 2003; Linke et al., 2010).

In addition, two studies found that ethanol fixation and storage for up to two years allows extraction of high quality nucleic acids, although at decreasing amounts (Kilpatrick, 2002; Ribeiro et al., 2004). However, incomplete fixation of thicker tissue samples has been identified as a potential source of nucleic acid loss in ethanol fixed tissue (Barnes et al., 2000). In addition, it can be speculated that improper storage and bacterial contamination of the ethanol solution may lead to its fermentation to acetic acid, thereby lowering the pH of the solution and again interfering with nucleic acid integrity.

HOPE fixative

Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) preserves the anatomical structure of tissue samples similar to formalin. It has a reduced, negative influence on the detection of protein epitops relevant for immunohistochemistry when compared with formalin fixation, mainly due to the absence of protein cross-linking (Vollmer et al., 2006). In addition, proteome studies confirmed that the majority of the proteins in tissues keep most of their biochemical features in terms of size and isoelectric point (Kahler et al., 2010). The lack of protein cross-linking and the almost physiologic pH of the HOPE solution RNA and DNA yields and quality is also superior to formalin-fixation (Vollmer et al., 2006). One of the major problems for the application of HOPE as a routine fixative in diagnostics and research are the, to the knowledge of the authors, so far unclear microbicidal, fungicidal and virucidal effects of HOPE and the potential biohazards of tissues fixed in it.

Nitrite pickling salt (Weigner`s solution)

Recently, nitrite pickling salt solution (NPS) supplemented with ethanol and Pluriol (Weigner's) has been suggested as an alternative to formaldehyde for the fixation of tissues and whole cadavers (Janczyk et al., 2010). In contrast to formaldehyde it has almost no negative health or environmental effects. Histologically, the texture of several tissue samples was well preserved after fixation for up to 12 months without any histological signs of autolysis. Microbiologically, NPS fixation was inferior to formalin but was nevertheless bactericidal for most bacterial pathogens, except a few halophilic species. In addition, NPS fixation did not induce nucleic acid degradation, at least after short term fixation (Klopfleisch et al., in preparation).

Other fixatives

It is almost impossible to completely cover all studies on potential non-formalin fixatives that were analyzed for their effects on nucleic acid. These studies mainly focused on the effects on mRNA or DNA, while studies on miRNA and proteins are in most cases not available. In the following some promising candidates and the knowledge on their effect on nucleic acids will be shortly summarized.

Methacarn fixation has been described as a potential tool for the analysis of gene expressions in paraffin-embedded tissue specimens (Shibutani et al., 2000). Methacarn is a non-cross-linking, protein precipitating fixative (Puchtler et al., 1970). It has only mild negative effects on the efficiency and quality of RNA and DNA isolated from microdissected rat tissues (Shibutani et al., 2000). Tissues fixed in this fixative also yield acceptable results in immunohistochemical assays and have acceptable histologic structure (Shibutani et al., 2000).

Mercurials such as B5 and Zenker's (Helly's) are fixatives with a so far unknown fixation mechanism that give excellent nuclear detail, for instance, of hematopoietic and reticuloendothelial tissues samples.

Mercurials penetrate tissues poorly and induce massive volume shrinkage. However, mercury is the main component of these fixatives and its detrimental effects on human health and environment almost prohibits their routine utilization. In addition, fixation of tissue samples with mercurial-based fixatives consistently impeded or drastically decreased amounts of isolated DNA and RNA (Ben-Ezra et al., 1991; O'Leary et al., 1994). Application of mercuric chloride-based fixatives should therefore be avoided when nucleic acid isolation is intended.

Similarly, Bouin's fixative, which mainly contains saturated picric acid supplemented with formaldehyde and glacial acetic acid also has detrimental effects on nucleic acids and should not be used when nucleic acid

extraction is an option. Almost all studies failed to isolate mRNA or DNA from tissue samples fixed in Bouin's (Ben-Ezra et al., 1991; O'Leary et al., 1994).

Several commercially available fixatives, such as Glyo-Fixx, FineFIX, ExcellPlus, Omnifix RCL2, Glyo-Fixx, have been tested for their effect on DNA, RNA and miRNA integrity (Ben-Ezra et al., 1991; Titford et al., 2005; Stanta et al., 2006; Lassalle et al., 2009). Of these, FineFIX, Omnifix and RCL2 were superior to formalin fixation while all others were similar to formalin fixation. In addition, most of these fixatives are less harmful to health and environment, but more expansive and mostly inferior in terms of structural preservation when compared to formalin (Ben-Ezra et al., 1991; Titford et al., 2005; Lassalle et al., 2009).

