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Pre-Analytics of Pathological Specimens in Oncology

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Contents

The Pre-analytical Phase in Surgical Pathology

Gianni Bussolati, Laura Annaratone and Francesca Maletta

Abstract

Several sequential passages are involved in the pre-analytical handling of surgical specimens from resection in the surgical theater to paraffin-embedding and storage. Each passage is highly critical and can significantly affect the preservation of morphology, antigens, and nucleic acids. Some key points in this process are still undefined and are subject to high variability among hospitals. High quality and standardization are demanded and pathologists should therefore work to comply with all novel clinical requests (such as genomic and antigenic testing for targeted molecular therapies). Under-vacuum sealing of surgical pieces can be a safe and reliable alternative to storage in large formalin-filled boxes; it prevents dehydration and favors cooling by removing air. Moreover, it implements tissue banking and preservation of nucleic acids. After transport of specimens to pathological anatomy laboratories, the next passage, fixation, has been the object of several attempt to find alternatives to formalin. However, none of the substitutes proved successful, and formalin fixation is still considered the gold standard for preservation of morphology and antigens. RNA has instead been found to be heavily affected by degradation and fragmentation in formalin-fixed tissues. Based on the hypothesis that RNA degradation would be inhibited by maintaining a low temperature, a protocol based on processing tissues with formalin at low temperature (cold fixation) was evaluated and proved useful in obtaining a reduction in RNA fragmentation. Finally, the problem of storage is discussed, in order to find ways to guarantee feasibility of molecular analyses even years after the original diagnosis.

Keywords

Ischemia · Under-vacuum · Cold fixation

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Contents

1 Introduction

Preparation of surgical specimens for histopathological examination requires a sequence of step-wise passages, leading from the biopsy removal up to the paraffin block (Fig. 1).

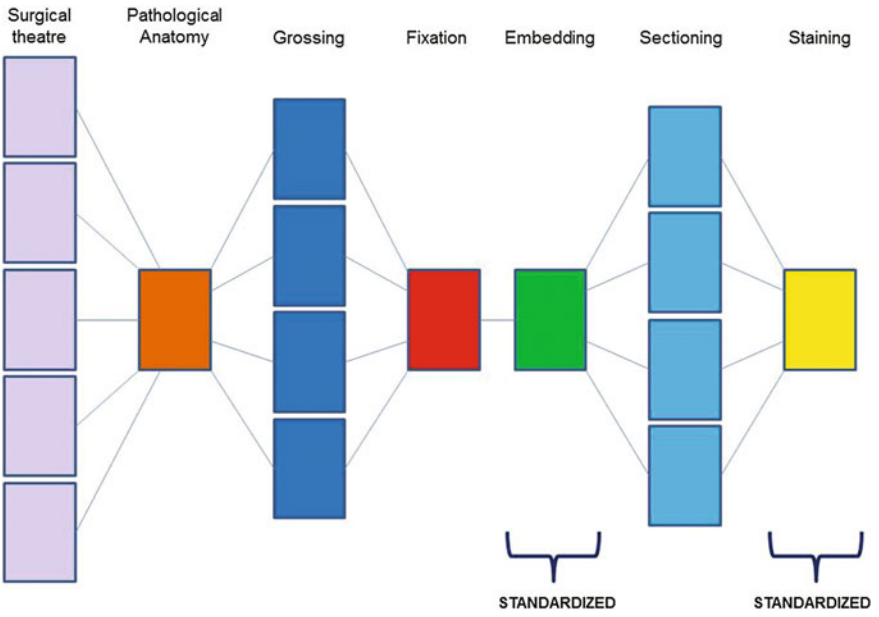

Fig. 1 Sequence of the pre-analytical steps. From the surgical theaters, tissue specimens are transferred to Pathological Anatomy. Grossing is immediately followed by fixation and paraffin embedding. After sectioning of the tissue blocks, sections are appropriately stained. So far, only paraffin embedding and staining are standardized being run with dedicated apparatuses, while for the other steps ample variations of modalities, times, and protocols are used in different laboratories

Local variations in the practice of these steps are common in pathology laboratories worldwide and depend on type of tissue, traditional habits, requests by clinicians and patients and on specific investigations. Moreover, the goal of shortening the turn-around time (TAT) has to be considered.

In common practice, diagnostic and TAT requirements, hence processing, are different for "small" biopsies (<2 cm in size) and large, surgical resection specimens. A fast and reliable pre-surgical diagnosis is requested for the former and, accordingly, these biopsies are best collected directly in fixative-filled vials and soon transferred to the laboratory for paraffin embedding and routine diagnosis.

The processing of "large" surgical specimens is instead more critical, variable, and problematic, involving steps significantly affecting the preservation of antigens and nucleic acids.

This implies that fixation and processing of "small", pre-surgical biopsies, which starts immediately after procurement, is relatively standardized. At variance, the handling of surgical specimens is more critical and undefined. This will be the topic of the present analysis.

The present interest on the analytical definition of the steps involved in the preparation of surgical specimens is linked to novel clinical requests, demanding genomic and antigenic testing for targeted molecular therapies. Indeed, requests for exploitation of archival formalin-fixed paraffin embedded (FFPE) tissues from surgical resections of breast, lung and colon cancers are exponentially increasing and Pathologists should get ready to play an important role in sample standardization in the years ahead. As a result, guidelines will have to be adopted by laboratories and hospitals on a global scale. Standardization of the pre-analytical phase of surgical specimens' preparation would be the right answer.

2 The Ischemia Time

The time interval between surgical intervention and proper fixation of the removed specimen is defined as "ischemia time" and is crucial, since ischemia allows activation of tissue enzymes, autolysis, and degradation of proteins and nucleic acids [1, 2]. Substantial RNA degradation may occur during this time interval [3].

The "ischemia time" is currently subdivided into the "warm ischemia time" which occurs during operation, after ligation of blood supply till removal of the specimen from the body, and the "cold ischemia time," afterwards. The former varies considerably (from a few minutes up to hours), depending on the complexity of the surgical procedure, ability of the surgeon, modality of intervention. The tissue remains alive and reactive, but will undergo progressive metabolic stress due to hypoxia. In an experimental study on colon cancer, Bray et al. [4] emphasized the need of protocols for tissue procurement and showed that significant variation in the level of expression (either increased or decreased) of transcripts was detectable already after 15 min, and by 120 min there was a fourfold increase in a number of genes with a more than twofold change in the level of expression. Indeed, this is bound to heavily influence

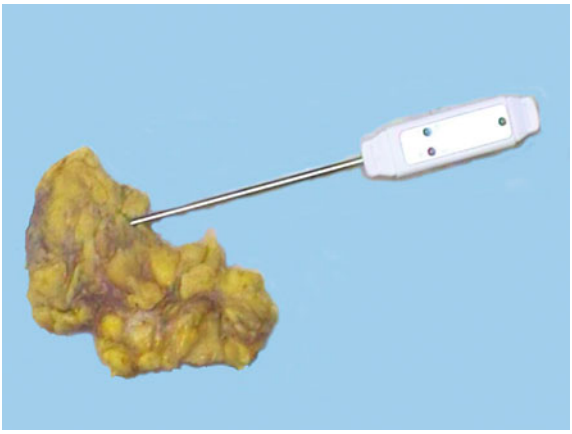

Fig. 2 Evaluation of the inner temperature in a surgical specimen, using a needle thermometer. Tissues (and notably fat tissue) are poor heath transmitters

the interpretation on the involvement of specific genes in the pathogenesis of diseases. For example, it is suggested that reduced expression of KLF6 may be implicated in the early stages of the molecular pathogenesis of approximately one-third of colorectal carcinoma cases, but the study by Bray et al. [4] shows that expression of KLF6 mRNA is significantly increased by delay in sample procurement.

The "cold ischemia time" is instead defined as the time interval between tissue removal from the patient and arrival in the pathology laboratory for grossing. During this interval it is mandatory to avoid autolysis and drying of the surface, which might damage tissue structure and components. The temperature of the specimen gradually reaches external temperature (either room temperature or 4 °C for tissues left in fridge), but this process is rather lengthy, as can be tested by using a needle thermometer (Fig. 2).

Methods of transfer of surgical specimens vary according to the architectural layout and distance between surgical theaters and pathology laboratories. The ideal situation is when physical location and hospital protocols allow for the immediate transfer of "fresh" tissue specimens for prompt grossing and fixation. Accordingly, it has been recommended the "cold ischemia time" to remain below 1 h so as to allow a proper processing of breast cancer specimens and permit a correct evaluation of both morphological and therapeutic-prognostic parameters [5, 6]. Preservation of HER2 reactivity seems less critical than that of Estrogen (ER) and Progesterone Receptors (PgR), since optimal results are still attainable after a 3 h delay [7]. Problems arise when the "cold ischemia time" cannot be kept into properly defined limits: depending on the hospital structure the transport of surgical specimens from the surgical theater to the pathology lab may prolong the ischemia time. In addition, transfer is currently performed wordwide using the most variable types of boxes, transport media, and at different temperatures. It is common practice in many hospitals to immerse surgical resection specimens and organs into large formalin-filled containers, which are then transferred to the pathology laboratory in due time, usually once everyday. This practice carries problems, since:

1. Plastic containers are large and relatively heavy; spilling may occur.
2. Immersion of the whole specimen into formalin prevents the collection of fresh material for tissue banking. In addition, fixation does begin, but only at the periphery. A delay in the transfer to pathology is somehow justified by the fact that "the tissue is already in formalin".
3. Surgical nurses are becoming increasingly concerned about potential toxicity and carcinogenicity of formalin, since the fluid has to be handled outside the hood.
4. When the container does arrive at the pathology lab, opening of the boxes, and handling of the specimen is major cause of diffusion of formaldehyde fumes.

Toxicity of formaldehyde is a matter of concern. This reagent demands caution since it is a skin allergen and produces irritating vapors that can cause asthma. Moreover, the International Agency for Cancer Research [8] has classified formaldehyde as a Class 1 carcinogenic agent. A positive relationship between formalin and respiratory symptoms has been reported not only in workers in match factories [9], but also in hospital staff members professionally exposed to this substance [10]. Statistical evidence has been presented for a possible link between formaldehyde exposure and lympho-hematopoietic malignancies [11], an observation that might fit with data reporting an excess of deaths due to cancer of the lymphatic and hematopoietic systems among British pathologists [12]. Still, the major concern for formaldehyde use is linked to the production of toxic, irritating, and allergenic vapors.

To ride over these problems, we adopted procedures designed to avoid the transfer of formalin-filled boxes in the hospital premises. We accordingly proposed to seal fresh surgical specimens under-vacuum in plastic bags in the surgical theaters, immediately after removal, and to keep them cooled at 4 °C until transfer to the pathology labs, where they are routinely processed [13]. Sealing of tissues in plastic bags is a quick procedure, taking approximately 15 s and is easily performed by nurses [14].

3 Under Vacuum Sealing

Under-vacuum sealing (UVS), per se, does not guarantee preservation, as experienced by Kristensen et al. [15]. Vacuum sealing, by removing air, prevents dehydration and favors cooling, the latter being the main preserving factor by blocking enzymatic autolysis. In fact, we experimentally demonstrated that cooling down at 4 °C is quicker in UVS-treated tissues [16]. Moreover, cooling down of under-vacuum sealed specimens can be speeded up by using a properly devised procedure (Fig. 3).

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 affect immune-phenotyping of the specimen, an issue that is presently regarded as mandatory for breast

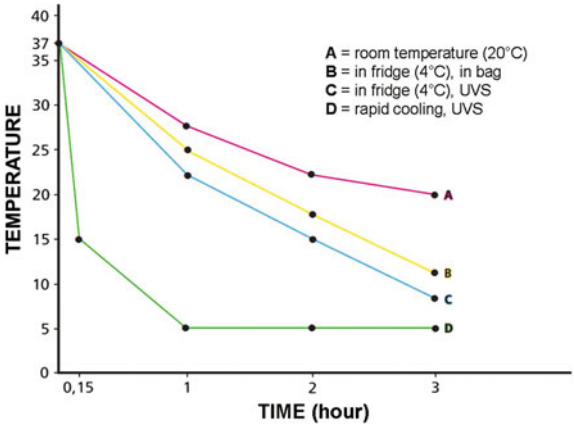

Fig. 3 Variations of the inner temperature in a surgical specimen (approximately 7 cm in diameter) in different conditions. From the body temperature, on surgical removal, if left at room temperature (*A*), it takes approximately 3 h for the specimen to stabilize at the external temperature. When the specimen is left, free, in a fridge (*B*), the inner temperature will gradually go down. The cooling process in Under-Vacuum Sealed (UVS) specimens (*C*) is facilitated by the lack of insulating air. By using cooling (-20°C) elements (*D*), the cooling of the UVS specimens is very rapid

cancer processing. A bonus of this under-vacuum Sealing and Cooling (UVSC) procedure is the preservation of RNA, which is permitted by the storage at 4 °C [17, 18], thus favoring tissue banking and gene expression profiling. Moreover, the UVSC procedure proved valuable for the preservation of hypoxia resistant cells, such as human stem/progenitor cells, which were successfully isolated and cultured from kidneys stored UVSC up to 48 h after surgery [19]. It derives that this procedure can be exploited to render the extraction of stem cells from human samples more practical and feasible. UVSC could also represent a reliable source for creation of primary cell cultures. The environmentally safe UVSC collection, preservation, and storage of surgical specimens may represent a strategy to bridge diagnostic and experimental pathology thus offering a new tool for bio-banking and for creation of clinically relevant models of neoplastic lesions (primary cultures) [16].

The UVSC procedure has extensively been adopted at the Azienda Ospedaliera Città della Salute e della Scienza di Torino (Italy), a large regional "pavilion" hospital where the distance between surgical theaters and pathology laboratories prevents an immediate transfer of fresh specimens. The transfer of surgical specimens across the Hospital in large formalin-filled boxes was a time-honored habit, but since 2009 the procedure of under-vacuum sealing and cooling has been adopted as the sole and routine procedure for the transfer of surgical specimens (larger than 2 cm). The experience accrued has been duly analyzed and reported [14]. The survey on the feasibility, compliance, and quality assurance of this new procedure for transferring surgical specimens was definitely positive. Dedicated apparatuses (TissueSafe®, Milestone srl, Sorisole, BG, Italy) were located in the premises of each of the six surgical theaters of the Hospital. The UVSC procedure

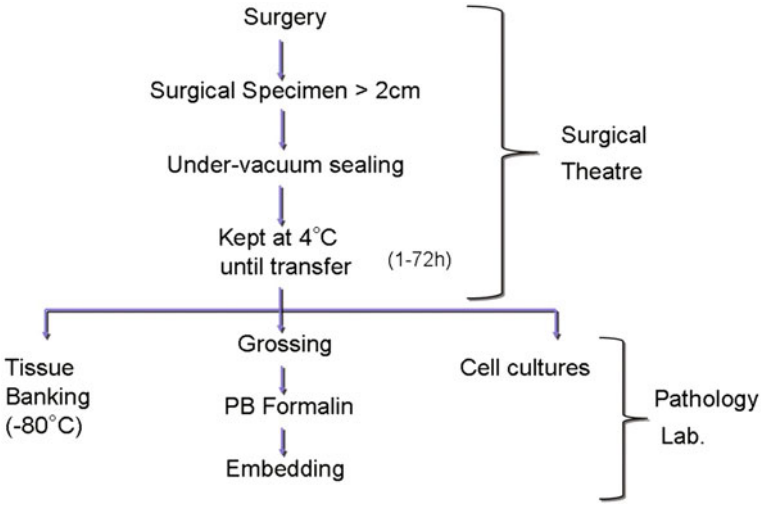

Fig. 4 Outline of the procedure for handling of the large (>2 cm) surgical specimens, as practiced in the Azienda Ospedaliera Città della Salute e della Scienza di Torino since year 2008. Under-Vacuum Sealed specimens are kept cool (in a fridge) in the surgical premises for a time variable from 1 up to 72 h (during weekends). Grossing, fixation, and histological processing follow. In alternative, material for tissue banking or culturing can be obtained from fresh tissues (*PB* phosphate buffered)

was favorably accepted by the staff and did not present special problems of practical or diagnostic interest and was therefore adopted as the standard in the Hospital.

