Critical Steps in Tissue Processing in Histopathology

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Abstract: Histopathological diagnosis using Formalin-Fixed Paraffin Embedded (FFPE) tissues is essential for the prognostic and therapeutic management of cancer patients. Pathologists are being confronted with increasing demands, from both clinicians and patients, to provide immunophenotypic and gene expression data from FFPE tissues to allow the planning of personalized therapeutic regimens. Recent improvements in the protocols for pre-analysis processing of pathological tissues aim to better preserve cellular details and to conserve antigens and nucleic acid sequences. These developments have been recently patented. The international protocol for the transporting of surgical specimens from the surgical theatre to the pathology department is to immerse the specimen in formalin. The alternative method of sealing the specimens into bags under a vacuum and then cooling is a well-accepted and environmentally safe procedure that overcomes the many drawbacks linked to transfer in formalin. Importantly, RNA is notoriously poorly preserved in FFPE tissue. Due to this, successful procedures for the extraction of genetic information from archival tissues have been the object of several studies and patents. Novel molecular approaches for RT-qPCR and gene array analysis on FFPE tissues are presented here. Moreover, a major advance is reported in this study, the observation that tissue fixation in cold conditions allows a much better preservation of nucleic acid sequences.

Keywords: Breast cancer, fixation, formaldehyde, formalin, genes, histopathology, neoplasms, nucleic acids, pathology, RNA, RT-PCR, sealing, tissues, tissues banks, under-vacuum.

INTRODUCTION

Histopathological diagnosis remains an integral component of the prognostic predictions and therapeutic planning of diseases. The field has been gaining further interest in recent years with the advent of personalized therapies for different tumor entities. Previously, diagnosis was only based on morphological features. Therefore, histopathological patterns, comprehensive of ultrastructural features, were the main basis for disease classification. The use of histochemical and immunohistochemical techniques has allowed pathologists to develop more precise, reliable and reproducible disease classifications. These techniques allow the pathologist to complement morphology with information regarding protein (antigen) expression and distribution.

Since the year 2000, pathologists have made critical steps forward in the knowledge of the pathogenesis and genetic profiles of several cancer types and this has made significant impact on prospects for both cancer prevention and the use of novel personalized therapeutic regimens. Progressively attention has moved toward gene analysis as a method of examining both origin and differentiation of various tumor types. Molecular analysis is thus emerging as an essential technique to complement conventional histopathology. This is reflected by the progressive evolution of the WHO Classification report outlined in the "Blue Books". This report initially only dealt with histological features, but more recent editions use the results of genetic analysis to complement, but never substitute for, the morphological characterization [1-3].

The consequence of this improvement is that cancer diagnosis for individual patients has become more complex and molecular tests have become mandatory, for example, for the identification of gene mutations responsible for familiar hereditary tumors of endocrine organs (MEN 1 and MEN 2 Syndromes) [4] or the assessment of microsatellite instability for the identification of carriers with increased risk for Lynch Syndrome [5-7]. Nowadays, morphological diagnoses are not sufficient for planning personalized therapies that need, for example, the detection of chromosomal translocations and aberrations in sarcomas and brain tumors [8-12]. the evaluation of mutations of EGFR and K-RAS genes in lung and colorectal adenocarcinomas [13-16], of cKIT and PDGFRA genes in cases of gastro-intestinal stromal tumors (GISTs) [17-19] and of BRAF and NRAS genes in melanomas [20, 21], and last but not least the evaluation of the status of the HER2 for breast cancer [22, 23].

These tests require a proper preservation of tissues, so that it can be analyzed in parallel for cell and tissue structural arrangement, protein (antigen) distribution and used for gene sequencing. The first, as well as the most crucial step of tissue processing in histopathology is the proper collection of the biological material. Microscopic diagnosis is strictly dependent on the technical preparation, the goal of which is obtaining thin sections optimal for analysis under the microscope, while at the same time preserving the integrity of both the structure and biology, particularly of the proteins and

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nucleic acids. For this purpose, the time-honored process of paraffin embedding, as originally devised by Klebs (1869) [24] maintains his value, since it allows the preservation of tissues for long periods (years) in a non-reactive environment. Paraffin embedding has to be preceded by fixation, which blocks and preserves the structure and the cell components as close as possible to the living condition. This is accomplished by chemical and physical procedures, and several alternatives have been proposed. Still, the most popular, dependable and reliable process remains formalin fixation, as originally proposed by Bloom [25, 26] in 1898. Variations on the conditions of formalin fixation and paraffin embedding heavily influence both structural and chemical preservation, thus bearing great impact on diagnostic definition and, ultimately on therapeutic treatment.

These preparatory processes which are completed before examination under the microscope are unfortunately poorly standardized in pathology laboratories world-wide. We agree with Groenen *et al.,* (2011) [27] in the claim that "optimal, standardized procedures are crucial if a high standard of test results is to be achieved, which is what each patient deserves".

The present report deals with improvements to the procedures of pre-analytical tissue processing which lead to a proper standardization and better preservation of tissue components. These improvements are specifically focused on two mandatory steps; (1) Transfer of tissues from the surgical theatre to the pathology laboratory and (2) Optimization of formalin fixation in order to improve preservation of nucleic acids in tissue specimens. These improvements were the object of separate recent patents.

UNDER-VACUUM SEALING AND COOLING (UVSC) FOR THE TRANSFER OF TISSUE SPECIMENS FROM THE SURGICAL THEATRE TO THE PATHOLOGY LABORATORY

A critical step in pre-analytical processing of tissue specimens is the transfer of tissues from the clinical wards to the pathology lab for histological processing. Small biopsies are collected directly into vials pre-filled with fixative (usually 10% buffered formalin), a process that guarantees rapid fixation and preservation. This universally practiced procedure is not a concern.