In summary, several alternative fixation methods to formalin have been tested. Some of them, alcohols, HOPE and several commercially available fixatives unequivocally have better performance in terms of nucleic acid preservation. However, other features like stability, costs, disinfectant properties or tissue preservation are in most cases inferior to formalin. It is therefore difficult to recommend a specific alternative to formalin since each of the broad range of potential fixatives may perfectly match in a specific organization or function.

Extraction, quality and quantification of DNA from FFPE tissue

DNA extraction protocols and DNA amplification by conventional polymerase chain reaction

Isolation of sufficient amounts of intact DNA from FFPE tissue samples is still challenging, despite the ever increasing number of commercially available kits and methods. This is mainly based on the fact that DNA-protein cross linking and DNA fragmentation is unavoidable, and in the case of DNA fragmentation irreversible. Protein-DNA cross linking can partly be overcome by a thorough deparaffination and digestion of FFPE tissues. Digestion with proteinases is an absolute prerequisite and may free most of the DNA from the linked proteins and make them available fir PCR amplification (Gilbert et al., 2007; Huijsmans et al., 2010). One crucial parameter for the satisfactory DNA yields during this step of DNA isolation seems to be the thickness of paraffin sections and the duration of digestion. Paraffin sections thicker than 2 μm and a digestion time less than 48 hours markedly reduced DNA yield (Weiss et al., 2010).

The degradation of DNA molecules into smaller fragments is nevertheless a problem when larger fragments are intended to be amplified. Usually it can be assumed that formalin fixation of several days to weeks leads to an almost complete degradation of DNA into fragments of 200 bp or less (Gilbert et al., 2007), resulting in the typical smear seen in agarose gels of PCR products of native extracted DNA (Fig. 1).

Several methods for extraction of DNA from FFPE tissues for genomic analysis or detection of infectious agents have been published (Werner et al., 2005; Dedhia et al., 2007; Vahlenkamp et al., 2008; Lin et al., 2009; Santos et al., 2009; Farrugia et al., 2010; Huijsmans et al., 2010; Okello et al., 2010a; Olias et al., 2010; Weiss et al., 2010b). In general, most of the protocols are based on commercially available DNA extraction kits that lead to mildly variable amplification success (Table 1). DNA yields were all low for all protocols when compared to yields from fresh tissue, but good enough for PCR analysis with all protocols (Table 1). Amplification of PCR fragments up to 200 bp was also uncomplicated for all protocols. However, suboptimal purity of DNA (Dedhia et al., 2007) or failure of effective amplification of DNA fragments beyond 300 bp (Dedhia et al., 2007; Lin et al., 2009; Farrugia et al., 2010; Huijsmans et al., 2010; Okello et al., 2010a) were reported, while some protocols allowed consistent amplification of fragments up to 600 bp (Lin et al., 2009; Muller et al., 2009; Santos et al., 2009; Weiss et al., 2010).

In a recent multicentre study that validated 13 commercially available or homemade methods for DNA extraction from FFPE tissues found that except for one homemade protocol, most protocols gave comparable results in terms of the quality of the extracted DNA in terms of length of amplifiable gene fragments by PCR