Moreover, the environmental goal of a progressive reduction of the exposure for nurses, pathologists, and technical personnel to formaldehyde vapors was met. The use of formalin has been reduced and restricted to dedicated areas in the pathology laboratory, and transfer of large boxes filled with fixative throughout the hospital ceased. The flowchart presented in Fig. 4 illustrates the procedures currently practiced in our Institution for tissue transfer and preservation.

In conclusion, the simple UVSC processing offered advantages in terms of staff satisfaction, tissue preservation and cost.

4 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 on the tissue components.

Several fixatives have been proposed, but those presently practiced are either of the aldehyde cross-linking category or alcohol-based, the latter producing coagulation by water subtraction. Alcohol-based fixatives have the advantage of lack of

toxicity and of a good preservation of nucleic acids, but show a poor penetration and reportedly result in an unsatisfactory preservation of morphological details [20]. Moreover, in our [21] and in others' [20, 22] experience, the performance of some immunohistochemical tests is impaired. The alcohol-based substitutes which have been proposed are generally non-cross-linking [21, 23–25] and allow a better preservation 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.

A 4 % formaldehyde solution in 0.1 M phosphate buffer pH 7.2 (phosphate buffered formalin, PBF) has been adopted as the fixative of choice in histopathology being (relatively) cheap, easy to use, and reliable (it does not over-fix). It guarantees, in appropriate conditions, an optimal morphological preservation. Still, the finality of formalin fixation has evolved 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 [26, 27]. 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 PBF of breast cancer tissue blocks for no less than 6 and no more than 48 h is now required in order to guarantee an optimal evaluation of ER, PgR, and HER2 expression by immunohistochemistry [28].

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 [2, 29]. Indeed, evaluation of proliferation activity and clinical evolution prospects in breast cancer are already attainable with molecular tests, and evidence has been presented that evaluation of predictive biomarkers (ER, PgR, HER2) by RNA analysis is fully matching that obtained by immunohistochemistry [2, 29].

The process of formalin fixation involves two separate and interrelated phenomena: (1) penetration into tissues by diffusion and (2) proper fixation by establishment of cross-links between different reactive groups. This latter phenomenon is slow (it takes hours or even days to be completed) and was extensively described and analyzed in numerous studies conducted on the reaction of formaldehyde with different tissue components [30, 31]. The main effect of formaldehyde on tissues is linked to the formation of methylol groups on amino groups first, followed by the establishment of cross-linking methylene groups that lead to proper fixation [32]. These links are partly reversible by the action of peptidases and heating [33], which seems to explain the extensively practiced procedures leading to "antigen retrieval", that is the restitution of immuno-histochemical reactivity in (FFPE) tissue sections.

The "penetration" step that is represented by the diffusion of the formaldehyde solution into tissues is instead poorly known in its analytical essence and reactions involved. It is only stated that it is "rapid", at a speed of approximately 1 mm/h [30] (see Fig. 5). The experience in surgical pathology testifies that the speed varies with

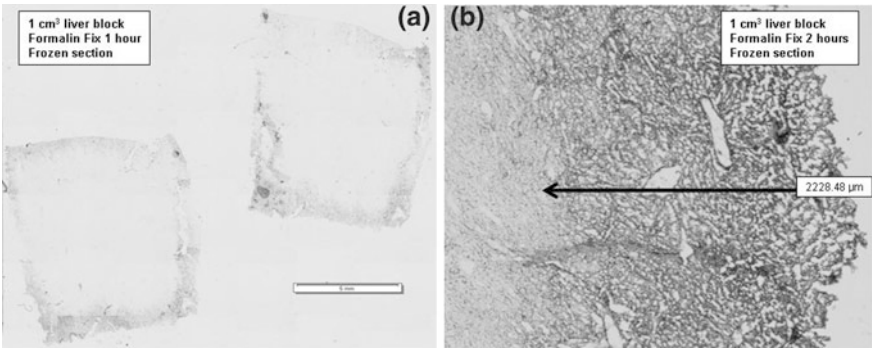

Fig. 5 Evaluation of the penetration of formalin in a square block of liver. After 1 h (**a**) or 2 h (**b**) of immersion in the fluid, the block is split, frozen, and sections are cut in a cryostat. The formalin-free area appears transparent, while a rough appearance marks the depth of penetration of the fixative (**b**). In **b**, the depth of formalin penetration after 2 h of immersion is indicated

tissues (being lower in fat tissues), that it is influenced by temperature, relative quantity of the fixative and agitation and that it can be enhanced by application of ultrasounds [34]. As a general rule, we can state that in a 3–4 mm thick tissue section (the tissue blocks produced by grossing) the center of the block is reached in 2 h while, on the contrary, in whole organs immersed in formalin overnight diffusion does not proceed over 1 cm depth. The above statement is referred to PBF which is the solution currently employed [30, 35]. The addition of a minute amount of mercaptoethanol [36] significantly speeds the penetration. As for the relative amount of fluid, common practice dictates that the quantity of formalin should be at least 10 times that of the tissue to be fixed. Experimental tests proved however that fixing tissues with a ratio of PBF volume to tissue volume of 2:1 for 48 h at 20–22 °C was enough to assure a proper fixation and infiltration of the tested tissues [37], fixation being improved by pressure and agitation.

The experimental measure of formalin penetration has not been pursued so far. As a test we exploited the phenomenon whereby frozen sections of fresh tissues are transparent, while instead those of formalin-fixed tissues are not and show a rough appearance. This experimental approach, when tested on 1 cm^3 liver tissue blocks, shows that indeed at room temperature the penetration is 1 mm in the first hour and approximately 2 mm after 2 h (Fig. 5).

Studies conducted on the preservation status of nucleic acids in FFPE tissues generally agree on the relatively good (though not optimal) preservation of DNA [3]. On the contrary, RNA has been found to be heavily degraded and fragmented so that only short sequences (approximately 100–200 nucleotides) can be recognized and amplified [32, 38–41]. The reasons for this effect are presently unknown, but the cross-linking properties of formaldehyde should be considered. Bases of nucleic acids are involved in this cross-linking process, resulting in the establishment of bonds with side-chain amino groups of proteins. However, this linkage is at least partly reversible following extensive treatment of FFPE tissue sections with

peptidases and high temperature [32, 33, 35, 38]. We may conclude that cross-linking of nucleic acid bases cannot be the sole responsible for nucleic acid fragmentation and degradation. The RNA degradation process may well continue during fixation. In fact, while formalin penetration is a rather fast process, tissue fixation is known to be a slow process that requires long time exposure [31, 33].

Based on these hypotheses, we have further considered that RNA degradation would be inhibited by maintaining low temperature through all the process of fixation. We have evaluated whether a protocol based on processing tissues with formalin at low temperature, a cold fixation (CF) process, would better preserve nucleic acid integrity, while preserving morphological and antigenic features as well.

The CF process [42] is based on a fixation process in PBF at 4 °C for 20–24 h, followed by dehydration in cold ethanol for 2 h. This was then followed by routine dehydration and paraffin embedding. 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 immuno-histochemical properties which make formalin the fixative of choice in histopathology. 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 Cold Formalin Fixed Paraffin Embedded (CFFPE) material from the same cases of breast cancer. The number of genes detected in RNA extracted from fresh tissues closely matches that of RNA extracted from cold formalin-fixed material from the same cases, the former being only 4.12 % higher [17, 18].

The improvement offered by the CF 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.

The ensuing step of embedding in paraffin wax following dehydration through a sequence of passages in ethanol is well standardized and generally processed with dedicated apparatuses. It has recently been demonstrated that even this process might involve variables affecting tissues and the performance of analytical tests. It is in fact well known that paraffin blocks stored for long times (years) undergo alteration affecting antigenicity as well as RNA preservation (*personal experience*). Xie and coworkers [43] revealed that inadequate tissue processing, resulting in retention of endogenous water in tissues, leads to antigen degradation and even tissues embedded in paraffin (a non-reactive medium) can undergo a slow degradation process.

Variables which so far attracted little interest, but in the experience of von Ahlfen et al. [44] might influence the status of RNA in the FFPE tissue blocks, are temperature and storage conditions. These authors showed that storage at different temperatures has a profound influence on the extent of RNA fragmentation. After 1 year storage at 4 °C, RIN values around 5–6 could be obtained while in contrast, RNA from blocks stored at room temperature (20–25 °C) or 37 °C, did not show clearly distinct rRNA bands anymore, and the mean fragment length was well below

100 nucleotides. Indeed a demanding topic of future interest would be to standardize the conditions of embedding and storage of FFPE tissue blocks, in order to guarantee feasibility of molecular analyses even years after the original diagnosis.

5 Conclusions

Morphological diagnoses are no longer the sole analyses requested to pathologists. Novel clinical demands, which require the evaluation of prognostic and predictive factors for targeted molecular therapies through genomic and antigenic testing, are pushing for the necessity to standardize handling of surgical specimens, so as to obtain high quality, reliable and reproducible results.

Technologies such as under-vacuum sealing and cold fixation will be implemented in future years, in an attempt to optimize and standardize on a global scale pathological diagnoses and analyses.

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Histologic Validation of Vacuum Sealed, Formalin-Free Tissue Preservation, and Transport System

Richard J. Zarbo

Abstract

We describe five validation trials of new vacuum sealing technologies that change the approach to the preanalytic "front end" of specimen transport, handling, and processing and illustrate their adaptation and integration into existing Lean laboratory operations with reduction in formalin use and personnel exposure to this toxic and potentially carcinogenic fixative. These trials provide histologic assessment by numerous pathologists of tissues processed in this new paradigm and define the financial advantages of applying this technology to the postanalytic or "back end" process of tissue storage. We conclude that the TisssueSAFE and SealSAFE vacuum sealing systems are both promising technologies for preserving fresh human specimens that can promote a safer environment by markedly reducing formalin use in operating room theaters and can minimize formalin use by laboratories.

Keywords

Formalin · Vacuum sealing · Tissue specimens · Histology

Contents

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With the advent of personalized medicine to individualize the profiling of patients for targeted therapies, the historical role of pathology to make an accurate tissue-based histologic diagnosis is now being challenged with new requirements. These include more timely diagnoses with triage and preservation of fresh tissues and documented quality control of the numerous currently uncontrolled preanalytic specimen variables of fresh and formalin fixed tissues that could affect the sensitivity of molecular based testing. These preanalytic variables will be important for assessment of biologic molecules at all levels of DNA, RNA, protein, and small signaling molecules. The new challenge for pathologists is to close the preanalytic "gaps" and identify molecular friendly techniques and technologies to be able to assure these requirements while relying on the advantages of formalin whose role as a fixative for morphologic based diagnoses is well over 100 years old [1].

In this chapter we describe five validation trials of new vacuum sealing technologies that change the approach to the preanalytic "front end" of specimen transport, handling, and processing and illustrate their adaptation and integration into existing Lean laboratory operations with reduction in formalin use and personnel exposure to this toxic and potentially carcinogenic fixative. These trials provide histologic assessment by numerous pathologists of tissues processed in this new paradigm and define the financial advantages of applying this technology to the postanalytic or "back end" process of tissue storage.

The technologies tested here are the TissueSAFE high vacuum biospecimen transfer system and the SealSAFE system, the latter capable of resealing specimens post-dissection and dispensing formalin into the vacuum-sealed bags based on a preset ratio related to specimen weight (Milestone Medical Srl, Bergamo, Italy). Experiences of others with the TissueSAFE system in Europe at a university based laboratory have been published previously [2, 3].

1 Validation Trial #1—Defining the Parameters of Temperature and Time

We initially tested the TissueSAFE device with human specimens from at the Main Hospital Operating Rooms (Ors) that arrived fresh in the adjacent Frozen Section Laboratory. We manipulated variables of temperature and time with vacuum sealed preservation of fresh specimens at 4, 7, 25 °C and held for variable times (24, 48, 72 h) at those temperatures before dissection and formalin fixation (Fig. 1). This was compared to samples from the same specimens that were immediately formalin fixed. Pathologists were blinded to the pairs being compared. Specimens were designated to any of 15 specialists and general surgical pathologists who evaluated the histologic features from hematoxylin and eosin stained glass slides based on a three part scheme of 1 = acceptable for diagnosis; 2 = inferior quality for diagnosis;

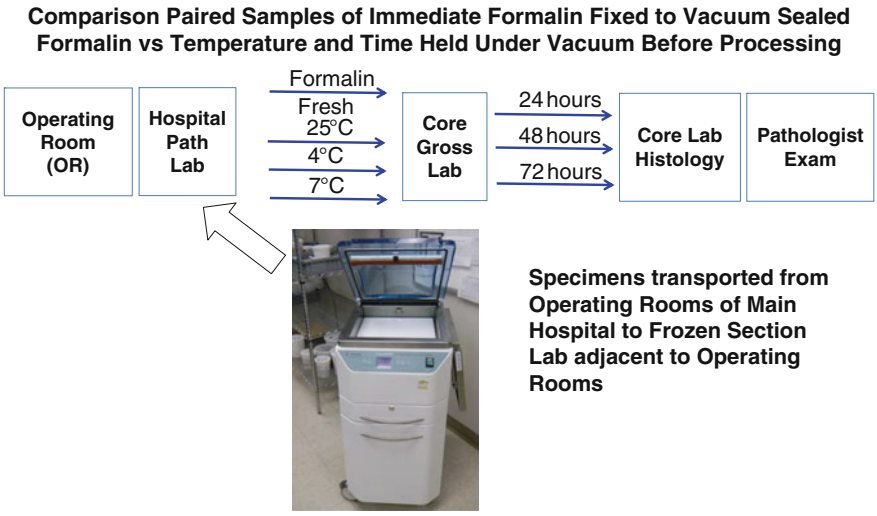

Fig. 1 Validation trial #1 main hospital specimens

3 = unacceptable for diagnosis. Of 50 blocks of tissue processed after sealing at 4 and 7 °C and held in simulated transport times of 24 and 48 h, 46 were found acceptable for diagnosis (Fig. 2). Four blocks of liver sealed at 4 °C were judged of inferior staining quality. These were considered under processed on review by the manager of the histology section. At a simulated transport time of 72 h, additional inferior quality stained slides were noted and therefore this prolonged time was no longer evaluated.