However, histopathological processing of large specimens (larger than 2 cm) requires grossing and selection of sections from significant areas. In order to favor penetration of reagents, into the tissue, sections that are to be processed in "bio-cassettes" should not be thicker than 3-4 mm. Grossing, which is immediately followed by fixation, can only be performed in pathology laboratories by qualified personnel and time taken and selection procedures vary between laboratories. The time interval between surgical removal of the specimen and proper fixation is defined as "hypoxia time" and is significant, since it allows activation of tissue enzymes, autolysis and degradation of proteins, as well as of DNA and RNA [28-30]. Interestingly, Chung *et al.,* [31] have demonstrated that substantial RNA degradation may occur during this "warm ischemia" time, but the RNA degradation due to warm ischemia may be slowed down by cooling the specimen.

Methods of transfer of surgical specimens vary according to the architectural layout and distance between surgical theatres and pathology laboratories. The ideal situation is if physical location and hospital protocols allow for the immediate transfer of "fresh" tissue specimens for prompt grossing and fixation. Accordingly, it has been recommended that the "hypoxia time" remains below 2 hours to achieve a proper processing of breast cancer specimens, permitting a correct evaluation of both morphological and therapeutic-prognostic parameters [32, 33]. Problems arise when this is not feasible; it is a common practice in many hospitals, to immerse large specimens and organs into large formalin filled containers, which are then transferred to the pathology laboratory in time, usually once every day.

Consequent problems with this practice can include:

1) Large plastic containers are large and relatively heavy, and therefore spilling can occur.
2) Immersion of the whole specimen into formalin prevents the collection of fresh material for tissue banking. Also, fixation does begin, but only at the periphery. Discoloration occurs, while a delay in the transfer to pathology is somehow justified by the fact that "the tissue is already in formalin".
3) Theatre nurses are becoming increasingly concerned about potential toxicity and carcinogenicity, since the fluid has to be handled outside the hood.
4) When the container arrives at the pathology lab, the opening and the extraction and handling of the specimen are major causes of the diffusion of formaldehyde fumes.

Formalin, a 4% solution of formaldehyde in water, extensively used world-wide as a fixative for histopathological specimens. Since its discovery at the end of 19th century [26], this aldehyde has been universally appreciated as a simple reagent that is a good antiseptic, penetrates tissues quickly (at a diffusion rate of 1 cm in 24 h) and is easy to handle. In tissues that are formalin-fixed, the morphology is well preserved, as is tissue antigenicity. Immunohistochemical procedures of diagnostic interest have routinely been adapted for use on formalin-fixed tissues [34].

The medical use of formalin as a tissue preserver and fixative is extensive, especially in pathology laboratories and its substitution with alternative fixatives does not currently seem likely [34, 35]. In fact, the volume used in public hospitals in the Piedmont region (Italy, inhabitants 4,500,000) alone for the preparation of approximately 300,000 histological exams is in the range of 100,000 litres annually.

Tissues preserved in formalin are even able to be sent by post, in the number of several thousands per year.

Despite its advantages, formaldehyde has some drawbacks that demand caution: it is a skin allergen and produces irritating vapors that can cause asthma. The International Agency for Cancer Research [36] has classified formaldehyde as a Class 1 carcinogenic agent, and statistical evidence has been presented for a possible link between formaldehyde exposure and lymphohematopoietic malignancies [37], an observation that might explain data reporting on excess of deaths due to cancer of the lymphatic and hematopoietic

systems amongst British pathologists [38]. Still, the major concern for formaldehyde use is linked to the production of toxic, irritating and allergenic vapors. A positive relationship between formalin and respiratory symptoms has been reported not only in workers in match factories [39], but also in hospital staff members professionally exposed to this substance [40].

Several attempts have been made to find a substitute for formalin, but so far all of the proposed alternatives have failed, being either ineffective or unreliable [41]. A more practical approach would be to limit the use of formalin to pathology laboratories, where this toxic agent is carefully handled with hoods and gloves in safe conditions, and to avoid its use in other less-protected areas of the hospital, such as in surgical theatres, where surgical tissues are commonly placed in boxes full of formalin until transfer to the pathology labs.

To overcome these problems, an alternative procedure is the sealing of tissues under-vacuum in plastic bags immediately after removal, and to keep them cooled at 4°C until transfer to the pathology labs, where they are routinely processed [42]. Sealing of tissues in plastic bags is a quick procedure, taking approximately 15 seconds and is easily performed by nurses in the surgical theatres [43]. An apparatus for under-vacuum sealing of tissue specimens has been patented with Number EP 2 070 410 A1 by Milestone srl (Sorisole, BG, Italy) [44].

Under-vacuum sealing, *per se*, does not guarantee preservation, as also experienced by Kristensen et al [45]. Vacuum sealing, by removing air, prevents dehydration and favors cooling, the latter being the main preserving factor by blocking enzymatic autolysis. In a series of experiments on rat liver tissue blocks, we checked the preservation of RNA at different time intervals and at different temperatures, in tissues preserved under-vacuum or not preserved under-vacuum. The results Fig. (**1**) indicate that the RNA Integrity Number (RIN) as an indicator of RNA preservation and evaluated with the Agilent 2100 Bioanalyzer, gradually decreases after a few hours at room temperature, while at 4°C (and, to a lower extent at 8°C) is still well preserved after several hours (up to 72 hours). These data are further confirmed by a consecutive series of breast cancer specimens which were surgically removed, immediately processed with under-vacuum sealing and cooling (at 4°C), then transferred to the pathology laboratory at different time intervals Fig. (**2**). During grossing, a tumor specimen was taken by a punch apparatus, put into RNARetain according to the recommended procedure and shipped for genetic evaluation to an external company (Agendia. Amsterdam, Nederlands). Before analysis, the specimen was tested for RIN value, and satisfactory data (above 7) were obtained in all specimens.