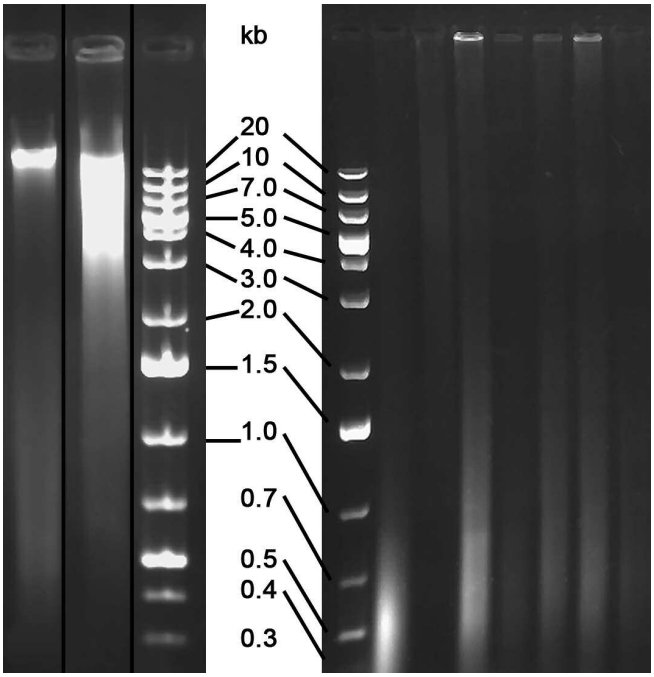

Fig. 1. Agarose gel electrophoresis of native DNAs. Lanes 1-2: DNA extracted from canine fresh-frozen tissues (Lane 1 - Tissue was snap-frozen immediately after excision, Lane 2 - Tissue was frozen two days after death). Lanes 3-9: DNA extracted from diverse FFPE-tissues. DNA is markedly degraded and size of DNA-fragments vary from 300 to 1000 bp.

(Bonin et al., 2010). However, for array-applications that need accurately determined DNA input, silica based adsorption columns were recommended over homemade protocols (Bonin et al., 2010).

(Fluorescent) in situ hybridization, CGH arrays and SNP analysis in FFPE tissues

Several other methods are used to characterize DNA sequences and amount in FFPE tissue besides PCR. In situ hybridization (ISH) is a method for detecting specific nucleic acid sequences within in its morphologic background. The advantage of this method is therefore to provide combined information on nucleotide sequence and the location of the nucleic acid. Several studies show that ISH can be used for detection of host, but also viral or bacterial DNA or RNA in FFPE tissue sections (Gruber et al., 1993). Fluorescent in situ hybridization (FISH) is a special form of ISH that is used to investigate genomic changes at the chromosomal and DNA level by hybridization of fluorescent labelled DNA probes in cell preparations (Gnanapragasam, 2010). FISH can also be directly applied on tissue sections and has been successfully used to in the analyze the presence of DNA fragments in FFPE tissue samples of bladder and prostate cancer (Gnanapragasam et al., 2003; Kruger et al., 2003). Similarly, comparative genomic hybridization (CGH), which is also used to analyse alterations in DNA copy number in tissue lysates, has been successfully applied on FFPE-derived DNA (Paris et al., 2007). A prior DNA amplification with random primers has been recommended to compensate for poor DNA yield and quality in FFPE tissue lysates for CGH arrays, but also for PCR analysis (Daigo et al., 2001; Baak-Pablo et al., 2010). However, a common problem of pre-amplification before quantification is the high probability of artefactual changes in the relative amounts of the randomly amplified DNA fragments.

Single nucleotide polymorphism (SNP) analysis has also been conducted in FFPE. SNP analysis allows allelic discrimination by very short genomic segments, thus allowing the use of highly fragmented FFPE DNA, although modified DNA extraction protocols and pre-processing steps for improved DNA quality may be improve results (Lyons-Weiler et al., 2008). In one study a SNP concordance rate of 96% between matched fresh and FFPE renal tumours were identified (Lyons-Weiler et al., 2008). This indicates that FFPE derived DNA, although fragmented, seems to have a high sequence preservation. However, other studies on mutational status in tumours found results from FFPE tissues to be less accurate than those from fresh frozen tissues, the gold standard (Verhoest et al., 2010). These artefacts may for instance be caused by the cross-linking of cytosine nucleotides. During PCR the Taq-DNA polymerase then fails to recognize the affected cytosine and incorporates an adenine instead of a guanosine, leading to an artificial C-T or G-A exchange (Williams et al., 1999). Up to one artificial mutation per 500 bases by this or other causes has been identified in FFPE derived DNA (Williams et al., 1999).

Extraction and expression analysis of mRNA from FFPE tissue

In contrast to DNA, mRNA analysis from FFPE tissues is much more focused on the quantification of relative or absolute mRNA amounts in different biologic tissues than the analysis of its sequence. Nucleotide exchanges in FFPE-derived mRNA are therefore less a problem than in FFPE-derived DNA that is used for mutational analysis, except when these changes take place in the primer binding site for the reverse transcription (RT)-PCR or affect the polyA-tail.

However, mRNA degradation and fragmentation which leads to decreased amounts of mRNA very much compromises the results of absolute mRNA quantification in FFPE tissues when compared to fresh

Table 1. Comparison of DNA yield and maximum PCR product in different DNA extraction protocols.