Variables Time and Temperature Under Vacuum

50 blocks evaluated-Formalin vs vacuum sealed
46 blocks acceptable after 24-48 hours at 4°C and 7°C

Large Specimens	1 acceptable	2 Inferior	3 unacceptable
Leg	1	0	0
Liver	1	1*	0
Lung	1	0	0
Lymph node	1	0	0
Ovary	1	0	0
Pannus	1	0	0
Uterus	2	0	0
Total	8	1	0

HEMATOXYLIN AND EOSIN Scoring Scale

- 1 = ACCEPTABLE FOR DIAGNOSIS
- 2 = INFERIOR QUALITY
- 3 = UNACCEPTABLE FOR DIAGNOSIS

*
•4 liver blocks under-processed
•Processed at 48 + 72 hrs at 4°C

Fig. 2 TissueSAFE histologic assessment of morphologic preservation

2 Validation Trial #2—Defining the Transport System from Community Hospital

One of our chief goals was to transport human tissues in the fresh state from a community hospital 25 miles away to a continuous flow core laboratory for dissection and processing (Fig. 3). Because we had to satisfy the requirement of five courier runs per day from the community hospital to the core laboratory, we innovated a means of transporting the vacuum sealed specimens in an insulated cooler between layers of plastic grids with conventional ice cubes in Ziploc bags place above and below the specimens (Fig. 4). An RFID card was included with each specimen run to record a temperature and time log for the transport run (Fig. 5). The optimal temperature achieved for transport with this mechanism was a stable 4 °C. In this pilot we evaluated 11 medium and large size tissue specimens assessed at transport delay times of 24 and 48 h on ice at 4 °C. All were judged to be acceptable for diagnosis by pathologists. The histologic assessment showed no differences in quality for specimens held in the chilled, airless state for 24 and 48 h (Fig. 6).

The TissueSAFE device has been subsequently located in the OR area and OR circulator nurses have been trained to triage and seal the specimens. We have now transported hundreds of specimens (roughly 56 % of OR based surgical volumes) from this community hospital in this manner for the past year without incident or pathologist complaint. The process receives very high marks from OR leadership and nurses. Large cubes of formalin are no longer stored and used in this OR suite to fill large specimen containers. Only small biopsy containers prefilled with small

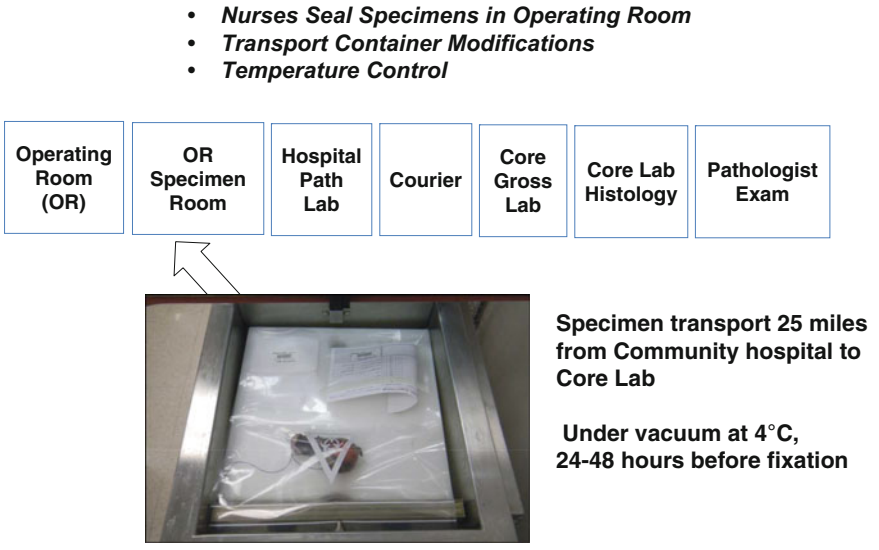

Fig. 3 Validation trial #2 transport system from community hospital

Fig. 4 Transport by insulated cooler with conventional ice cubes in Ziploc bags

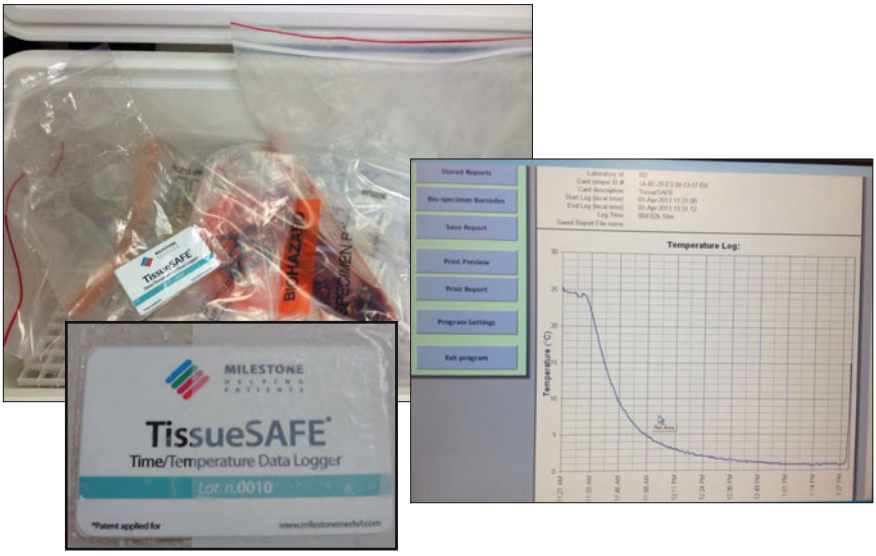

Fig. 5 RFID card records the run temperature

amounts of formalin are used in these ORs for small specimens and needle biopsies. The enhanced safety from reduced exposure of OR personnel to formalin is considered priceless by OR leadership.

Based on this TissueSAFE process, this community hospital laboratory has used 135 fewer gallons of formalin for an annual cost savings of $1,688. The cost of consumables, plastic sealing bags replacing plastic bucket, was neutral. Additional savings not calculated are the courier fuel costs of transporting heavier specimen containers that would have been filled with formalin.

24 and 48 hours under vacuum at 4°C before fixation

Large Specimens	1 Acceptable 24 hours	1 Acceptable 48 hours	2 Inferior or 3 Unacceptable
Fallopian tube	1	n/a	0
Fistula soft tissue	1	1	0
Gall bladder	1	1	0
Placenta	1	1	0
Small bowel	2	2	0
Stomach	3	3	0
Thyroid	1	1	0
Uterus	1	1	0
Total	11	10	0

Fig. 6 TissueSAFE histologic assessment of morphologic preservation after transport in coolers on ice at 4 °C

3 Validation Trial #3—Evaluating the Histology of Wider Variety of Large Specimens

In this trial we obtained a wider variety of tissues from the ORs of the Main Hospital for histologic evaluation after vacuum sealing with the TissueSAFE device located in our Pathology specimen receipt adjacent to the large theater of 32 ORs. These sealed specimens were then transported at intervals throughout the day at 4 °C to the core gross lab within another building at transport times of 1–10 h.

Of 122 medium and large size specimens transported using the TissueSAFE, 97 % were assessed by 15 pathologists as histologically acceptable for diagnosis (Fig. 7). Only four specimens were considered inferior for histologic assessment and none were found to be unacceptable. The specimens considered inferior for diagnosis were one each of kidney, prostate transurethral resection chips, small bowel, and uterus. There was no root cause in the first two specimen types however in the latter two specimen types we have since created standards that require hollow organs to be opened before vacuum sealing.

Large Specimens	1 acceptable	2 Inferior	3 unacceptable
Appendix	4	0	0
Brain	1	0	0
Colon	11	0	0
Gall bladder	14	0	0
Heart	1	0	0
Kidney		1	0
Liver	4	0	0
Lung	3	0	0
Prostate TURP	1	1	0
Skin	3	0	0
Small bowel	3	1	0
Stomach	20	0	0
Soft tissue	2	0	0
Thyroid	7	0	0
Uterus	28	1	0
Tonsils	13	0	0
Miscellaneous*	7	0	0
Total	122	4	0

* Miscellaneous: hemorrhoid, hydrocele, heart valve, artery, endometrium

Fig. 7 Validation trial #3 TissueSAFE histologic assessment of large specimen morphologic preservation

4 Validation Trial #4—Evaluating the Histology of Needle Biopsies

We separately assessed the feasibility of transporting needle biopsies in the fresh state with the TissueSAFE device. We simulated clinical biopsies with a pathologist's assistant using a Biopty gun to take needle biopsies from 35 freshly delivered resection specimens from the main ORs. The needle cores were placed in specimen

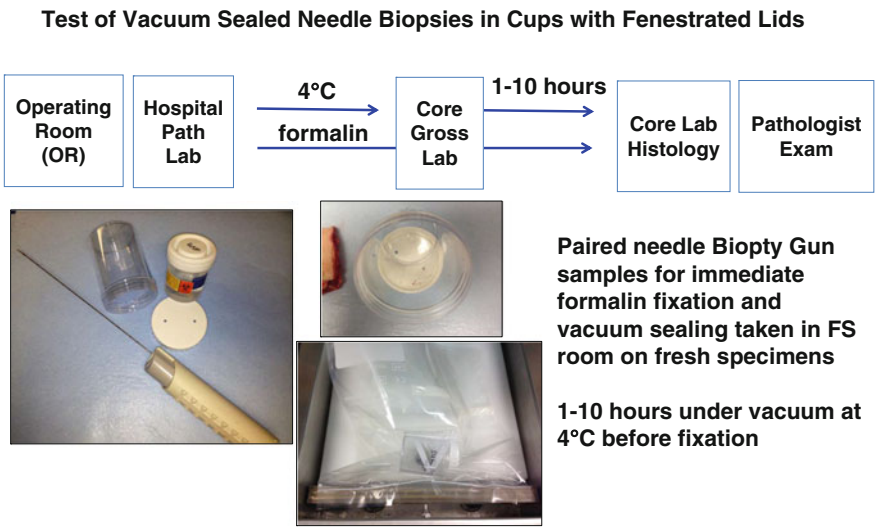

Fig. 8 Validation scheme #4 needle biopsy evaluation

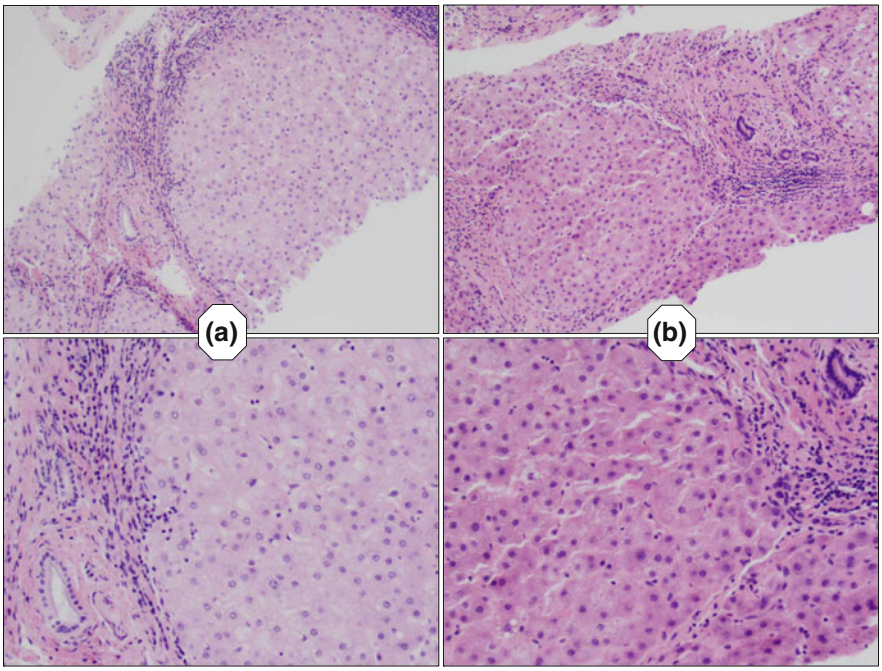

Fig. 9 Liver needle biopsies-**a** immediate formalin fixed, **b** vacuum sealed

Paired Needle Biopsy Comparison	Number cases	Vacuum Sealed Score Avg.	Immediate Formalin Score Avg.
Colon cancer and adenomas	6	1.7	1.7
Liver cancer and cirrhosis	4	1.4	1.0
Kidney cancer			
Stomach cancer and gastritis	2	1.9	1.5
Thyroid- cancer and goiter	6	2.0	1.5
Uterus cancer and myomas	14	1.6	1.3
Total	35	1.7	1.4

HEMATOXYLIN AND EOSIN
Scoring Scale

- 1 = ACCEPTABLE FOR DIAGNOSIS, OPTIMAL HISTOLOGY
- 2 = ACCEPTABLE FOR DIAGNOSIS, LESS THAN OPTIMAL HISTOLOGY
- 3 = UNACCEPTABLE FOR DIAGNOSIS

Fig. 10 TissueSAFE histologic assessment of needle biopsy morphologic preservation

collection cups with fenestrated lids that were sealed under vacuum at 4 °C and transported to the core gross lab over 1–10 h. These test cases were assessed histologically in comparison to Biopty gun needle cores that were taken at the same time and fixed immediately in formalin (Fig. 8). Histologic assessment of suitability for diagnosis was more variable for the same test biopsies between the 15 pathologists. This was ascribed to inconsistent differences in deeper nuclear staining intensity and cytoplasmic eosinophilia seen in the sealed needle biopsy specimens (Fig. 9b) compared to the immediately formalin-fixed pairs (Fig. 9a). This is illustrated from liver core biopsies in Fig. 9. Because of the wider range of pathologist assessment, the scores were averaged and showed a small preference for the immediate formalin fixed biopsies (average score 1.4) compared to the vacuum sealed biopsies (average score 1.7) among the group of 15 pathologists who evaluated cancers and non-neoplastic diseases of colon, liver, kidney, thyroid, and uterus (Fig. 10). None were judged to be unacceptable for diagnosis.

5 Validation Trial #5—Evaluating Reduced Formalin for Tissue Storage

The SealSAFE device differs from TissueSAFE system in that this vacuum sealing device can dispense formalin into the sealing bags based on a preset ratio related to specimen weight determined on its platform scale (Fig. 11). This device is therefore capable of resealing specimens in formalin post-dissection.

Fig. 11 SealSAFE

Dictums abound related to optimal tissue fixation and the importance of excess volume of fixative in relation to the total volume of tissue. Fixative to tissue ratios ranging from 10:1 to 50:1 can be found. The generally accepted but unscientific rule of thumb for formalin fixation is to immerse a specimen in a volume that is 10 times its weight. Unfortunately this results in large, heavy buckets of formalin that require expensive disposal. However, our paradigm for tissue handling is to receive specimens in the fresh state, to dissect specimens fresh and to fix in appropriate amounts of formalin only what tissue will be processed. Therefore we seek to use minimal formalin compared to the weight of the entire specimen in the initial processing and we subsequently desire to continue that miserly use of formalin into specimen storage.