The San Giovanni Hospital in Torino, Italy is a large regional "pavillon" hospital where the distance between surgical theatres and pathology laboratories prevents an immediate transfer of fresh specimens. Here, it was a time-honored habit to transfer specimens in large formalin-filled boxes, since 2009 the procedure of under-vacuum sealing and cooling has been extended to the transfer of all surgical specimens (larger than 2 cm). The experience accrued has been duly analyzed and reported [43]. The survey on the feasibility, compliance and quality assurance of a new procedure for transferring surgical specimens was definitely positive. Dedicated apparatuses (TissueSafe^{R}, Milestone srl, Sorisole, BG, Italy) were located in the premises of each of the 6 surgical theatres of the Hospital. The Under Vacuum Sealing and Cooling (UVSC) procedure, which was met with favor by the staff and did not present specific problems of practical or diagnostic interest, has been adopted as the standard in our Hospital.

Additional benefits are linked to the possibility of standardizing fixation times and of implementing tissue banking. In fact, we can now determine the starting time of fixation in formalin, thus avoiding over-fixation, which can cause less effective immuno-phenotyping of the specimen, an issue that is presently regarded as mandatory for breast cancer processing. An additional bonus of the novel UVSC procedure is the preservation of RNA, which is enhanced by the storage at 4°C [46], thus permitting tissue banking and gene expression profiling.

RNA Integrity Number (RIN) in rat liver specimens preserved Under-Vacum at different temperatures

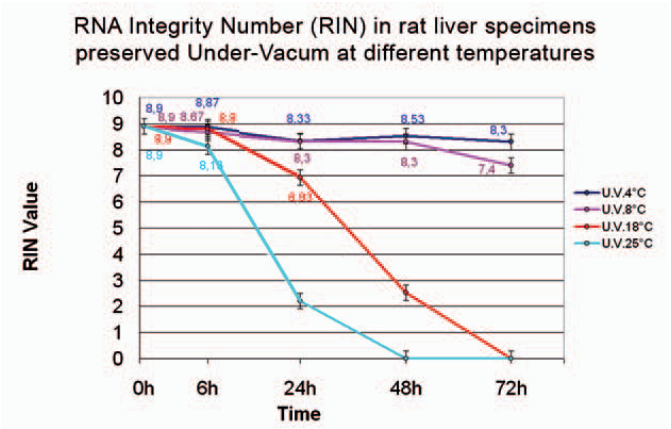

Fig. (1). The RNA Integrity Number (RIN), an indicator of RNA preservation, gradually decreases after a few hours at room temperature, while at 4°C (and, to a lower extent at 8°C) is still well preserved after several hours (up to 72 hours).

One hundred and twentynine consecutive breast cancers processed with the UVSC procedure.
Cases with difference in gap range are analysed in separate settings.

Histo ID	gap	RIN
1285/09/3	1h	9
1271/09/3	1h	7,8
1844/09/3	1h	8,7
1886/09/3	1h	8,5
2241/09/3	1h	8,9
3216/09/3	1h	7
3673/09/3	1h	9,6
5598/09/3	1h	7,9
1422/09/3	2h	8,7
6226/09/3	2h	6,9
273/09/D	2h	8,6
7249/09/3	2h	9,1
4014/09/3	2h	7,6
4617/09/3	2h	8,9
305/10/3	2h	7,2
650/10/3	3h	6,8
7250/09/3	4h	9,6
9182/09/3	4h	8,1
9687/09/3	4h	7,1
1945/10/3	4h	7,4

Histo ID	gap	RIN
2238/09/3	6h	8,4
2574/09/3	6h	7,9
6054/09/3	6h	8,6
7004/09/3	6h	8,3
7129/09/3	6h	8,1
7362/09/3	6h	9,4
7405/09/3	6h	7,9
7613/09/3	6h	7,3
7782/09/3	6h	7,7
4073/09/3	6h	9,1
506/10/3	6h	8,6
1677/10/3	6h	9
2032/09/3	7h	7,5
3365/09/3	7h	8,1
3368/09/3	7h	7,7
8153/09/3	7h	7
4275/09/3	7h	9,4
5661/09/3	7h	7,1
8687/09/3	7h	8,6
9183/09/3	7h	6,9
9461/09/3	7h	7,5
1267/10/3	7h	7,8
1775/10/3	7h	9,2
1944/10/3	7h	8
7781/09/3	8h	7,9
2665/09/3	20h	7,8
4122/09/3	20h	7,3
649/10/3	23h	6,8

Histo ID	gap	RIN
1498/09/3	5h	7,5
1469/09/3	5h	7,9
1267/09/3	5h	8,2
1992/09/3	5h	7,3
2237/09/3	5h	8
6263/09/3	5h	7,8
7489/09/3	5h	9,1
7805/09/3	5h	7
7886/09/3	5h	7,9
8542/09/3	5h	7,2
8623/09/3	5h	7,6
4075/09/3	5h	7,9
4831/09/3	5h	8,3
5525/09/3	5h	7,8
5764/09/3	5h	6,2
5822/09/3	5h	7,4
9452/09/3	5h	7,9
108/10/3	5h	7,7
143/10/3	5h	8
435/10/3	5h	8,2
651/10/3	5h	8,5
1316/10/3	5h	7,7
2033/10/3	5h	9,1
2082/10/3	5h	6,6