	Extraction methods	Maximal fragment size	Yield of DNA (µg / mg tissue)
(Dedhia et al., 2007)	Heat pretreatment	< 250bp	n.r.
(Farrugia et al., 2010)	Qiamp DNA Mini Kit	< 300bp	0,3
(Hennig et al., 2010)	Iron oxide beads (modif. Versant® kPCR System)	n.r.,	suitable amounts from one section
(Huijsmans et al., 2010)	Heat-treatment	400bp	
	QIAamp DNA-blood-mini	400bp	
	EasyMAG NucliSens	400bp	
	Gentra Capture-Column	unsuitable	n.r.
(Lin et al., 2009)	DNeasy Blood & Tissue	606bp	n.r.
(Okello et al., 2010b)	None (Phenol/Chlorophorm)	< 200bp	0.04
(Santos et al., 2009)	QickGene Tissue	600bp	1.3 – 5.2
(Weiss et al., 2010)	Heat pretreatment Gentra® Puregene® Tissue	600bp	2.0 – 3.1
(Werner et al., 2005)	DNeasy Blood & Tissue	300bp	n.r.

frozen tissues (Fig. 2). In a diagnostic scenario, this may decrease the sensitivity of the detections of viral pathogens (Gruber et al., 1993, 1994).

For the quantification of mRNA expression the absolute amount of mRNA in a certain tissue sample is normalized and compared to a reference tissue or so called housekeeping genes which are expressed at stable levels independent from the specific cell status (Vandesompele et al., 2002; Klopfleisch et al., 2010c). If it can be assured that mRNA degradation proportionally affects all mRNAs, i.e. the different mRNA of interest and the housekeeping genes, formalin fixation should not be a problem for relative mRNA quantification. Unfortunately, one study reported, that the relative amount of different mRNA species changes during formalin fixation when compared to the initial relative amounts in fresh tissues (von Smolinski et al., 2005). This effect even obliterates the effects of autolysis on the relative mRNA amounts in FFPE tissues.

The limited availability and the cost intensive storage of fresh tissues, however, lead to an ever increasing number of studies on how to overcome these adverse effects of formalin fixation on mRNA quality and quantity. Hence, several protocols that are supposed to diminish the adverse effect of formalin fixation on mRNA amounts have been published.

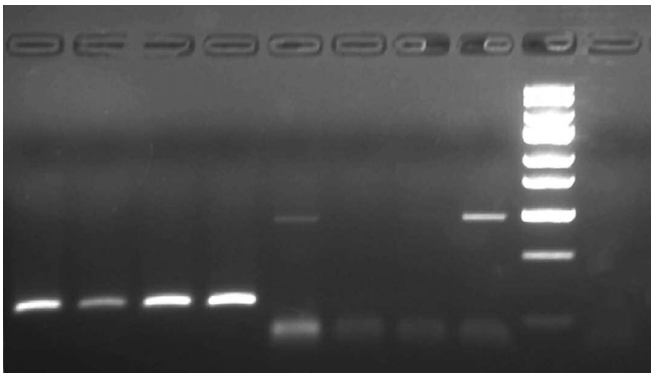

Fig. 2. RT-PCR-Amplification of two RNA-fragments from four different FFPE-samples. Lanes 1-4 depict a 133 bp PCR product. Lanes 4-8 depict a 470 bp PCR product amplified from the same samples. While amplification of a 133 bp product was successful in all cases, the 470 bp fragment was amplifiable only in 3 of 4 samples and yielded markedly smaller amounts of PCR product.

RNA extraction and RT-PCR

Several approaches to improve yield and quality of mRNA extraction from FFPE have been reported. Consistently throughout all reports, tissue lysis by proteinase K digestion has been reported as the best approach for maximum mRNA yield, while other dissolving agents like chaotropic substances failed to do so (Jackson et al., 1990; Masuda et al., 1999). However, poor mRNA quality due to degradation and fragmentation is still a major problem for proper quantification of mRNA from FFPE. It only allows the consistent amplification of small targets up to 120 bp while attempted PCR products larger than 200 bp should be avoided (Jackson et al., 1990; Farragher et al., 2008). In some instances it is nevertheless possible to amplify RNA of up to 470 bp in length (Figure 3) (Weiss et al., in preparation). In addition, formalin induced methylene bridging that randomly inhibits cDNA synthesis randomly affects mRNA types and thereby changes relative composition of the transcriptome (Masuda et al., 1999). These changes may be reversible and can be removed by incubating of the mRNA in basic buffers at 70°C (Jackson et al., 1990).