In this trial of the SealSAFE device, we tested reduced formalin volumes related to specimen weight to preserve tissues in storage that may potentially require return dissection for morphologic assessment of the stored tissues by pathologists. We evaluated 10 specimen types (colon, gall bladder, small bowels, thyroid and uterus) at specimen: formalin weight ratios of 2:1 and 1:1. Specimens were sealed with formalin, held under vacuum at room temperature and sampled, with resealing, for histologic assessment after 24, 48, and 72 h with minimal formalin. Morphologic assessment demonstrated that all tissues at both formalin ratios were acceptable for diagnosis with no degradation in histology noted at 72 h (Fig. 12).

2:1 and 1:1 formalin to weight ratio
24, 48, 72 hours under vacuum at room temperature

Specimens	1 Acceptable 24 hours	1 Acceptable 48 hours	1 Acceptable 72 hours	2 Inferior or 3 Unacceptable
Colon	1	1	1	0
Gall bladder	2	2	2	0
Small bowel	1	1	1	0
Thyroid	1	1	1	0
Uterus	5	5	5	0
Total	10	10	10	0

HEMATOXYLIN AND EOSIN Scoring Scale

- 1= ACCEPTABLE FOR DIAGNOSIS
- 2= INFERIOR QUALITY
- 3= UNACCEPTABLE FOR DIAGNOSIS

Fig. 12 SealSAFE histologic assessment of morphologic preservation in storage

6 Analysis of Upstream and Downstream Savings

A number of savings have been identified here, not the least of which is a safer formalin-free environment for all employees, especially in OR theaters where a spill clean up can close a room for many hours of hazardous waste containment and removal. Although it is a very good fixative and the basis of the histology artifact upon which pathologists define most microscopic diagnoses, formalin is well known to be a toxic and potentially carcinogenic substance. Restriction of its use should be strongly considered and is now possible.

7 Financial Savings

In our experience, using this combined process of vacuum sealing specimens at the "front and back end" processes of anatomic pathology resulted in reduced formalin usage at the community hospital of 135 gal/year, reduced formalin for tissue storage in the core laboratory of 468 gal/year, 43 % less storage shelf space used for specimens and a financial reduction to the institution of $51,000 for routine rather than hazardous disposal of stored formalin fixed tissues because of the minimal formalin used.

8 Conclusions

We have successfully integrated these technologies into existing Lean operations to consistently obtain our goals of controlled preanalytic transport and fixation variables, rapid turnaround times, preservation of fresh tissues for biobanking, and reduction in use of formalin at the front end process of initial tissue fixation and the back end process of excess tissue storage before discard.

We conclude that the TissueSAFE and SealSAFE are both promising technologies employing vacuum sealing of human specimens that can promote a safer environment by markedly reducing formalin use in OR theaters and can minimize formalin use by laboratories. The investment in these technologies is offset by the perpetual savings associated with disposal expense of tissues that would ordinarily be stored in higher volumes of formalin.

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The Evolution of Pre-analytic Factors in Anatomic Pathology

Stephen M. Hewitt

Abstract

Anatomic Pathology has continuously evolved since launch by Virchow in Berlin. The era from 1990 to 2010 saw the rise of immunohistochemistry and its application for diagnosis, prognosis, and prediction of response to therapy. Currently the next wave of evolution is ongoing; molecular pathology, with emphasis on alterations to DNA, and expression of mRNA as biomarkers. The interrogation of biomolecules by specific probes is more demanding on specimens than the traditional application of histologic stains to tissue. This issue is juxtaposed to the fact that the majority of specimens are purely evaluated by histomorphology, for which current specimen practices are adequate. The capacity to identify a priori which cassette of tissue is appropriate for molecular analysis is difficult, if not impossible, the goal is to improve the quality of all pathology specimens in an economically viable model to enable advanced assay, when applicable.

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1 Introduction

Anatomic Pathology has continuously evolved since launch by Virchow in Berlin. The era from 1990 to 2010 saw the rise of immunohistochemistry and its application for diagnosis, prognosis, and prediction of response to therapy. Currently the next wave of evolution is ongoing; molecular pathology, with emphasis on alterations to DNA, and expression of mRNA as biomarkers. The interrogation of biomolecules by specific probes is more demanding on specimens than the traditional application of histologic stains to tissue. This issue is juxtaposed to the fact that the majority of specimens are purely evaluated by histomorphology, for which current specimen practices are adequate. The capacity to identify a priori which cassette of tissue is appropriate for molecular analysis is difficult, if not impossible, the goal is to improve the quality of all pathology specimens in an economically viable model to enable advanced assay, when applicable.

2 Specification for Fixed Tissue

The specification of tissue preparation for pathologic examination is relatively simple. The primary goals are:

1. Preservation of tissue in such a manner that it is a permanent record, available for re-evaluation with new diagnostic modalities.
2. Economics are critical, as a large volume of tissue is collected and stored. The pathology laboratory is paid once, at time of diagnosis, but must store the material for a minimum of a decade. Volume of specimens drives low cost for preservation, and payment model drives low cost for storage.
3. The collection and preservation of tissue is a labor-intensive process with multiple staff involved across sites from patient care to the laboratory. Safety must be a primary concern in handling reagents.
4. The fixative is an anaseptic, preventing the growth of microorganisms in the tissue.
5. The methods must be applicable worldwide, to support the fund-of-knowledge employed by pathologist, as well as allow consultation.

Although far from perfect, the use of formalin fixation and paraffin embedding has evolved as the preferred method of tissue preservation. Alternative fixatives abound, most commonly containing acids, alcohols, and/or glycols. In the past these fixatives

have been limited to specialty uses, or limited geographic distributions, but have seen diminished use with the introduction of large tissue processors, and the demands for immunohistochemistry. With formalin now acknowledged as a (likely) carcinogen, it remains unclear what the fixative in widespread use 25–50 years from now will be (http://www.cancer.gov/cancertopics/factsheet/Risk/formaldehyde).

3 Evolution/Fit-for-Purpose

Although the application of formalin fixed, paraffin embedded tissue to diagnostic histopathology appears static, and relatively unchanged over the last 120 years, this is not accurate. The fixation of tissue has been carried out for centuries. Prior to the introduction of formaldehyde as a fixative in 1893 [1], alcohols and other organic-solvent based fixatives predominated. The introduction of buffers to formaldehyde solutions dates from the mid-twentieth century, and were applied to reduce the formation of "formalin-pigment", iron-formaldehyde precipitates. These first buffers were commonly formulations of calcium. Multiple buffers have been applied since that time, largely consolidating into the use of phosphate buffers, contributing both buffering of pH as well as modification of osmolarity of the buffer. As demonstrated by Chung et al. the evolution of buffers for formalin supported the nascent development of molecular pathology, with improved RNA preservation compared to other buffers, and undoubtedly improved immunohistochemical assays, although the benefit was obscure to most investigators [2].

Concurrent with the evolution of fixation, impregnation evolved, with the introduction of the first instrumentation for the serial dehydration, clearing, and impregnation of tissue introduced in first half of the twentieth century, and the development of vacuum processing by Lillie in the 1940s [3] (SMH Library). Although beneficial, vacuum impregnation is not universally used. Many low volume laboratories, as laboratories in Africa, and other underdeveloped nations continue to rely on rotary "dip and dunk" tissue processors. Concurrent with this, reagents became more refined and standardized. Paraffins underwent a substantial advancement from preparations derived from beeswax to synthetic, lower-melting point paraffins which provide better impregnation, and result in better section quality with microtomy [4].

4 High-Throughput Pathology—A Turning Point

Until the early twenty-first century the impact of pre-analytic variables was largely obscure to pathologist. Although immunohistochemistry had entered clinical practice, antigen retrieval was still viewed more as magic than science, and little standardization or systematic approach was applied. Histology laboratory practice was derived from a limited number of manuals, and local practice generally followed local variations based on pathologist preference and availability of equipment and reagents.

The tissue microarray (TMA) was described by Kononnen in 1998 [5]. Although a refinement on the work of Battifore [6] (Can find in a SMH paper), it represented the turning point. The modern TMA was high-throughput, with hundreds of samples per recipient block, and used primarily for immunohistochemical analysis. Studies jumped from tens of samples from a single site and unit numbers of immunohistochemical markers to hundreds of samples collected from multiple sources, or over longer periods of time with tens of immunohistochemical markers. The direct impact was that suddenly variables that had been invisible to investigators because of the small scope of studies now became glaring, when they could be visualized within a single experiment, and not simply dismissed as a failure of the investigator to accurately replicate the assay [4] (SMH review).

5 Quality—Subjective

The challenge remains how to quantify quality in tissue. The historic, and still most commonly used approach is cyto- and histomorphology observed on a Hematoxylin and Eosin (H&E) stain. This process is inherently subjective, based on the experience and preferences of the pathologist as well as the tissue evaluated. Furthermore, *breaking* and H&E is very difficult—when a section can be obtained from a block, it is generally of acceptable quality, and in fact it is the difficulty of obtaining the section that is a far better metric [2].

Other metrics remains as complex. Protein quality, generally evaluated by immunohistochemistry, results in sample bias, especially when an immunohistochemical assay is optimized for an undefined pre-analytic matrix [7]. The evaluation of these assays remains manual and qualitative, with limited dynamic range. Evaluation of nucleic acids is equally challenging—either relying of fragment length and quantity recovered, or dependent on specific assays for which the two metrics demonstrate poor correlations [8].

6 Immunohistochemistry as the Gatekeeper of Quality

Over the last decade, with the increased use of immunohistochemistry, the baseline for the evaluation of tissue, and specification on which pre-analytic variables are defined has shifted from producing a specimen adequate for diagnosis by histologic stains, and is stable over time, to a specimen adequate for immunohistochemistry of prognostic/predictive biomarkers. Although this shift appears small, it is in fact substantial, as it adds critical specifications to those already described. Until this shift, it was not uncommon for histology laboratories to utilize different fixatives, based on preferences based on cyto- and histo-morphology to shift everything to a single fixative to support the validation of IHC. Phosphate-buffered formalin became essentially universal [2].

Immunohistochemistry also rose to dominance ahead of the other molecular assays, and because of the breadth of specific targets that can be measured, the simplicity of the assay, and the economics of the assay has become a gate-keeper before the application of other molecular assays (including FISH and PCR-based assays, but also sequencing applications and advanced proteomic assays).

7 Steps Forward

With the unmasking of the lack of pre-analytic standardization and a lack of science to support more advanced rigorous specifications, a number of studies have been carried out to better define the impact of pre-analytic variables. Below are some of the advances, however more glaring are the "black spaces" that have yet to be investigated.

8 Quantity of RNA

In a paper published in 2006, Chung was the first to show quantitative data on RNA recovery from tissue, comparing frozen, fixed, and paraffin embedded tissues [9]. This data was a substantial step forward in appreciating how to leverage RNA from FFPE tissue. The authors demonstrated that the fixation process was the period during which RNA "loss" occurred, and that tissue processing had minimal impact on RNA quantity. The authors also showed that ethanol fixation, and its absence of crosslinking retained 70 % of the RNA in tissue, compared to frozen tissue, and formalin fixation resulted in a pool of 30 % of the RNA as measured by quantity per mg of starting tissue.

9 Fixation Time, Buffers, and Processing Time

Carrying this effort forward, the authors evaluated three variables and demonstrated at the RNA level, but by evaluation of total RNA, as well as transcript-specific measurements optimal conditions for the length of formalin fixation, the impact and choice of buffers in formalin, and the impact of time (isolated as a single variable) in the process of impregnation of tissue [2]. This study resulted in a new model of tissue fixation. The authors, using PCR were able to demonstrate that in formalin fixed paraffin embedded tissue, mRNA integrity was preserved mid-gene, with degradation originating from both the 5′ and 3′, while frozen tissue, RNA integrity was relatively flat across the gene, when reverse transcription is mediated by random hexamers. Although not altering the chemical models of fixation, the authors put for a model in which the tissue suffers a terminal hypoxia event, when immersed in fixative, and the artifact embodied by the RNA was a result of the cells response to hypoxia.

10 Storage

In their latest studies, this same group has investigated the stability of formalin fixed, paraffin embedded tissue [7]. Although the degradation of RNA in FFPE blocks, and proteins in cut sections has been well documented, no mechanistic models existed, and all prior efforts were empiric, and of dubious value. The authors demonstrated that degradation was dependent largely on impregnation, rather than fixation, and that residual water, from inadequate dehydration, was responsible for a substantial proportion of the degradation. The authors also demonstrated that this process was temperature dependent.

There remains a long list of variable yet to be tested, including for example reagent quality and substitution. Most concerning, no variable yet examined to date, has not been demonstrated not to be important, and new potential variables continue to come to light.

11 New Models

Based on the examples above, a new model for how tissues cease to be vital emerged. The cessation of cellular activity in frozen tissue is fairly obvious—water freezes and cellular activity comes to a halt, including oxidative phosphorylation and glycolysis. With a shift in temperatures that is sufficiently rapid, the cessation of cellular processes is nearly immediate and the cell has little to no time to activate protective or apoptotic programs. There is a period of hypoxia/ischemia between removal of blood supply and freezing that does result in measurable alterations in biomolecules. In the case of fixation in a liquid the process is vastly different. The hypoxia/ischemia functionally has no end until the penetration of the fixative, which in the instance of larger fragments of tissue is long after metabolic activity has ceased. With the use of liquid fixatives, this is a function of diffusion, and with formalin is estimated at 1 mm/h. Fundamentally, the process is that of drowning, during which oxidative phosphorylation comes to an end quickly, as a result of a lack of oxygen, and then quickly switches to glycolysis, again which is limited by a lack of glucose. The cells activate their survival/apoptotic mechanisms. The model is well supported at the protein level by the alterations in the phosphorylation of protein. The original assumptions of RNA degradation in FFPE tissue were that the damage to the RNA would be “democratic” and random, without respect to location within a mRNA transcript, as would the crosslinking of proteins [4]. Rather, as demonstrated by the RT-PCR data, RNA integrity was greatest mid-transcript and diminished at both the 5′ and 3′′ ends of the mRNA due to the hypoxic related signals to remove the 5′ cap and degrade the 3′ poly-A tail [2, 9]. This model was supported by other independent data, and was critical toward a better understanding of optimal probe design for RNA obtained from FFPE tissue.

12 The Future of Formalin

Clearly diagnostic pathology continues to evolve. Although it is not entirely possible to speculate what the next substantial change in the preservation of tissue will be, the subject engenders the greatest investigation remains the replacement of formalin as the primary fixative of diagnostic pathology. The pressures include the fact that it is a carcinogen, costly to dispose of, and results in complex damage to DNA, RNA, and proteins. Bolstering the continued use of formalin is the substantial fund-of-knowledge, and some diagnosis which are dependent on *formalin artifact*. The concepts of what constitute the specification of formalin have been discussed in the preceding sections. The challenge remains the issues to be defined. Formalin could well live on, applied in a different manner. Alternatively formalin could be replaced with a new solution that offers benefits (less damage to biomolecules) and fewer drawbacks. The path forward will remain chaotic until a new approach arrives via consensus, of which a critical element will be the economic cost of the solution in a total-cost model accounting for reagent cost, disposal cost, staffing cost, and infrastructure demands.