Histo ID	gap	RIN
1385/09/3	60h	7,9
1394/09/3	60h	7,9
1599/09/3	60h	7,4
1783/09/3	60h	7,9
1793/09/3	60h	7,6
2163/09/3	60h	8,1
3850/09/3	70h	7,4
4221/09/3	70h	7,4
4211/09/3	70h	6,2
1995/09/3	72h	8,1
2712/09/3	72h	7,4
2743/09/3	72h	6,4
6368/09/3	72h	7,6
6369/09/3	72h	8
6849/09/3	72h	7,8
8215/09/3	72h	9
8413/09/3	72h	6,5
3657/09/3	72h	7,5
3889/09/3	72h	7,8
3888/09/3	72h	8,2
3993/09/3	72h	7,6
4594/09/3	72h	6,2
4937/09/3	72h	8
5118/09/3	72h	6,2
1022/10/3	72h	8,6

Histo ID	gap	RIN
1698/09/3	24h	8,7
1904/09/3	24h	7,3
2621/09/3	24h	7,6
2622/09/3	24h	6,8
2820/09/3	24h	7,2
2817/09/3	24h	7,9
6056/09/3	24h	7,3
6058/09/3	24h	7,9
6422/09/3	24h	7,1
6520/09/3	24h	7,7
6552/09/3	24h	7,1
6753/09/3	24h	7,8
6907/09/3	24h	8,7
7514/09/3	24h	8
7904/09/3	24h	8,6
3400/09/3	24h	8,7
3701/09/3	24h	7
4298/09/3	24h	6,6
4668/09/3	24h	8,6
5038/09/3	24h	8,2
5357/09/3	24h	7,3
9000/09/3	24h	7,4
9112/09/3	24h	7,8
9324/09/3	24h	8,7
9585/09/3	24h	8,6
1453/10/3	24h	6,5
2036/10/3	24h	7,4
2096/10/3	24h	9,5
4553/09/3	30h	8
3764/09/3	48h	6,6
4471/09/3	48h	8,6
9248/09/3	48h	8

Gap range	N°cases	RIN (mean)
1h - 4h	20	8,2
5h	24	7,8
6h - 23h	28	8,0
24h - 48h	32	7,8
60h - 72h	25	7,5

Fig. (2). A consecutive series of 129 breast cancer specimens, were immediately processed with Under-vacuum sealing (UVS) and Cooling (at 4°C), then transferred to the Pathology laboratory at different time intervals. During grossing, a tumor specimen was taken by a punch apparatus, put into RNARetain according to the recommended procedure and shipped for genetic evaluation to an external Company. Before analysis, the specimen was tested for RIN value, and data deemed satisfactory (above 7) were obtained in all specimens.

Moreover, we met the environmental goal of a progressive reduction of the exposure of nurses, pathologists and technical personnel to formaldehyde vapours. The use of formalin has been restricted to dedicated areas in the pathology laboratory, and transfer of large boxes filled with fixative throughout the hospital ceased. In addition, the simple UVSC processing offered advantages in terms of staff satisfaction, tissue preservation and cost.

TISSUE FIXATION

Fixation is the process whereby cell and tissue structures, as well as chemical components are preserved in their integrity. This process is most commonly accomplished by immersion into a fluid, which gradually penetrates and acts chemically and/or physically.

Several fixatives have been proposed, but those presently practiced are either of the aldehyde cross-linking category or alcohol-based, producing coagulation by water subtraction. The alcohol-based fixatives [27] have the advantage of lack of toxicity and of a good preservation of nucleic acids, but have a poor penetration and result in an unsatisfactory preservation of morphological details. Moreover, in our [47] experience and that of others [27, 48], the performance of some immunohistochemical tests is impaired. A patent for a new fixative agent, bis-maleic anhydride, which has little impact on the later detection of proteins and nucleic acids, has recently (Feb. 2011) been presented by Roche Diagnostics GMBH (Int. Publ. Number WO 2011/020612 Al) [49].

As already stated, 4% formaldehyde solution in water (formalin) has been adopted as the fixative of choice in histopathology as it is relatively cheap, easy to use. It is also reliable because it does not over-fix and it guarantees, in appropriate conditions, an optimal morphological preservation. Several alcohol-based substitutes have been proposed, either cross-linking or non-cross-linking [47, 50-52]. These fixatives allow for a better preservation of Nucleic Acids, and especially of RNA sequences. However, they are inferior as far as morphological (and immunohistochemical) preservation is concerned, and we have to conclude that substitution of Formalin with alternative fixatives cannot be foreseen at present [34, 35].

However, the uses of formalin-fixed tissues have varied over time. Originally, optimal morphological preservation was the sole requirement, but in more recent times, with the advent of immuno-histochemical typing, reliable antigenic preservation is also required [34, 53]. As a consequence, the protocols of formalin fixation have become stricter.

This issue is particularly relevant in onco-pathology for the evaluation of factors predicting responsiveness to therapeutic treatments, and thus, fixation in phosphate buffered formalin (PBF) of breast cancer tissue blocks for no less than 6 and no more than 48 hours is now required in order to guarantee an optimal evaluation of Estrogen (ER) and Progesterone Receptors (PgR) and HER2 expression by immunohistochemistry [32, 33].

In more recent times, a crucial request in cancer pathology has been nucleic acid preservation for gene expression profiling, with the goal of generating new and reliable diagnostic and prognostic parameters [29, 54].