Initially, guanidinium thiocyanate or phenol chloroform extraction has been successfully applied to extract mRNA from FFPE tissues (Chomczynski and Sacchi, 1987). In recent years commercial kits specifically designed for the extraction of FFPE (Macherey&Nagel, Qiagen, Gentra, Life Technologies, Ambion) have become available and are most commonly used. They mostly yield reasonable quantities of RNA that are qualitatively acceptable for RT-PCR amplification. Several additional treatment options for increased mRNA amounts have been suggested and they are reviewed in (Lewis et al., 2001). They include sonification and the use of oligo(dT)25 paramagnetic beads (Houze and Gustavsson, 1996) and RNA binding to glass beads in guanidinium salt solutions (Koopmans et al., 1993). Generally, a formalin fixation time of less than 48 hours at 4°C before paraffin embedding has been recommended, since this causes the least amount of nucleic acid degradation (Abrahamsen et al., 2003). Prolonged storage of paraffin-embedded tissues blocks does not affect mRNA quality while storage of paraffin sections mildly degenerates RNA by oxidation (Ribeiro-Silva et al., 2007).

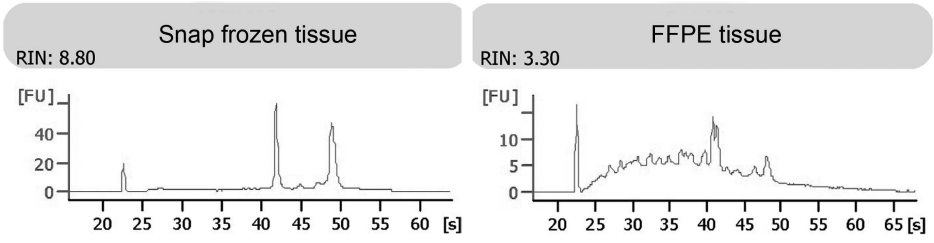

Fig. 3. RNA integrity number (RIN) as measured by an Agilent bioanalyzer. Well preserved RNA integrity of mRNA extracted from a snap frozen tissue sample of a canine mast cell (left). In contrast, mRNA extracted from the same tumor after FFPE results in pronounced degradation that is reflected in the loss of the typical two peak appearance of the electropherogram (right).

None of the above mentioned methods are able to restore damage caused by improper handling of tissues before fixation. As mentioned above, RNA degrades fast due autolysis when fixation is delayed. Proper handling of tissue samples by surgeons or pathologists is therefore the basis for all further steps in mRNA analysis and this requires a significant contribution and effort by these "first hand on" collaborators. In addition, small tissue samples that allow complete penetration of the fixative in an appropriate time are irreplaceable.

Successful reverse transcription of mRNA extraction from FFPE tissues can be considered another crucial step for mRNA quantification. Failure of successful RT-PCR at later stages is usually due to failure to establish sufficient cDNA amounts (Hewitt et al., 2008). It is assumed that loss of the mRNA polyA tails may lead to the failure of polyT oligomer binding that is commonly used for reverse transcription (Hewitt et al., 2008). Random hexamer or the specific antisense primer of the final PCR have therefore been recommended as a possible alternative (Lewis et al., 2001; Xiang et al., 2003). In addition, contamination of the mRNA with diethyl pyrocarbonate (DEPC), a commonly used RNAse inhibitor, may also hamper subsequent PCR (Lewis et al., 2001).

Finally, the design of an RT-PCR should be adapted to the fact that that the total RNA extracted from FFPE tissue is significantly degraded despite the improved methods (Figs. 2, 3) and this includes design of primers that enclose fragments smaller than 200 bp (Jackson et al., 1990). Contamination of mRNA extracts with genomic DNA and Trizol-residue after mRNA extraction from FFPE have been reported to interfere with successful real-time quantitative reverse transcriptase PCR, while RNA quality might nevertheless be sufficient for endpoint PCR (Foss et al., 1994; Weiss et al., in preparation).