13 Conclusions

The preservation of tissue for diagnostic purposes has followed a "fit-for-purpose" evolution in which changes in process and reagents have been driven by the diagnostic needs. The same basic process is used world-wide, supporting a singularity of diagnostic paradigms. Although the advancement of diagnostic tools and schema is readily apparent to pathologist, the slow evolution of the biospecimens they work with occurs largely unnoticed. The last shift in tissue preservation was the introduction of buffers in formalin, to reduce the presence of formalin pigment, but inadvertently improving the capacity to perform immunohistochemistry on tissue.

With the advancement of molecular biology and the capacity to evaluate DNA, RNA and proteins with greater precision, our understanding of tissue preservation has advanced moving beyond the simple models of chemistry of fixation. These models are driven by the demand that the specimen deliver more in the diagnostic space. Histo- and cyto-morphology, although not replaced, are now the starting point for such ancillary tools as immunohistochemistry, FISH, RNA in situ, and sequencing of RNA and DNA. Formalin fixation remains a subject of much debate —economic, robust, and a link to all pathology supports its continued use. Pressures for a new fixative are driven by formalin's status as a carcinogen, and the chemical reactions that it undergoes with biomolecules, damaging our capacity to measure these biomolecules.

Concurrent with this, are ongoing research to improve tissue impregnation. Numerous alternative tissue impregnation methods have been demonstrated, but none have become dominant. Currently there are a lack of data on the variables associated with impregnation, other than that the removal of water is critical.

Ultimately tissue preservation will continue its constant, albeit slow, evolution, with changes in both fixation and impregnation, to result in a better biospecimen for diagnosis.

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Tissue Heterogeneity as a Pre-analytical Source of Variability

Giorgio Stanta

Contents

A low level of reproducibility is the shortcoming of clinical studies where human tissues are used, especially in oncology [1, 2]. This could be due to the high variability of the pre-analytical conditions of tissue managing and preservation, but also to other two causes. One is the low level of standardization of the methods that is pertinent to the analytical phase. Heterogeneity of tissues is the other problem and it can be considered to be actually related to pre-analytical procedures. Indeed, it is a pre-condition that has to be taken into consideration before the specific analysis. Not all the consequences of heterogeneity can be more or less easily avoided by carefully choosing the tissues to be analyzed. Some types of heterogeneity are strictly related to the complexity of the carcinogenesis phenomena and are not easy to localize with a proper micro-dissection. It is anyway possible to improve reproducibility of tissue molecular analysis by taking into consideration at least some of the aspects of tissue heterogeneity. It is also important to recognize that

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different types of molecular analyses can be differently affected. Gene expression analysis is even more perturbed than DNA sequencing by which only different genetic cell populations can be recognized in the same sample.

1 Different Types of Heterogeneity

Different types of heterogeneity have to be considered when analyzing human cancer tissues for diagnostic or clinical research purposes. We have to consider macroscopic, microscopic, and molecular types of variation. Macroscopic heterogeneity is the clinical variation in patients, such as type of tumor and treatment, or patients' age and gender. This type of clinical heterogeneity is strictly related to the design of the study and, if properly driven, it does not affect the result reproducibility of a study because these characteristics have been chosen and well known from the beginning of the research. In most cases, this clinical variability is the reason why the study was performed and is not considered in this paper.

Other types of heterogeneity are more insidious, like heterogeneity in cancer tissues or the more recently detected molecular heterogeneity related to clonal evolution or autocrine, paracrine cell interaction (Box 1). These types of heterogeneity can heavily affect the results of molecular analyses at the clinical and research level and should be considered as the most important cause for the scarce reproducibility of clinical research. On the other hand tissue related heterogeneity is well recognized but very often under-evaluated as a source of analytical variability, especially in clinical research but sometimes also at the diagnostic level and it sure can be improved. Molecular heterogeneity is more complex and still in a research phase. At the moment we do not have sufficient information to manage the problem properly.

Heterogeneity in cancer tissues is one of the characteristics that suggest a multidisciplinary approach. Clinical heterogeneity must be evaluated mostly by oncologists, tissue related heterogeneity can only be tackled by an experienced pathologist and molecular heterogeneity is still at the center of a research process and must be evaluated by experts in molecular biology, oncology, and pathology.

Box 1: Different Types of Heterogeneity Affecting Diagnosis or Clinical Research

Clinical Heterogeneity related to different patients' conditions (different tumor type, ethnicity, age, therapy, etc.)

Tissue Related Heterogeneity

- Related to tissue complexity (fibrosis, inflammation, necrosis, normal residual tissues…)
- Related to histological heterogeneity (different differentiation pattern of the same tumor)
- Different functional areas (border vs center of the tumor)

Molecular Heterogeneity

- Genetic clonal evolution
- Epigenetic clonal evolution
- Phenotypic plasticity
- Heterotypic interaction

1.1 Tissue-Related Heterogeneity

Tissue-related heterogeneity is a well-known pattern that is always detectable in cancer tissues at a more or less high degree. Sometimes this variability is of very low level and does not affect molecular analysis, but sometimes it can give highly contradictory results depending on the analyzed area. It is possible to recognize at least three types of this kind of heterogeneity. The first one is related to *tissue complexity*. Tumor tissues do not only contain cancer cells but a variable degree of fibrosis that is characteristic of the tumor type as a desmoplastic reaction. It could also be related to the size of the tumor with central necrosis substituted by a dense fibrosis due to insufficient neo-angiogenesis and related hypoxia. In most tumors there are also cells with normal genome, such as reactive inflammatory cells that are part of the carcinogenetic process, or residues of normal tissues involved in the invasion process. It is easy to realize how the presence of one or more of these cancer tissue components, especially if they are quantitatively relevant, can completely alter the results of molecular analysis, giving false positive or negative results. This is usually the only tissue heterogeneity taken today into account during tissue micro-dissection, when histological examination is performed and mechanical or laser dissection is suggested. In many tumors such as breast and colon cancer, it is common to find normal tissue together with the neoplasia in histological specimens and this mixture is often accepted in the sample analyzed for diagnostic molecular signatures such as Mammaprint, Oncotype DX, PAM50. It has been shown that the presence of normal tissue can modify, even if in a limited quantity, the category of risk to a less aggressive than the one detected in the pure breast tumor tissue [3].

A more complicated type of heterogeneity is the one related to *different histological patterns* in the same tumor, which sometimes could also represent different levels of molecular differentiation. At the moment this phenomenon is still badly defined and deserves more attention by pathologists and molecular biologists. For example, we could expect to find differences when analyzing differentiated and anaplastic areas of the same tumor that can often be found in human cancers. We need to better define those characteristics not only in a general way but also for

specific types of tumors, by evaluating the importance of this factor in the reproducibility of molecular analysis in diagnostics and clinical research.

For quite a long time we have been aware that there are *different functional areas* in a tumor such as borders and the center of an invasive neoplasia that display different gene expression patterns [4]. Usually the central part of the tumor is more affected by hypoxia and cellularity is low with a higher fibrotic component, whereas the border can be crowded with cells with activated proteolytic enzymes and a more frequent interaction with reactive cells. The border itself can give better information on the aggressiveness of the tumor. When the tumor is large and the position of the analyzed tissues is unknown, this can heavily affect molecular diagnostics and research analyses.

Of course there are differences in tissue heterogeneity among tumour types and a more complete analysis is necessary to establish specific characteristics. However it is possible to consider some general rules that could help a higher level of standardization like those reported in Box 2.

Very short time between cutting micro-dissected area sections and the extraction of nucleic acids (especially for RNA) should pass, otherwise this could be another pre-analytical source of variability due to possible further degradation of nucleic acids by contamination of environment nucleases.

Box 2: Suggestions for a Practical Approach to Tackle Tissue Heterogeneity

Small biopsies

1. Histological evaluation of tissues
2. When possible micro-dissection (including border of the tumor and avoiding stroma, normal t. residues)
3. Digital record of the selected tissues

Surgical specimens

1. Histological evaluation of the tissues with topographical definition (identification of the infiltrative border)
2. Micro-dissection: single or multiple sampling, depending on the type of lesion and the histological pattern:
   a. in single sample, this should be taken from the infiltrative border, with a minimal stromal component
   b. for multiple sampling the topographical location of the micro-dissected areas should be recorded.
3. Digital record of the micro-dissected areas as integral part of result evaluation (specific IHC could help a morphometric cancer cells versus stroma evaluation)

1.2 Molecular Heterogeneity

Molecular heterogeneity in cancer is related to cancer progression. It is well known that phenomena like microsatellite instability or chromosomal instability occur in many types of tumors. Accumulation of genetic errors of sequence in DNA or gene copy number alterations are always present in cancer. These alterations are not uniform in the tumors and sometimes they are present in localized cell clones. Molecular heterogeneity is not only related to structural damages of DNA. Silencing of gene expression by promoter methylation is also on the basis of tumor progression and these phenomena are also clonal. So we have to consider a genetic and an epigenetic clonal evolution that can affect only part of the tumor and sometimes very few cells.

More unpredictable and difficult to detect is the so called phenotypic plasticity. This is related to autocrine-paracrine phenomena between cancer cells that show the same genotype but with evident different patterns of functional gene expression.

Another type of phenotypic plasticity seems to be stochastic, for which the level of gene expression and the time of expression can vary from cell to cell according to efficiency of the single cell machine.

Of note is also the heterotypic interaction between cancer cell and stromal component cells such as lymphocytes, macrophages, fibroblasts, etc., for which cytokines can stimulate tumor progression. Phenotypic plasticity and heterotopic interaction are strictly related and sometimes undistinguishable mechanisms and they will be discussed together.

2 Genetic Clonal Evolution

Genetic clonal evolution in human tumors is strictly related to genomic instability in cancer. Instability can give different alterations in different cells and the clones with growth advantages tend to expand. These alterations can be common to all the tumor cells or are restricted to same cells only. This process was presented as a Darwinian phylogenetic evolution in cancer [5].

It is well known that there are clonal genotypes with expansion or decline of clonal populations over time. From these clones, rare or frequent in the tumor, the metastatic process can originate. Some of the clones derived from random genetic drift can also show a neutral relationship without discernible phenotypic consequences [6]. There are also tumors that do not present an evident clonal structure based on genome aberrations (Box 3).

Box 3: Molecular Heterogeneity and Genetic Clonal Evolution in Cancer [6]
Existence of different clonal genotypes in the same tumor
Expansion and decline of clonal populations over time
Existence of internal spatial variation in tumor composition
Emergence of drug-resistant malignant cells
Metastatic cells from sub-clones (rare or common)
Absence of clonal structure based on genome aberrations in some cancers
Existence of neutral clonal relationships (from random genetic drift without discernible phenotypic consequences).

One of the most relevant consequence of genetic clonal evolution is the evidence that it is related to acquired resistance to new target biological treatments of cancer. It was shown that after anti-EGFR therapy in metastatic colon cancer the majority of the cases that were KRAS wild turned out to be mutated if detected after some time in circulating tumor cells. On the other hand the cases treated with traditional chemotherapy only continued to be KRAS wild type [7]. This can be related to the expansion of mutated minor clones driven by the specific therapy.

3 Epigenetic Clonal Evolution

For epigenetic clonal evolution in cancer the considerations to be made can be similar to those for genetic clonal evolution. It is well known that silencing of tumor suppressor genes can be due to promoter methylation and this process is common to most of the tumor types. Also some mechanisms are known in tumors that highly increase the frequency of hyper-methylation of gene promoters. The so called CpG island methylator phenotype (CIMP) was first recognized in the hyperplastic polyps of the colon as a new type of carcinogenesis process [8]. Hypermethylation of the CpG-island promoter of tumor-suppressor genes and of miR genes inactivates transcription and the epigenetic changes are inheritable, for this reason they are strictly part of clonal evolution of cancer. It is important anyway to underline that not hypermathylation but hypomethylation of DNA is a landmark of malignant cells and this can reactivate intragenomic endoparasitic DNA repeats (L1 and Alu). These undermethylated transposons can be transcribed or translocated to other genomic regions with the promotion of chromosomal rearrangements.

Also other epigenetic mechanisms like deacetilation or methylation of histones can silence tumor-suppressor genes even without hypermethylation of the promoter CpG islands (Box 4).

Box 4: Molecular Heterogeneity and Epigenetic Clonal Evolution
Epigenetic changes are inheritable and part of clonal evolution
It is possible to modify epigenetic alterations
Hypomethylation of DNA in malignant cells can reactivate intragenomic endoparasitic DNA repeats (L1 and Alu). These undermethylated transposons can be transcribed or translocated to other genomic regions with chromosomal rearrangements.
Hypermethylation of the CpG-island promoter of tumor-suppressor genes and of miR genes inactivates transcription
Deacetilation or methylation modification of histones can silence tumor-suppressor–genes with or without hypermethylation of the promoter CpG island.

4 Phenotypic Plasticity and Heterotypic Interaction

A specific tumor genotype can express a wide range of phenotypic manifestations, called phenotypic plasticity, in response mostly to the surrounding microenvironment stimulations. These are the result of autocrine and paracrine mechanisms related to the same tumor cells or to reactive normal cells. In this latest case it is called heterotypic interaction. In most of the cases the two mechanisms are almost undistinguishable, and very often tumor cell autocrine activity cooperates through the heterotypic interaction with the reactive cells, taking to very complex functional relationships [9, 10]. Similar examples are very well known. Phenotypic plasticity is frequent as an adaptation of the cell to common aspects related to cancer, such as hypoxia, which is related to tumor proliferation and aggressiveness. There is also cooperation between hypoxic cells and non hypoxic ones in the same tumor because the lactate produced by hypoxia can be transformed into pyruvate and utilized by the non hypoxic areas [11, 12]. In breast cancer, hypoxia induces lymphangiogenesis around the tumor tissues, facilitating the lymphatic way of diffusion of the cancer cells [13].

Also “stemness” is related to autocrine and paracrine phenomena [14, 15] and the same epithelial-mesenchymal transition (EMT). These are facts that show how progression of the tumor is related not only to genetic and epigenetic clonal mechanisms but also to different functional phenotypes that can coexist in tumor cells with the same genotype. This is true not only for coding genes but there is increasing and wide evidence also for non-coding RNAs like microRNA expression [16, 17].

There is another type of phenotypic plasticity related to the different efficiency of the cell machines, especially those of transcription and translation that appear to be stochastic and different from cell to cell of the same tumor [18]. It was shown experimentally in cell lines that different cells with the same genotype have different

time for programmed death when the process of apoptosis is stimulated simultaneously [19]. Pathologists have known this phenomenon for a very long time. Indeed, in immunohistochemistry the positivity for any antigen hardly ever appears to be uniform in the same type of cells, but it varies a lot in intensity. This variation is taken as a criterium to evaluate specificity of a new antibody.