EXTRACTION AND PREPARATION OF NUCLEIC ACIDS

The success of nucleic acid–based diagnostics is related to the quality of the specimens from which the nucleic acids are obtained and to the procedures adopted for RNA/DNA extraction and analyses.

Any process concerning RNA manipulation must be performed by blocking endogenous ribonucleases and by avoiding the contamination of the sample by exogenous nucleases by using clean gloves, autoclaved tubes and tips, and nuclease-free or DEPC-treated water.

Nucleic acids extracted from frozen or FFPE specimens is usually performed starting from tissue sections. The number of sections needed to obtain a nucleic acid yield adequate for molecular analysis is related to the type of tissue samples (fibrosis, cellularity), on the sample dimensions and on its exposed surface. On equal sample characteristics, the number of sections is higher for paraffin embedded tissue because of the expected decrement in RNA and DNA yield from fresh to formalin fixed tissues.

The choice of the sample to be analyzed is pivotal; for example, in cancer specimens, one must strictly avoid sampling into the necrotic area of the lesion, which is rich in autolytic enzymes. In cases where the area corresponding to cancer tissue is under-represented, selection can be performed using a laser microdissection system (Leica DM 6000) or a core from an area of viable tumor, as proposed by Franklin *et al.*, 2010 [55]. According to these authors, on the Hematoxylin and Eosin-stained section of a case, a suitable area of at least 2mm in diameter is selected. The same area is then identified and marked on the corresponding tissue block, and a core of tissue is obtained by introducing the needle of a Tissue Arrayer. Following a similar approach, in the experience of our laboratory, a core of suitable tissue could easily obtained using an 18 or 16 gauge disposable needle (BD Microlance 3; Becton Dickinson, Franklin Lakes, NJ). To extract the core we used a 20 gauge BD Insyte™ peripheral venous catheter (1.1 mm x 48 mm; Becton Dickinson) as a sleeve. Our procedure is easy and fast and avoids the risk of cross-contamination by using disposable and sterile material. The core of paraffin-embedded tissue thus obtained was of 0.9 to 1.2 mm. in diameter and approximately 2 mm long and the material so obtained was consistently found fit for nucleic acid extraction. The paraffin-embedded material, either sections or micro-dissected samples or cores, should be de-paraffinized and processed following recommended protocols [56].

EVALUATION OF GENE EXPRESSION IN FFPE TISSUES

Modern requirements of gene analysis on surgical specimens of different tumor types, as demanded by recent therapeutic regimens should ideally be performed on nucleic acids from fresh frozen tissues. However, a number of factors prevent an extensive and routine use of such material. In fact, clinical requests are often advanced a long time, sometimes years, after the surgical removal of cancer specimen and several practical, legal and ethical issues should be resolved for the widespread use of tissue banks for clinical purposes [57].

The prospect of using archival (FFPE) tissues for molecular analysis appears instead both attractive and practicable [56] and several investigations open prospects for the extensive and reliable exploitation of this material.

Studies conducted on the preservation status of nucleic acids in FFPE tissues generally agree on the relatively good (though not optimal) preservation of DNA [58]. On the con-

trary, RNA has been found to be heavily degraded and fragmented so that only short sequences, approximately 100–200 nucleotides long, can be recognized and amplified [31, 46, 58-61].

For gene expression profiling on FFPE tissues from cancer patients, 3 types of strategies have been pursued.

The first is the use of Real Time quantitative PCR analysis, focusing on short amplicons, in order to circumvent RNA fragmentation. Sequences as short as 150 bp can be detected and evaluated [62]. This type of procedure has extensively been used to evaluate proliferation levels and prospects of response to chemotherapy in breast cancer (Oncotype Dx® Genomic Health, Redwood City, CA)[63-65], as well as to determine values of ER, PgR and HER2 in breast cancer [62]. This approach is very promising for clinical applications, since values obtained are closely matching those assessed with classical immunohistochemical procedures, while offering the additional bonus of a proper quantitative evaluation.

We conducted preliminary experiments on breast cancers processed in parallel as frozen and FFPE samples. The RNA extracted from either frozen or FFPE tissues was profiled by RT–PCR-based RT^2 Profiler™ PCR array PAHS-131 (SABiosciences, Frederick, MD, USA) [66]. RT-qPCR was performed in a 96 wells format using the Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). The Human Breast Cancer RT^2 Profiler™ PCR Array tests the expression of 84 key genes commonly involved in tumor classification, signal transduction, epithelial to mesenchyimal transition, angiogenesis, adhesion, cell cycle, DNA damage, xenobiotic transport and transcription factors. Data analysis is based on the $\Delta\Delta C_t$ method with normalization of the raw data to housekeeping genes and allows comparing all of the groups side-by-side. In a RT-qPCR reaction the expression is detected by accumulation of a fluorescent signal. The Ct value is defined as the number of cycles required for the signal to cross the threshold, that is, the cycle number at which the fluorescence signal generated within the reaction is significantly above the background. The software measures for each samples is the cycle number at which the fluorescence crosses the arbitrary line. More dilute samples will cross at later Ct values. The higher the Ct, the less the mRNA detected is present, because more cycles of amplification are required to detect the fluorescence. Lower Ct values (< 29) represent strong positive reactions.