Quantification of FFPE-derived mRNA by microarray technology

Microarray technology is an efficient method to analyze the up- and down-regulation of mRNA expression levels of several genes concurrently (Klopfleisch et al., 2010a). Although it is not as sensitive as RT-PCR it allows the comparison of almost the complete transcriptome of cells or tissues (Klopfleisch et al., 2010b). The analysis of up to several thousand genes in one tissue sample interferes apparently with the sensitivity, and to a minor extent with the specificity of the results when compared to RT-PCR assays. Array technology therefore requires large quantities of high quality mRNA. Fresh frozen tissue has therefore often been recommended as a prerequisite for microarray analysis, where FFPE-derived mRNA may inconvenient (Elkahloun et al., 2002). This is supported by other studies that identified short term formalin fixed FFPE tissues may be of potential use for mRNA analysis by microarray technology, while mRNA derived from extensively formalin fixed tissues is not suitable for gene expression profiling (Paik et al., 2005; Penland et al., 2007).

Formalin fixation itself, and also the different fixation protocols of different tissues were suggested as major causes for the insufficient results in microarray analysis of FFPE samples (Penland et al., 2007). In contrast, other studies found a good correlation between data retrieved from matched fresh frozen and FFPE material that was stored for up to 19 years (Coudry et al., 2007; Frank et al., 2007; Ravo et al., 2008). Coudry et al showed that a short pre-fixation time and mRNA pre-amplification before reverse transcription led to almost comparable results between FFPE and fresh frozen material. Nevertheless, sensitivity is reduced to 50% of the transcripts identified in FFPE-mRNA when compared to matched fresh frozen samples (Linton et al., 2008). On the other hand, cross-hybridization due to degeneration of mRNA and consequent falsely high numbers of genes present on the microarrays has been identified as a major problem when FFPE-derived mRNA is hybridized on microarrays (Coudry et al., 2007; Farragher et al., 2008). Several alternative protocols have been suggested to circumvent these problems with FFPE-derived mRNA in microarray

Table 2. Comparison of RNA yield and maximum PCR product in different RNA extraction protocols.

	Extraction methods	Maximal fragment size	Yield of RNA (µg/mg tissue)
(Gruber et al., 1993)	Proteinase K digestion , Phenol/Chlorophorm	402bp	n.r.
(Koopmans et al., 1993)	Proteinase K digestion , Phenol/Chlorophorm	249bp	suitable amounts
(Foss et al., 1994)	TRIZOL	168bp	0.5
(Abrahamsen et al., 2003)	RNeasy Mini Kit	136bp	n.r.
(von Smolinski et al., 2005)	Proteinase K digestion, TRIZOL	130bp	0,1 – 0,5
(Ribeiro-Silva et al., 2007)	RecoverAll™ High Pure RNA Paraffin Kit Absolutely RNA® FFPE Kit FormaPure™ Kit	151bp (242bp)	25 ng/µl* 64 ng/µl* 26 ng/µl* 180 ng/µl*
(Weiss et al., in preparation)	Heat-treatment, Proteinase K digestion, TRIZOL	115bp (470bp)	0.9

*: amount of elusion buffer not reported

analysis and the reader is referred to Farragher et al. who comprehensively reviews the current state of knowledge in this field (Farragher et al., 2008). Independently from the sample type, array data have to be validated by other methods, like RT-PCR and at the protein level, in order to verify their significance and this is even more important when FFPE tissue are used.

Extraction and expression analysis of miRNA in FFPE tissue

MicroRNAs (miRNAs) are short, non-coding RNA of 20-22 nucleotides in length and influence several biological processes, including carcinogenesis and differentiation (Ambros, 2004). Due to their important effects on the regulation of mRNA transcription and translation there is an ever increasing number of studies that focus on the quantitative expression differences between tissues with different biologic features.

Needless to say, miRNA is after all chemically a RNA that is affected by all described forms of degradation and this may hamper their quantification in the worst case. Fresh frozen tissues are therefore certainly the gold standard for the analysis of miRNA as is true for the extraction of every biomolecule from tissues. However, very early in the rather young history of research on miRNA it has been proposed that miRNA is an eminently suitable, i.e. stable, target molecule for analysis in FFPE. Primarily its shortness and close association with large proteins may increase its resistance against the fragmenting effect of formalin (Liu et al., 2009). In addition, most miRNA reverse transcription assays are based on direct end-labelling and do not rely on the formalin fixation sensitive polyA-tails that are commonly used for reverse transcription of mRNA (Castoldi et al., 2006). It can also be speculated that the uniform miRNA structure may also cause a similar and proportional degradation of the different miRNA types and thus keep their relative amounts to some extent equal.