An example of cell machine efficiency is the fact that chaperon proteins can modulate the effect of a mutation at the expression level and the result is again referred as stochastic with often a very wide range of overlapping phenotypes between mutated and wild cells [20]. Tyrosine kinase receptors are client of a chaperon protein HSP90 and the efficiency of HSP90 modulates the cellular response of these receptors. This could also be the reason why different tumors or sub-clones of a tumor can react differently to the receptor inhibition in cancer treatment. For this reason inhibition of HSP90 was proposed as a developing cancer treatment [21].

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Standardized Processing of Native Tissue in Breast Pathology

M.E. Dämmrich and H.H. Kreipe

Abstract

In breast surgery, replacement of intraoperative frozen section by core needle and vacuum biopsies hampers collections of unfixed breast specimens. We practice immediate intraoperative macroscopic analysis of resection margins and vacuum-cooling of breast specimens to enable native tissue asservation for assessment of biological markers and tissue banking of tumor tissue. In addition, slicing of native tissue before formalin fixation guarantees a standardized and uniform fixation. Starting in 2013, more than 350 breast specimens were processed as native specimens in the Institute of Pathology of Hannover Medical School. Breast specimens with an invasive carcinoma and request of an intraoperative resection margin assessment were processed with an immediate intraoperative pathological analysis. All other breast specimens without assessment of an intraoperative resection margin were vacuum-fixed processed. In all cases, native tissue for biomarker analyses and tumor banking could be preserved.

Contents

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Table 1 Usefulness of frozen sections in breast pathology

Indications and contraindications for a frozen section in mammapathology		
	Applicable	Non-applicable
Clarification of dignity in preoperative uncertain situations (e.g., multicentricity)	+	
Clarification of representativeness (dense breast parenchyma, dislocation of wire mark)	+	
Histological examination of the retromammillary region	+	
Resection margins to invasive carcinoma	+	
Sentinel lymph node (invasive carcinoma)	+	
Clarification of dignity in core biopsies		+
Clarification of dignity in B3-lesions (core biopsy)		+
Resection margins to DCIS		+
Sentinel lymph node (DCIS) and invasive carcinoma in DCIS		+

In breast surgery intraoperative frozen sections for the clarification of dignity and the diagnosis of malignancy have lost impact because of usually preceded preoperative core needle and vacuum biopsies. Particularly, advantageous of preoperative biopsies is an optimized strategy for surgical intervention and an individualized neoadjuvant therapy planning, but less significance of frozen sections hampers in turn the availability of native tissue for biomarker analysis and tumor banking.

Indications for native tissue specimen handling (including frozen sections) and the possibility of fresh tissue banking (Table 1) are given for the histological investigation of sentinel lymph nodes (invasive carcinoma, not DCIS), the clarification of dignity in preoperative uncertain situations, the assessment of representativeness, the histological investigation of the retromammillary region, and the investigation of the surgical resection margins (Fig. 1).

1 Investigation of Sentinel Lymph Node

Intraoperative investigation of sentinel lymph node has the advantage to circumvent a secondary surgery. In cases with histological sentinel lymph node metastases, a simultaneous axillary dissection can be performed by the surgeon.

According to the literature, different analyses assigned frozen sections of sentinel lymph nodes a false negative rate of results [1–5]. Based on reduced specificity and sensitivity as well as limited possibility to supplementary immunohistochemical analysis, the S3-guidelines do not recommend a layered cut of sentinel lymph nodes in frozen sections [6, 7]. In contrast, a combined procedure of alternating frozen section and paraffin reprocessing guarantees a high specificity of nodal status assignment. Referring to this, a sentinel lymph node is laminated in slices of 0.2 cm vertical to its longitudinal axis and alternating slices are either paraffin embedded or frozen sectioned. From each slice at least 8 layered cut were produced (Fig. 2). In a

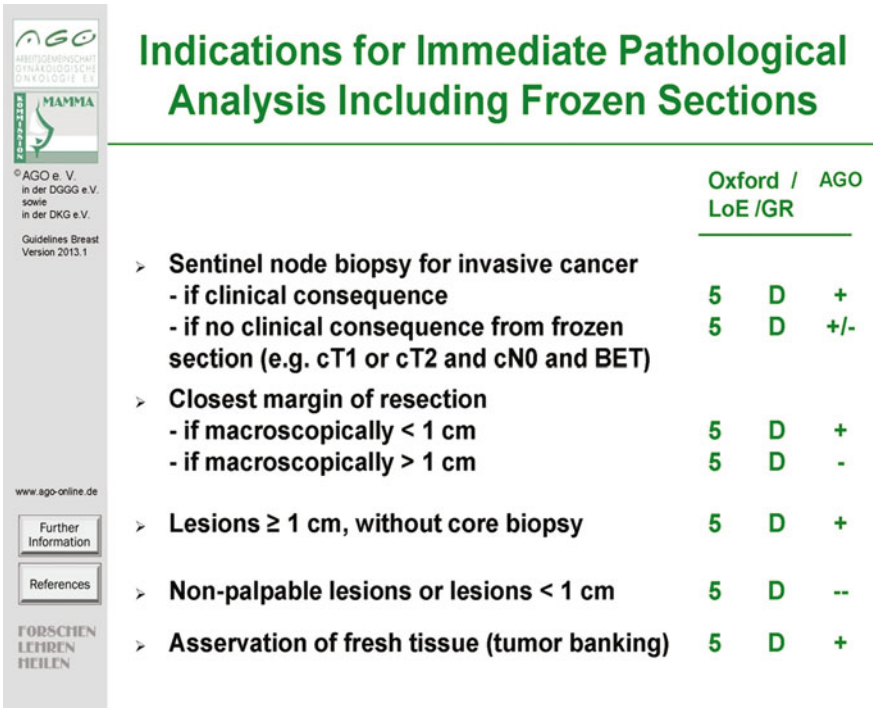

Fig. 1 Indications for immediate pathological analysis including frozen sections. Evidence-based recommendation of the Arbeitsgemeinschaft Gynäko-logische Onkologie (*AGO*), differentiating between Oxford criteria "Level of Evidence" (*LoE/GR*) and AGO criteria "Grade of Recommendation" (++, +, +/-, −, and −−)

series of 229 sentinel lymph nodes, a concordance in the technique of alternating slices of 97.9 % could be realized, in contrast to the so called OSNA-technique with a concordance of 90 % between lysate and frozen section as well as lysate- and paraffin-embedded tissue.

2 Investigation of Breast Specimen

For prognostic molecular biologic analysis like ELISA-produced proof of uPA and PAI-I as well as for innovative diagnostic testing, asservation of unfixed native tumor tissue is recommended [8]. According to interdisciplinary guidelines, asservation of native tissue is restricted to institutions of pathology to warrant a standardized investigation of the tumor (Fig. 3).

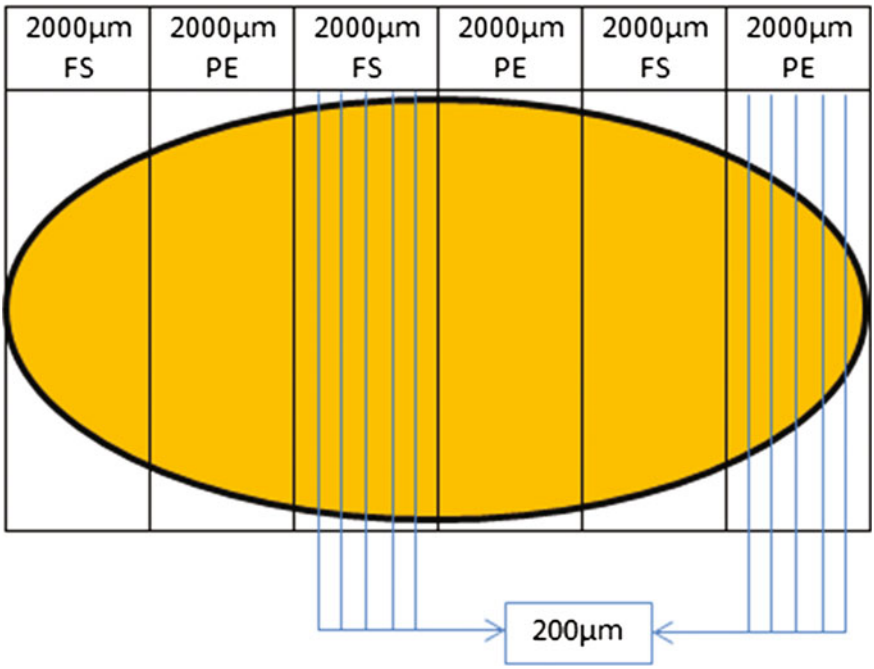

Fig. 2 Standardized investigation of sentinel lymph node. *Vertical* to the *longitudinal axis* of a lymph node alternating slices of frozen sectioned (*FS*) or paraffin-embedded (*PE*) tissue are sectioned, each of them measuring 2,000 µm. Of each slice 8–10 layer cuts are produced, each of them measuring 200 µm

3 Investigation of Native Breast Specimen—Immediate Pathological Analysis

According to the aforementioned Oxford criteria and guidelines of the Arbeitsgemeinschaft gynäkologische Onkologie (AGO)—an indication for immediate pathological analysis (including frozen sections) is given in cases with a lesion taller than 1 cm without a core biopsy, to clarify dignity in preoperative uncertain situations, the assessment of representativeness, the histological investigation of the retromammillary region, and the investigation of the surgical resection margins (Fig. 1 and Table 1).

Particularly, the investigation of resection margins by immediate pathological analysis helps to circumvent secondary surgery—which is declared with 15–50 % [9, 10]. Also therapeutic options like immediate reconstruction or intraoperative radiotherapy (IORT) depend on reliable information of surgical resection margins [11, 12] and especially IORT needs—according to the Targit study [9, 13]—a residual tumor-free resection in a one-time intervention.

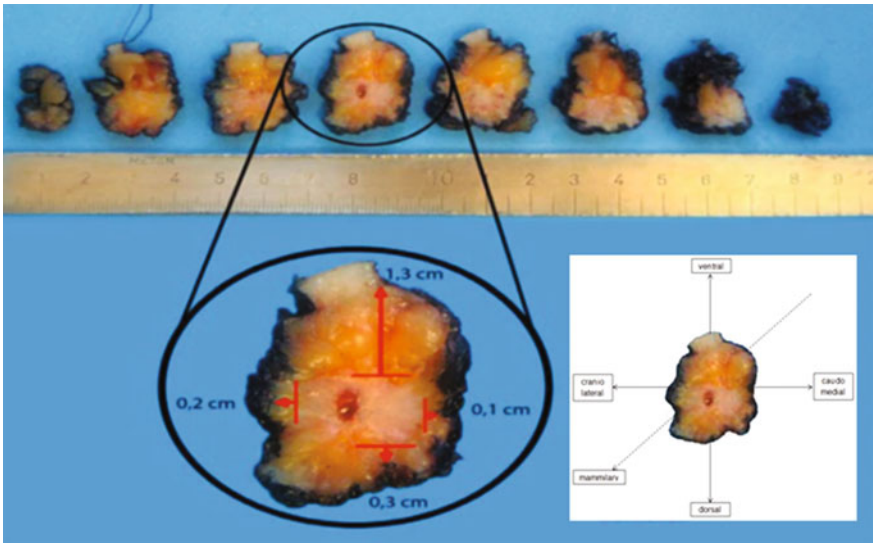

Fig. 3 Standardized investigation of breast specimen. A breast specimen with an invasive carcinoma is vacuum-fixed in the operation room and sent within 5–10 min to pathology. Surgeons receive immediate information by telephone about width of resection margins and tumor diameter. In case of narrow margins, surgeons can either demand histological verification by frozen section or they opt for further tissue removal from the resection cavity. Native breast specimens are inked and sectioned *vertical* to their *longitudinal axis* in slices from mammillary-far to mammillary-near and orientated *ventral* (12.00), *caudomedial* (3.00), *dorsal* (6.00) and *craniolateral* (9.00). *Thickness* of each slice is 0.3–0.5 cm. In the depicted example, the specimen is sectioned into eight slices. The tumor can be seen in the slices 3–6 with a maximal diameter of 1.6 cm and a minimal distance to the resection margins of 1.3 cm (*ventral* (12.00)), 0.1 cm (*caudomedial* (3.00)), 0.3 cm (*dorsal* (6.00)), 0.2 cm (*craniolateral* (9.00)), and 0.8 cm (mammillary-far and -near)

Different methods are exercised in immediate pathological analysis [9, 10, 12, 14–19]. In our experience a timesaving macroscopic supported (Fig. 3) safety distance of 1 cm could reduce the rate of secondary surgery from 21.4 to 9.1 % with a negative predictive value of 97 % [11, 20].

4 Investigation of Native Breast Specimen—Vacuum-Fixed Instead of Formalin-Fixed Tissue

According to the aforementioned Oxford criteria and guidelines of the Arbeitsgemeinschaft gynäkologische Onkologie (AGO)—no indication for immediate pathological analysis is given in cases with a non-palpable or less than 1 cm measured lesion, a clarification of dignity in corebiopsies and B3 lesions as well as an investigation of resection margins in DCIS (Fig. 1 and Table 1). Moreover, a

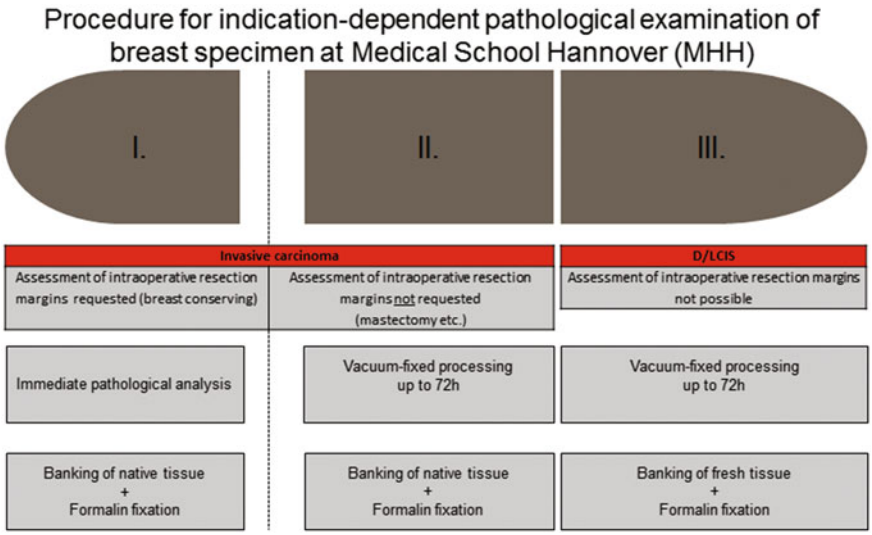

Fig. 4 Indication-dependent pathological investigation algorithm of breast specimen. Since 2013 the Medical School Hannover (*MHH*) examines breast specimen indication-dependent according to the current guidelines. Referring to this, it has to be differentiated between breast specimens with a premalignant lesion (*D/LCIS*) and an invasive carcinoma. Breast specimens with a premalignant lesion are generally vacuum-fixed processed, assessment of intraoperative resection margins is not possible. In breast specimen with an invasive carcinoma the assessment-request of intraoperative resection margins is trend-setting. If the surgeon needs an investigation of resection margins width, an immediate pathological analysis is performed. If no assessment of intraoperative resection margins is requested, a vacuum-fixed processing analogous to Fig. 5 is performed. In all cases, native tissue for biomarker analyses and tumor banking can be preserved

recently published study showed a guideline controversy surgeon-dependent variety of practice patterns for handling surgical margins in breast conservation treatment [21]. In all these cases, formalin-fixed specimen have replaced native tissue specimen investigation and thus fresh tissue asservation was hampered in the last years.