In our experience, the expression profiles obtained with PAHS-131 PCR Array on mRNA from FFPE breast cancer specimens are comparable to those of fresh-frozen samples Figs. (**3** and **4**). Moreover, no substantial difference in expression of the 84 key genes was noticed when RNA extracted from breast cancer specimens processed following

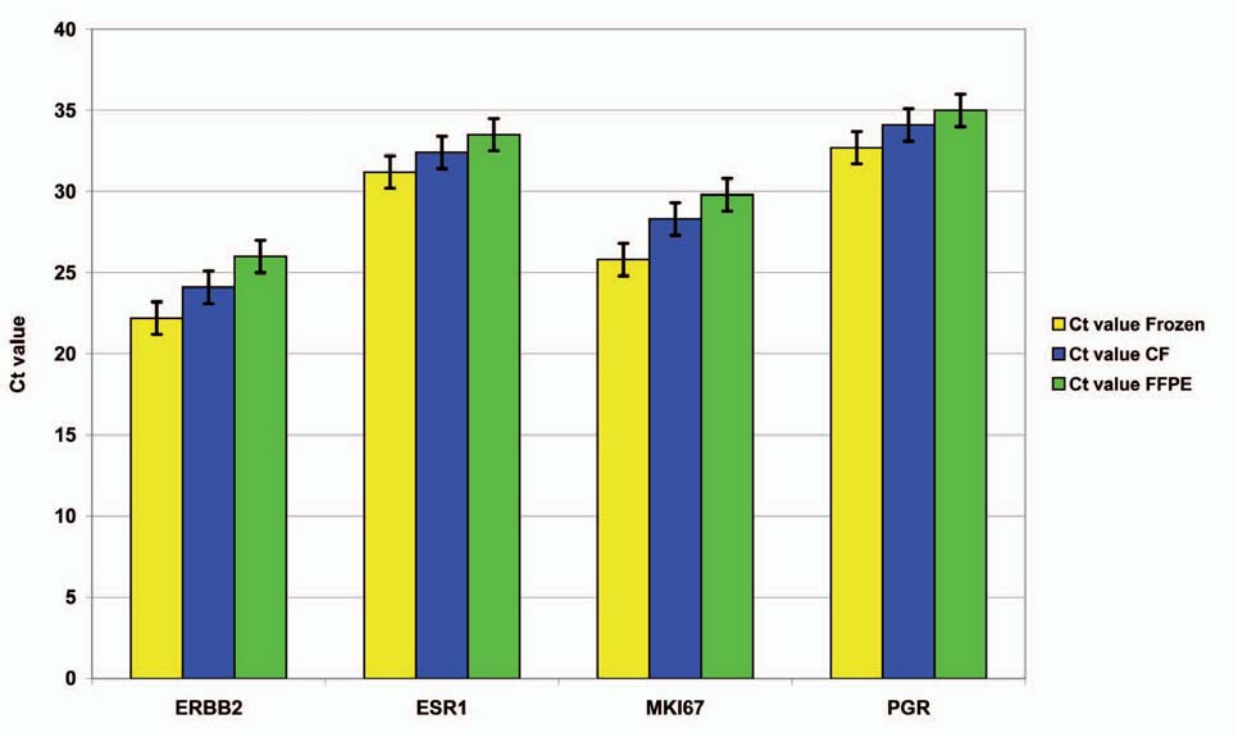

Fig. (3). Represents the Ct values obtained for the four main prognostic factors (ERBB2, ESR1, MKI67 and PGR), from a single breast cancer case processed following three different methods (Frozen, Cold Fixed and FFPE). The graph shows that the highest Ct values were obtained from the FFPE samples, while the best results (lowest Ct values) were obtained from the frozen samples, as expected. As one can see from the graph, the two most similar Ct values referred to a specific gene were obtained from Frozen and Cold Fixed samples. Ct values from FFPE samples varied greatly from the other two methods, but were still consistent with the level of expression. (All the Ct values were obtained setting the same threshold). Despite the differences in Ct values, within each prognostic factor, the gene expression level is consistent among the three different methods.