These hypotheses were confirmed by several well structured studies that demonstrated a surprisingly good correlation of miRNA expression analyses in FFPE and fresh frozen tissues (Glud et al., 2009; Liu et al., 2009). Moreover, comparison with mRNA expression analysis found that miRNA outperforms mRNA when compared to fresh frozen tissues in RT-PCR analysis (Li et al., 2007; Doleshal et al., 2008), microarray analysis (Glud et al., 2009; Liu et al., 2009) and also by deep sequencing (Weng et al., 2010). In addition, different formalin fixation times did not change the stability of miRNA based on real-time qRT-PCR analysis (Xi et al., 2007).

Sensitivity, however, is a problem of miRNA quantification in FFPE tissue, especially when laser capture microdissection is employed, due to the inherent small amounts of miRNA in tissues. Andreasen et al. developed a method that uses locked nucleic acid (LNA)-enhanced primers in quantitative RT-PCR that enables accurate and reproducible quantification of microRNAs in scarce clinical samples (Andreasen et al., 2010). They furthermore recommend the addition of small carrier RNA prior to total RNA extraction, which improves miRNA quantification in blood plasma and laser capture microdissected (LCM) sections of FFPE samples (Andreasen et al., 2010).

In situ detection of mature miRNA can also be accomplished in FFPE sections by in-situ hybridization while inactive precursor miRNA can be visualized in FFPE sections by in situ RT-PCR with a sensitivity of up to one copy per cell (Nuovo, 2008). By these methods information on the quantity and the subcellular localization of a given miRNA type can be combined and may provide new perceptions of these important regulatory RNA.

Proteom analysis in FFPE tissue

Immunohistochemistry on FFPE tissue sections or Western Blot analysis of FFPE lysates are the common standard method to analyze protein expression in FFPE tissue. Major problems for both methods are the problematic absolute or relative protein quantification and that they are strictly hypothesis-driven approaches, in which the target protein to be analyzed has to be known a priori (Hood et al., 2006). In addition, formalin fixation induces severe protein-protein cross linking and the effect of unbuffered, acidic solutions on the chemical characteristics of the proteins often requires antigen retrieval of cross-linked protein epitopes by digestion of tissue sections with proteinase K or heat treatment in acidic or basic buffers (Kunkel et al., 1981; Ikeda et al., 1998). Alternatively, other fixatives that less affect protein structures like FineFix, RCL2 and HOPE have been suggested to alleviate protein expression analysis in long term stored tissues (Stanta et al., 2006; Mange et al., 2009).

The development of methods for explorative, not necessarily hypothesis driven, quantitative and qualitative analysis of virtually all proteins in a tissue, the proteome, has tremendously changed the approach to protein biomarker identification. Proteome analysis is usually based on a first separation of all proteins in a given sample according to their chemical properties (i.e. chromatography, two-dimensional gel electrophoresis (2D-GE)) or their size (2D-GE) which is followed by their quantification (fluorescence differential gel electrophoresis (DIGE), Isotope-Coded Affinity Tags (ICAT)) and a final identification of differentially expressed proteins by mass spectrometry (MS) (Michael et al., 2006; Klopfleisch et al., 2010d). Commonly, fresh frozen samples are used for proteome analysis and immediate freezing is used to avoid any unnecessary changes in the protein composition of the tissues (Klopfleisch et al., 2010d).

A first study on the proteome of fixed tissues compared proteins extracted from FFPE, ethanol-fixed, and fresh-frozen tissues by 2D-GE (Ahram et al., 2003).

They identified a comparable protein expression pattern in ethanol fixed tissues and fresh frozen tissues but failed to identify any proteins from FFPE tissues, most probably due to severe, intra- and interprotein covalent cross-linking (Ahram et al., 2003). This cross-linked protein "mesh" seems to generally hamper the proper separation of proteins by 2-DGE or chromatographic methods (Hood et al., 2006). Nevertheless, it has been shown that enzymatic digestion, heat incubation and application of high concentrations of detergents such as SDS can break up this "mesh" and produce peptides that, however, may be randomly modified by formalin fixation (Ikeda et al., 1998; Hood et al., 2005; Shi et al., 2006). The mechanisms of heat induced antigen retrieval are unclear, but at least a partial thermally-driven hydrolysis of methylene bridges has been suggested (Shi et al., 2006; Guo et al., 2007). Hood et al. speculated that a substantial portion of a given protein is solvent, inaccessible and therefore protected from formaldehyde modification (Hood et al., 2006). However, Nirmalan et al. stated that the use of detergents like SDS is required for efficient protein extraction from FFPE but significantly interferes with downstream trypsin digestion and mass spectrometric analysis (Nirmalan et al., 2008). The necessary removal of the detergent however leads to unpredictable losses of smaller peptides that are the dominating fraction in FFPE protein extracts. This inconsistency in protein extraction most probably precludes a gel-based analytic approach for FFPE proteomics (Nirmalan et al., 2008).