In order to warrant fresh tissue asservation for biomarker analysis and tumor banking of all mammary resection specimens, the Medical School Hannover (MHH) uses in addition to immediate pathological investigation the possibility of vacuum-fixed processing of every native breast specimen for an indication-dependent pathological investigation algorithm (Fig. 4).

Every breast specimen of D/LCIS and invasive carcinoma without immediate intraoperative assessment of resection margins is vacuum-fixed processed to warrant the possibility of fresh tissue asservation. Hence vacuum-fixation guarantees also asservation of native tissue in mastectomy specimen as well as in specimen with an invasive cancer of less than 1 cm, specimen with a nonpalpable lesion, specimen with a B3-lesion, and every breast specimen with a premalignant lesion (D/LCIS) (Fig. 1 and Table 1). Furthermore, vacuum-fixation of specimen warrants availability of native tissue for tumor banking and measurement of biomarkers

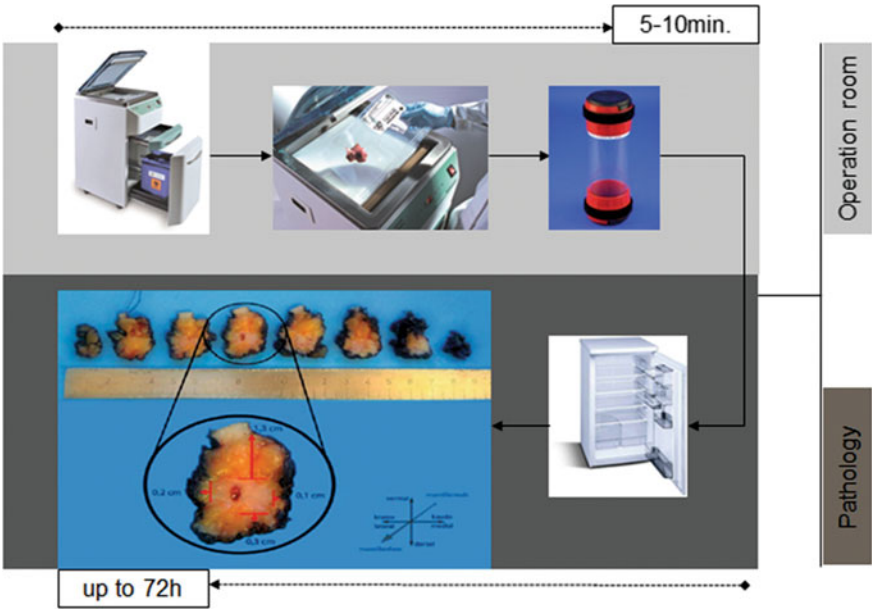

Fig. 5 Investigation of native breast specimen–vacuum-fixed tissue. The processing shown in Fig. 3 can be delayed up to 72 h by vacuum fixation and refrigerated asservation (4 °C) of the specimen in case that immediate intraoperative evaluation of resection margins did not take place

without pressure of time. According to preceded testing, vacuum-fixation with refrigerator asservation of the specimen offers a time-frame of 72 h—in our institution, the specimens are normally processed within the next 20 h.

Since January 2013, all breast specimens without indication of immediate pathological analysis are handled with the vacuum-fixation technique in the MHH —including meanwhile more than 350 specimens of tumor and premalignant lesions. Advantageous for vacuum-fixed specimen is an unfixed "natural" consistence of the tissue for investigation, cutting and teaching—also after a period of more than 1 day post-surgery. Especially, in larger resection specimens like mastectomies, with a diameter of more than 5 cm, the loss of degradation and formalin-associated fixation artifacts is notable. Sole deformation of tissue through vacuum-fixation aggravates in some cases the orientation of an unmarked tissue with complicated conclusion to its physiological diameter. In such cases, a prevacuum-fixed marking could be helpful, especially in cases with macroscopic difficult to uncircumscribable breast lesions like in D/LCIS. According to immediate pathological analysis, a slice thickness of less than 0.5 cm can be assured in practiced hand—rather a better handling of the lipomatous tissue can be observed through the refrigerated specimen. In accordance with previously published results [13, 15, 22], no significant differences between formalin and vacuum-fixation technique could be detected in indication-dependent immunohistochemical or molecular-pathological

reprocessing. Furthermore, the workplace exposure to formalin is eliminated and formalin-associated artifacts of specimen are reduced with the possibility of a standardized formalin fixation after grossing.

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Short Formalin Fixation and Rapid Microwave Processing Do Not Affect HER2 Testing

Isabella Sassi, Francesca Invernizzi and Claudio Doglioni

Abstract

Correct and consistent results in estrogen and progesterone receptors, HER2 and Ki67 proliferation rate testing are a basic prerequisite for selecting therapy and individualizing prognosis in patients with breast carcinoma. Preanalytic factors, including time from excision to fixation and time and type of fixation, are critical to obtain reproducible and reliable results in these immunohistochemical assays and their relevance has long been stressed. The ASCO-CAP guidelines on HER2 testing indicated that histologic material including both biopsies and surgical specimens must be fixed for at least 6 h in order to obtain reliable results; however, there is a very limited scientific support regarding the setting at 6 h the minimum fixation time. We demonstrate that with a short fixation time (30′) and rapid processing with MW technology (69′), it is possible to achieve an adequate and reproducible assessment of HER2 status. We obtained similar results in HER2 evaluation in breast carcinoma biopsies treated with this short protocol and in the corresponding surgical specimens processed routinely with a 24 h formalin fixation time—i.e., within the guidelines interval time.

Contents

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1 Introduction

The evaluation of prognostic and predictive factors is of paramount relevance in selecting therapy and individualizing prognosis in patients with breast carcinoma. Correct and consistent results in estrogen and progesterone receptors, HER2 and Ki67 proliferation rate testing are a basic prerequisite for a proper clinical and therapeutic decision [1].

Preanalytic factors, including time from excision to fixation and time and type of fixation, are critical to obtain reproducible and reliable results in these immunohistochemical assays. The relevance of preanalytical variables has long been stressed, with particular reference to limit cold ischemia time and correct formalin fixation time. Tissue fixation is an important process in which type and time of fixation play a critical role in ensuring accurate results. Improper fixation has been related to many false-negative results in breast cancer hormone receptor tests and to lack of reproducibility of HER2 testing in clinical trials [2]. Several studies highlighted the negative effects of delays in fixation (cold ischemia time) and prolonged fixation times on the adequacy of prognostic and predictive breast carcinoma markers analysis [3–10]. Less investigated is the minimum fixation time necessary for reliable results with only few papers addressing this point [11–15].

At our Institution, we have been working in setting up a same day diagnosis service for women suspected of breast cancer. There are several potential advantages for patients with a clinical and/or imaging suspect of breast cancer to have a rapid diagnosis: limiting stressful days of waiting for biopsy results and more importantly, providing with immediate supportive and comprehensive counseling on the spectrum of available treatments, including neoadjuvant chemotherapy and conservative surgery. However, breast carcinoma histologic diagnosis based on biopsy is not enough for a complete and accurate characterization of this complex disease; in fact, for a proper patient management the most relevant biologic factors determining the therapeutic selection, including ER and PR and HER2 and Ki67 proliferation index, must be integrated with the histology report. The first ASCO-CAP guidelines on HER2 testing [16] indicated that histologic material including both biopsies and surgical specimens must be fixed for at least 6 h; the updated version of these guidelines [17] maintains this minimum fixation time, with 72 h as

the upper limit for an adequate fixation interval. Nevertheless, there is a very limited scientific support regarding the setting at 6 h the minimum fixation time.

Following these guidelines render practically impossible to complete the diagnostic and prognostic-predictive work up of a breast needle biopsy in the same day. Therefore, we performed a pilot study to verify if a short MicroWave (MW) fixation time followed by rapid MW processing could permit a correct assessment of these relevant markers in breast biopsies.

2 Materials

We evaluated different formalin fixation times in cell lines with known ER, PR, and HER2 status and then processed with a rapid MW protocol; the same MW protocol was applied to bioptic fragments obtained from a consecutive series of surgically treated breast carcinoma specimens, after a short formalin fixation.

3 Cell Lines

MCF7, T47D, and SKBR3 cell lines were grown at confluence, detached, collected, and fixed with different interval times 30′–1–6–24 h in 10 % neutral buffered formalin and then processed as cytoblocks with a short MW protocol (1 h; see Table 1) with the Logos MW processor (Milestone Serisole Italy) or with a routine protocol (9 h).

Table 1 Microwave protocol for rapid needle biopsies (*NBF* Neutral Buffered Formalin)

Rapid MW protocol logos™ (1 h 39 min)				
Reagent	Time	Phase	T °C	Pressure (mBar)
Formalin 10 % NBF	25′	1 = 10′	50°	
		2 = 15′		
Ethanol 60°	2′			
Ethanol 99°	3′			
Ethanol 99°	16′	1 = 8′	65°	
		2 = 8′		
Isopropyl alcohol	16′	1 = 8′	68°	
		2 = 8′		
Vaporization	1′ 30″			600
Paraffin	35′ 30″	1 = 30″	66°	995
		2 = 15′	66°	400
		3 = 15′	70°	300
		4 = 5′	65°	200
	Total: 1 h 39′			

4 Tumor Specimens

40 consecutive cases of T1c-T2 surgically treated breast carcinomas (age range 41–67; 39 F,1 M) were evaluated. The fresh resected specimens were received from the operating room (cold ischemia time 10–15 min) and immediately dissected by a pathologist. This series included 34 invasive ductal/NST carcinomas and 4 lobular, 1 tubular, and 1 mucinous carcinomas. All of them were primary tumors, without any previous treatment. At our institution, most patients with either triple negative or HER2 positive tumor are treated with neoadjuvant therapies; therefore, these types of tumors are underrepresented in this series. Two or more fragments no more thicker than 1 mm were obtained from the tumor mass and immediately processed with the protocol outlined in Table 1 in a Logos MW processor (Milestone Serisole Italy). This protocol includes 25′ fixation time in NBF reaching a 50 °C plateau after 10′ and is followed by a 60′ cycle with dehydration in ethanol, clearing with isopropyl alcohol and embedding in paraffin. The main part of the tumor was left in fixative for 24 h and processed the following day with a routine 9 h protocol in a Leica ASP300 instrument. Written informed consent for the procedure was formally obtained from all the patients.

5 Immunostaining

HER2 evaluation was performed on 3 micron sections from cell lines paraffin blocks, bioptic fragments, and routine breast specimens with manual Herceptest™ kit K5204 (Dako) and with HER2 Pathway™ on a Benchmark Ventana Ultra autostainer. In each case, rapid biopsy and routine block sections were immunostained in the same run and with the same standard protocol (HIER, primary ab, detection system). All the samples were also immunostained for ER (clone 6F11, dil 1/200 Novocastra), PR (clone 312, dil Novocastra), and Ki67 (clone MIB1, dil 1/600, Dako) with Refine (Novocastra) as DAB-based polymer detection system in a Bond III automated immunostainer (Leica-Microsystems);

6 FISH

HER2 FISH analysis was performed in eight cases defined as score 2 plus by immunohistochemistry, in both biopsy and surgical sample, with the PathVysion™ HER2 DNA Probe Kit (Abbott Molecular) according to the manufacturer's instructions. Slides were analyzed using NIKON 90i fluorescence microscope with both a single pass (green and orange) and a triple-pass filter band (DAPI/green/orange); images were captured by Genikon software (Nikon). A total of 100 neoplastic nuclei were observed per each sample.

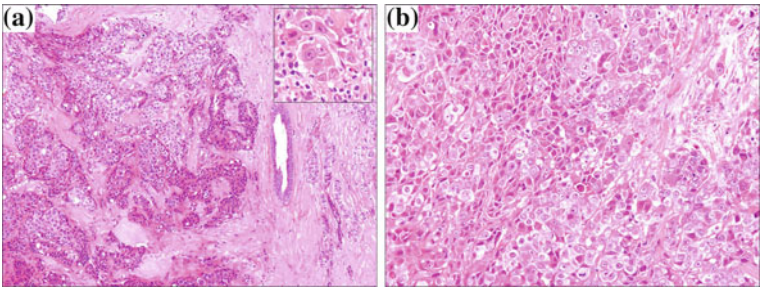


Fig. 1 H&E staining of breast carcinoma rapid biopsy sections demonstrating good morphological quality (**a** original magnification 100x; insert 400x; **b** original magnification 200x)

7 Slides Evaluation

Immunostained slides were evaluated independently by two pathologists (C.D. and I.S) with experience in breast pathology, without knowledge of the pairing (biopsy vs surgical) of the samples. The updated ASCO-CAP guidelines were utilized for HER2 scoring.

8 Results

All the shortly fixed and MW-processed biopsies showed a good morphology on H&E stained sections, with excellent nuclear details and without any artifact (Fig. 1a, b).

9 Cell Lines

Intense and complete membranous staining for HER2 was detected in the majority of SKBR3 cells, whereas weak, focal, and incomplete staining was present in a limited percentage of MCF7 and T47D cells, irrespective of the fixation time (Fig. 2). In MCF7 and T47D cell lines, a similar percentage of cells with the same intensity, stained for estrogen and progesterone receptors with all the fixation times (30′, 1 h, 6 h, 24 h); SKBR3 cell line, as expected, was negative for ER and PR. A very similar percentage of cells was immunostained at all fixation times with Ki67 in each of the three cell lines.