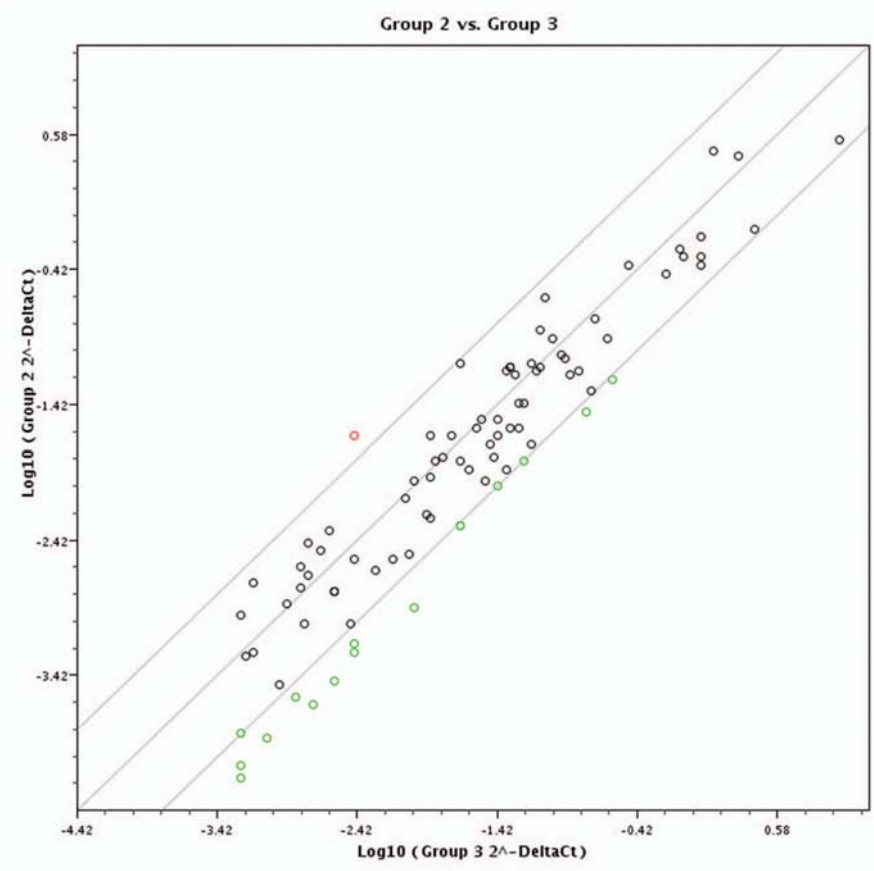

Fig. (4). The scatter plot puts in comparison 2^-DeltaCt values obtained from a fixed sample with 2^-DeltaCt values obtained from the same sample, but frozen, both evaluated with Breast Cancer RT^2 Profiler PCR Array (QIAGEN) and analyzed with QIAGEN Web-Based PCR Array Data Analysis Software. The expression of the majority of the genes is consistent in the two methods, as shown by the black dots. Given the differences in the methods used to process the sample, hence in the quality of the starting material, it is acceptable to see a few genes fall out of the correlation range (red and green dots).

different protocols were compared using the scatter plot analysis tool. Specifically, we compared material either routinely fixed in formalin or processed using the Cold Formalin fixation procedure (see below). Only a few genes (out of 84) slightly trespassed the fold regulation cut-off, thus confirming the suitability of FFPE tissues for this gene analysis.

The second approach, recently proposed by Illumina Inc. afforded gene expression profiling in FFPE tissues using an artificial reconstruction of degraded RNA. The complementary DNA-mediated Annealing, extension, Selection and Ligation (DASL®) assay (Illumina; San Diego, CA) [67] is a gene expression profiling system suitable for use with degraded RNAs such as those derived from FFPE tumor samples. DASL assay provides a reliable approach to gene expression profiling in FFPE tumors [68]. Mittempergher and colleagues [69] demonstrate that data generated from FFPE material with the DASL assay, if properly processed, are comparable to data extracted from the fresh frozen counterpart. Specifically, gene expression profiles for a known set of prognostic genes for a specific disease are highly comparable between two conditions. This opens up the possibility of using both FFPE and fresh frozen material in gene expressions analyses, leading to a vast increase in the potential resources available for cancer research [69]. Also, Louding and colleagues [70] demonstrate that high-throughput gene expression profiling from formalin-fixed, paraffin-embedded tissues has become a reality. They showed that the whole-genome complementary DNA–mediated annealing, selection, extension and ligation assay (Illumina Inc.) is a powerful assay for profiling degraded RNA from archived specimens when combined with quantitative reverse transcriptase polymerase chain reaction validation [70].

A similar approach was followed by Turner *et al.,* [71] who presented a new technology that enables fast and successful analysis of samples previously thought to be too degraded for gene expression analysis. NuGEN Technologies WT-Ovation™ FFPE System linearly amplifies RNA from FFPE samples through a robust and simple whole-transcriptome approach using as little as 50ng total RNA isolated from FFPE samples [72]. The amplified material may be labeled with validated kits and/or protocols from NuGEN for analysis on any of the major gene expression microarray platforms, including Affymetrix, Agilent, and Illumina gene expression arrays. Results compare well with those obtained using RNA from fresh-frozen samples [71].

We have recently proposed a variation in formalin fixation resulting in improved preservation of RNA [73]. This is based on a fixation process in formalin at 4°C, followed by dehydration in cold ethanol and paraffin embedding (CFFPE). Using this procedure, we succeeded in obtaining a substantial reduction in RNA fragmentation in FFPE tissue blocks, as assessed by RT-PCR and gene array analysis, while at the same time preserving the morphological and immunohistochemical properties which make formalin the fixative of choice in histopathology. A patent request for an apparatus allowing a cold fixation-dehydration sequence for histological tissues has been filed by Milestone srl (Sorisole, BG, Italy) (European Depository Number EP 10 425 360.4).

Using cases of colon, breast, pancreas and gastric cancer as a model, we explored the nucleic acid integrity allowed by the presently described CFFPE procedure compared to that of frozen and routinely fixed specimens from the same cases. To study the preservation degree of mRNA we amplified the PBGD/ABL/B2M genes (128 bp, 193 bp and 334 bp, respectively), *G6PD* gene (660 bp) and specific markers for colon and breast tissues (*CK20*, 329 bp and 500 bp for colon; mammaglobin, 331 bp and *ER* alpha, 346 bp for breast). The CFFPE procedure allowed RT-PCR amplification of relatively long RNA fragments, up to 660 bp, with a notable improvement over routinely processed tissues. In addition, all fresh-frozen and CFFPE-treated samples, were subjected to gene expression analysis using a microarray high-throughput method from Illumina (HumanHT-12_V4 Expression BeadChip; Illumina, Inc., San Diego, CA). We observed that gene expression profiling analysis showed that RNAs from cold fixed (CF) samples are significantly less fragmented than those from standard FFPE samples, with high amounts of more than 500-base long fragments. The performance of CFFPE-treated samples were comparable to that shown by fresh-frozen samples, which are, at present, the sole specimens considered suitable for microarray gene expression analysis.