However, several newer studies established protocols that allow proteome analyses of FFPE tissues despite the negative effects of formalin on protein structure (Hood et al., 2005; Prieto et al., 2005; Shi et al., 2006; Guo et al., 2007). In these studies, optimized antigen-retrieval technique and MS were applied to reverse cross-linking in FFPE samples. For instance, boiling of FFPE sections during heat-induced antigen retrieval and re-suspension of proteins led to a significant overlap between proteins from FFPE and matched fresh frozen tissue after gel electrophoresis or liquid chromatography and MS (Prieto et al., 2005; Shi et al., 2006). A mild decrease in the total number of proteins, but no covalent peptide modifications attributable to formaldehyde chemistry were detected by comparison of the proteome of FFPE- and fresh frozen tissues in most of the studies (Sprung et al., 2009). Fixation of tissue for up to two days in neutral buffered formalin did not adversely impact protein identifications, while storage of FFPE tissues for up to ten years increased in methionine oxidation (Sprung et al., 2009).

Imaging mass spectrometry (IMS) of proteins direct on the FFPE slide is another new and promising methods, especially for morphologists. In contrast to the methods described above, proteins are in situ trypsin digested and identified by MS directly from the slide during IMS (Groseclose et al., 2008). Thus, information on the location and the nature of proteins can be combined during proteome analysis, but sensitivity and spatial resolution of IMS are still low (Groseclose et al., 2008).

Conclusion

Fresh frozen tissues will always be the gold standard and best tissues to use for the analysis of nucleic acids and proteins. However, the demand for studies on large cohorts and the almost infinite resources of FFPE tissues in the archives of pathology and histology departments world wide have, and will, certainly force the development of proper methods of biomelecule extraction from FFPE tissues. It is imaginable that in the future the explorative search for biomarkers is in fact still based on small groups of fresh frozen tissues in the initial first phase of such studies to avoid any interference with artefacts due to formalin fixation. In a second phase, however, properly established protocols for extraction of biomolecules from FFPE tissues will allow the use of large groups of FFPE tissues to confirm the initial findings.

For instance, the development of methods for DNA analysis from FFPE tissues has already reached a level that allows the integration in the second or even the first phase of explorative studies on mutational status in diseases or the search for infectious agents. Amplification of PCR products of up to 200bp in size from FFPE tissue-derived DNA is consistently possible in routine applications. However, caution is recommended in terms of reduced sensitivity of the detection of infectious agents and the potential detection of pseudo-mutations due the effect of formalin fixation on DNA sequence.

Analysis of mRNA is mostly focused on the comparison of absolute expression levels in different disease states and health. Formalin fixation has an unambiguous degenerative effect on the different mRNA species in a cell. These effects are disparate between different mRNA species, such as housekeeping genes and genes of interest, and therefore hamper relative quantification. Fresh frozen tissue samples are still recommended for initial explorative studies on mRNA expression. Nevertheless, several methods for improved mRNA extraction have been developed that may allow robust second phase expression analysis of mRNA in FFPE in the near future.

In contrast to mRNA, regulatory miRNA seems to be an interesting and well preserved analyte for evaluation in FFPE tissues due to its robustness against the effects of formalin fixation. The observation that miRNA expression profiling is more accurate for distinguishing disease states in FFPE tissues than mRNA expression analysis indicate that miRNA may be a better choice for expression analysis of FFPE samples even in high-throughput methods like microarray. Future studies will show whether FFPE-derived miRNA may even be sufficient for the identification of diagnostic biomarkers or disease-relevant molecular mechanisms in the first phase of explorative studies.

On the other, the use of FFPE tissues for proteome analyses is highly propagated at the moment, but several issues concerning the rather random effects of formalin fixation on protein structure, efficiency of protein extraction and separation and the general reproducibility of those experiments remain to be resolved. Experimental data, although promising, are at the moment not good enough to finally and fully recommend FFPE samples for retrospective and prospective proteome studies. More detailed studies defining the protein biochemistry of formalin fixation of current extraction protocols are therefore needed.

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