These data were further confirmed by investigations using whole genome arrays (Agilent Technologies, Inc. Santa Clara, CA). We investigated in parallel RNA extracted from fresh-frozen and CFFPE material from the same cases of breast cancer. A VENN diagrams shows that the number of genes detected in RNA extracted from fresh tissues closely matches that of RNA from CFFPE material from the same cases, the former being only 4.12% higher Fig. (**5**).

The improvement offered by the presently proposed procedure is therefore linked to a definitely lower degree of nucleic acid fragmentation, especially of mRNA, while keeping the basic advantages that make formalin the fixative of choice in diagnostic histopathology.

CURRENT AND FUTURE DEVELOPMENTS

The study of pathological tissues has always been and remains the basic source of information for planning therapy for individual patients and, in more general terms, to understand nature and causes of diseases and to foster health progresses. Analysis of the different cellular and chemical components requires preservation. Given the practical problems linked to frozen tissue banking, archival FFPE tissue blocks, accrued in millions in Pathology laboratories world-wide, constitute the material of choice for the above purposes.

Venn diagram comparing the genes detected in the fresh sample (red) and the cold fixation sample (blue)

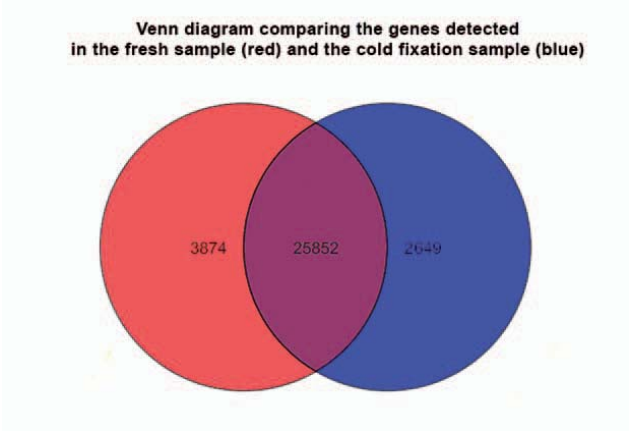

Fig. (5). Venn diagram comparing in parallel the genes detected in 2 fresh samples (red) and 2 cold fixed (CFFPE) samples (blue) from the same cases of breast cancer. Using the software Agilent Genespring 11.5.1 we generated lists of genes detected in cold fixed and fresh sample. The number of genes detected in the fresh samples was 29726 and in the cold fixed samples was 28501 genes. The number of genes detected in the fresh samples was only slightly (4.12%) higher than in the cold-fixed ones. Please notice that using the present scheme, to prepare Venn diagrams, the numbers are not proportional to the circle area indicated in the drawing. The relative quantities are specified by numbers inside each area.

In the last 20 years, an increasingly significant role in tissue analysis has been paid by "ancillary" techniques as a complement to pure morphological examination. The presence and distribution of proteins are mainly investigated on tissue sections with immuno-histochemical procedures, but a number of dedicated protocols have been devised allowing extraction and analysis of proteins from FFPE tissues (see reference 56). In recent times, modern molecular techniques through the analysis of nucleic acids have been opening prospects for a detailed and clinically useful evaluation of gene expression profile in tissues, especially in the very demanding field of tumors.

Progresses in personalized therapeutic regimens for cancer require molecular analyses of tissue specimens from individual patients. Present-day and foreseeable future requests demand a proper preservation of protein and nucleic acid components and standardization of pre-analytical processing of formalin-fixed paraffin embedded tissues. Recent advances in this field, meeting the pressing demand from both, clinicians and patients, have in part been the object of patents related to the treatment and fixation of tissues and to the processing of extracted RNA.

Optimal preservation of structural details, antigenic components and nucleic acid sequences has thus become mandatory and a major attention has now to be paid to pre-analytical processes for the preparation of FFPE tissues.

The time interval between surgical removal of tissue specimens and transfer to Pathology laboratories for grossing (s.c. warm ischemia time) is critical. Prevention, of drying, autolysis and RNA degradation is achievable by Under-Vacuum Sealing and Cooling of tissues [44], a process adopted in our hospital and found a well-accepted, environmentally safe procedure, overcoming the many drawbacks linked to transfer in formalin.

In order to improve RNA preservation, thus permitting gene expression profiling, novel fixatives have been proposed [49], but the fixative of choice remains buffered Formalin (4% Formaldehyde). We have recently reported [73] that fixation in Cold Formalin results in a satisfactory preservation of RNA sequences. Along the same line, numerous patents have been presented for extending the length of RNA sequences [67, 72] and for achieving a quantitative evaluation of gene expression by Real Time PCR procedures [63-66].

This approach and foreseeable development of novel procedures for the exploitation of archival tissue banks is going to provide Pathologists with valuable parameters, complementing diagnoses and permitting to expand the flourishing era of personalized therapies.

CONFLICT OF INTEREST

No conflicts of interest of the Authors with patents and Companies mentioned in this paper. Gianni Bussolati was originally responsible for inventions, but is not owner of the patents and does not receive royalties or other forms of payments.

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ABBREVIATIONS

FFPE = Formalin-Fixed, Paraffin Embedded

RT-qPCR = Real Time quantitative Polymerase Chain Reaction

WHO = World Health Organization

MEN = Multiple Endocrine Neoplasia

EGFR = Epidermal Growth Factor Receptor

PDGFRA = Platelet Derived Growth Factor Receptor Alpha

HER2 = Human Epidermal growth factor Receptor 2

RIN = RNA Integrity Number

UVSC = Under Vacuum Sealing and Cooling

PBF = Phosphate Buffered Formalin

ER = Estrogen Receptor

PgR = Progesterone Receptor

CFFPE = Cold Formalin Fixed Paraffin Embedded

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