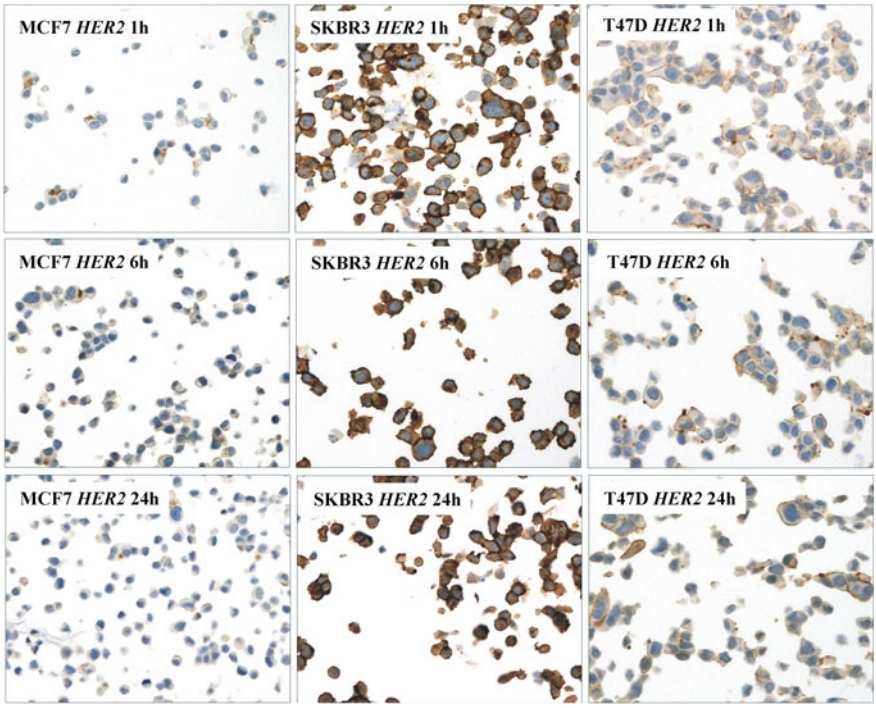


Fig. 2 Breast carcinoma cell lines (MCF7, SKBR3, and T47D) immunostained with HER2—Herceptest™—after different fixation times (1, 6, 24 h) and rapid MW processing, displaying similar staining intensity (original magnification 200x)

10 Immunohistochemical Results in Rapid Biopsies and Surgical Specimens

The quality of immunohistochemical staining was equivalent in biopsies and paired surgical samples (Fig. 3a–f). No differences were detected in HER2 score between rapid and routine samples, utilizing the Herceptest™ and the HER2 Pathway™ kit. (Table 2) with 3 minor exception: 1 case 1+ in biopsy and 2+ in surgical sample (not amplified by FISH), 1 case 2+ in biopsy and 1+ in surgical sample (not amplified by FISH), and the third case score 0 on biopsy and score 1+ in surgical sample. Some minor scoring differences were observed in some cases, in paired biopsy and surgical sample, also with Herceptest™ versus HER2 Pathway™ kit. Four cases scored 1 plus with the Herceptest™, but had a score 0 with HER2 Pathway™ kit. Normal breast tissue present in biopsies was always negative with HER2 Pathway™ kit; incomplete faint membrane staining of normal tissue was observed in some of the breast biopsies with the Herceptest™. In these cases, a similar staining intensity was present also in the paired surgical sample. For estrogen and progesterone receptors, no statistically significant differences in the

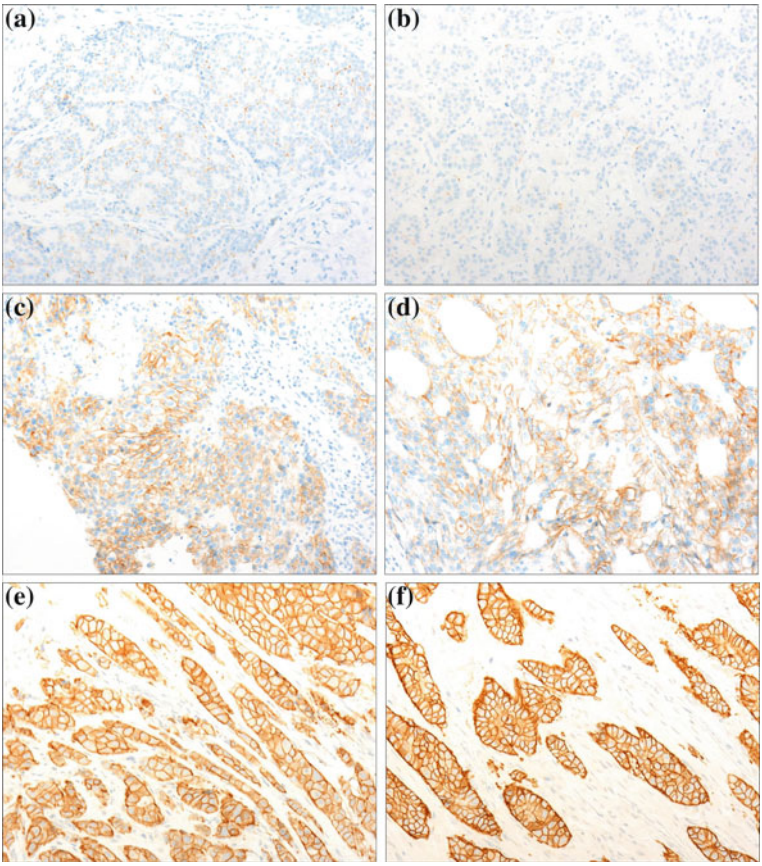


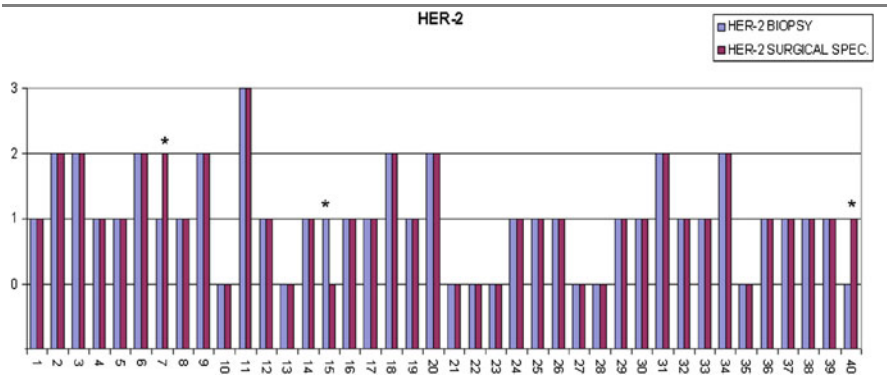
Fig. 3 Similar scoring results for HER2—Herceptest™—in three paired samples: short MW formalin fixation and MW-processed biopsy (**a**–**c**–**e**) and corresponding routinely processed surgical sample (**b**–**d**–**f**): **a**–**b** score 0, **c**–**d** score 2+ (FISH amplified case); **e**–**f** score 3+ (original magnification 200x)

percentage of stained cells and in the intensity of the reaction was observed in rapid biopsies compared with the routine surgical specimen, both by visual evaluation and by Aperio Digital Image software analysis, with no more than 1 point difference in the Allred score in paired samples (data not shown).

11 FISH

The same FISH results were obtained in both rapid biopsies and surgical specimens, without any significant modification of the protocol, with the exception of a slight reduction in digestion times for rapid biopsies slides. Two out of the eight 2+ IHC scoring cases displayed *HER2* gene amplification.

Table 2 HER2 evaluation in paired biopsy and surgical specimen



Discordant cases are noted with *asterisk*

12 Discussion

Short fixation time and rapid processing with MW technology permit an adequate and reproducible assessment of the relevant biologic factors affecting therapeutic decisions in breast carcinoma. We obtained overlapping results in HER2 evaluation in breast carcinoma biopsies treated with this short protocol and in the corresponding surgical specimens processed routinely with a 24 h formalin fixation time —i.e., within the guidelines interval time [17]. We obtained the same results in HER2 testing with the two most commonly utilized kits, Herceptest™ and Pathway HER2™. The evaluation of estrogen and progesterone receptors was also similar both biopsies and surgical samples not only by visual analysis but also by image analysis.

Preanalytical variables are important for obtaining the most reliable results. Many papers analyzed the preanalytical factors influencing breast markers testing: cold ischemia time and prolonged formalin fixation were the most intensely investigated topics, reaching an almost unanimous conclusion that a long ischemia time—longer than 2–4 h and prolonged formalin fixation are deleterious for ER and HER2 testing [3–10]. On the contrary, very limited data are available if and how, short fixation times and rapid MW processing affects breast markers evaluation [12–14].

A threshold of 6 h fixation is considered the gold standard in all guidelines for ER and HER2 testing; probably the paper of Goldstein et al. [11] must be credited for establishing this limit as the minimum fixation time. In this study, it was suggested that the minimum formalin fixation time for reliable immunohistochemical ER results is 6–8 h; however, the protocol utilized introduced relevant variations into routine fixation procedure: after different formalin fixation times (3–6–8–10–12–24–48 h and 7 days), the specimens were placed in cold absolute ethanol (time not specified) and after that, further fixed for 2 h in 20 % formalin at

40 °C before dehydration. The results of this analysis, limited only to the evaluation of estrogen receptor, demonstrated suboptimal results with a 3 h fixation. The 2007 ASCO-CAP guidelines [16] were the first to officially set this threshold; however, also in this publication there were some discrepancies regarding this aspect. In Table 2, it was reported that needle biopsies fixed less than 1 h are not suitable for HER2 testing, whereas in appendix E, under the heading of Tissue Handling, there was a statement that "fixation time for needle biopsies have not been addressed." These conflicting indications were eliminated in the most recent ASCO-CAP guidelines [17] where the 6 h threshold was stated as the minimum fixation time for any breast carcinoma specimen, without any further comment and no more scientific support. Furthermore, although rapid MW processing is routinely utilized in several laboratories, its effects on the immunohistochemical evaluation of these markers have been the subject of very few studies [13, 14], and none of them evaluated HER2. In our study, we demonstrated that short formalin fixation and rapid MW processing permit an accurate assessment of HER2 expression as well as of all the other markers at present necessary for breast cancer treatment selection. The rapid MW-assisted procedure allows an adequate and complete evaluation of prognostic–predictive markers permitting to complete biopsy, histologic diagnosis, prognostic and predictive markers evaluation diagnosis, and oncological counseling in the same day. The availability of results from diagnostic biopsies in short and predefined times may display additional clinical values in terms of patient anxiety reduction, opportunity for automatic planning of further diagnostic procedures, quick access to the fit oncological treatment and logistical aspect solution (i.e., Friday's biopsy).

The 6 h interval could therefore be not a waiting time, but an active and complete reporting time.

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Current Projects in Pre-analytics: Where to Go?

Anna Sapino, Laura Annaratone and Caterina Marchiò

Abstract

The current clinical practice of tissue handling and sample preparation is multifaceted and lacks strict standardisation: this scenario leads to significant variability in the quality of clinical samples. Poor tissue preservation has a detrimental effect thus leading to morphological artefacts, hampering the reproducibility of immunocytochemical and molecular diagnostic results (protein expression, DNA gene mutations, RNA gene expression) and affecting the research outcomes with irreproducible gene expression and post-transcriptional data. Altogether, this limits the opportunity to share and pool national databases into European common databases. At the European level, standardization of pre-analytical steps is just at the beginning and issues regarding biospecimen collection and management are still debated. A joint (public–private) project entitled on standardization of tissue handling in pre-analytical procedures has been recently funded in Italy with the aim of proposing novel approaches to the neglected issue of pre-analytical procedures. In this chapter, we will show how investing in pre-analytics may impact both public health problems and practical innovation in solid tumour processing.

Keywords

Standardisation · Pre-analytic · Innovation · Fixation · Ischaemia · Funding opportunities

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Contents

1 Why New Projects in Pre-analytical Phases of Surgical Pathology

The current clinical practice of tissue handling and sample preparation is multifaceted and lacks strict standardisation: this scenario leads to significant variability in the quality of clinical samples. Technological innovations that pursue standardisation of pre-analytical processes in surgical pathology represent fundamental tools to assure correct diagnosis, which in turn will enhance treatment decisions. Standardisation of bioptic and surgical specimens is not only a need of patients but also of researchers. The detrimental effect of poor tissue preservation (i) leads to morphological artefacts; (ii) hampers the reproducibility of immunocytochemical and molecular diagnostic results (protein expression, DNA gene mutations, RNA gene expression) and (iii) affects the research outcomes with irreproducible gene expression and post-transcriptional data, thus limiting the opportunity to share and pool national databases into European common databases.

Moreover, fostering an upgrade in surgical pathology is not easier than in other healthcare fields, due to the escalating costs, long product development cycles and protracted regulatory approval. It is well known that the difficulties in economics in Europe involve also the healthcare public system thus inhibiting the innovation process. Finally, pathologists are not keen to change their habits. Procedures for preservation and processing of human tissue obtained from biopsies and surgical specimens date back to Rudolf Virchow in the nineteenth century, anachronistically known as the 'father of modern pathology'.

On one hand, pathologists should start thinking about the possibilities to apply for funding in order to create a network open to different stakeholders. On the other hand, institutions should be better informed of our work and thus understand that our everyday diagnosis may be not scientifically sexy, but needs to be as precise, reliable and up to date as possible for the patient care.

A recent 'viewpoint' article [1] calls on pathologists to consider that as genomic testing becomes part of routine care and patients become increasingly informed, the workflow of pathology lab will have to adapt to meet the demands of the 'next generation of patients'.

Pathologists are also expected to facilitate procurement, preservation and distribution of tissues to qualified biomedical researchers. Dr. Compton, Director of the National Cancer Inst. Office of Biorepositories and Biospecimen Research, said that billions of dollars have been wasted in the past because researchers developing biomarkers supposed to be predictive of cancer and response to therapies relied on tissue samples that were utterly useless; tissues had been subjected to careless handling, in addition storage and sampling procedures were missing, so that results were not reproducible.

Changing practices within both the pathology and oncology communities is not a trivial task but it is bound to happen, since the field has no better choice.

2 Examples of Funding Opportunities and Projects Financed in Pre-analytics

In the USA, the Cancer Diagnosis Program of the National Cancer Institute (NCI) has recently invited applications for cooperative agreement awards for the NCI-supported Collaborative (currently "Cooperative") Human Tissue Network (CHTN) (http://www.chtn.nci.nih.gov/).

The goal for CHTN is to collect and distribute high-quality human tissue specimens to facilitate basic and early translational cancer research. The CHTN is designed as a unique biospecimen resource as it is based on prospective collection and distribution of samples upon specific investigators' requests.

This Funding Opportunity Announcement (FOA—Funding Opportunity: RFA-CA-13-007) solicits applications for CHTN awards from institutions/teams capable of contributing to the mission of CHTN by: (1) providing prospective investigator-defined procurement of high-quality malignant, benign, diseased and uninvolved (normal adjacent) tissues and fluids from patients throughout North America and elsewhere; (2) assisting individual investigators with regard to specific specimen needs of their research; (3) assisting in developing and disseminating knowledge on high-quality practices for successfully operating a biospecimen repository and (4) educating the community about the importance of the availability of high-quality human tissue specimens for medical research.

At the European level, standardisation of pre-analytical steps is just at the beginning and issues regarding bio-specimen collection and management are still debated.

Of note, in the last 7th Framework Programme (7FP) the EU funded some programs that pertain to pre-analytic issues and biobanking. As an example, SPIDIA (http://www.spidia.eu/) is a 4.5 year project, funded by the European Union FP7 programme to the value of 9 million Euros, which brings together a consortium of 16 leading academic institutions, international organisations and life sciences companies. The project is coordinated by QIAGEN-GmbH. It aims at tackling the standardisation and improvement of pre-analytical procedures for in vitro diagnostics. The proposed research and standardisation activities cover all steps from

creation of evidence-based guidelines to creation of tools for the pre-analytical phase to testing and optimisation of these tools through the development of novel assays and biomarkers.

3 Current Projects and Goals in Italy

In 2012, the Ministry of Health in Italy co-funded together with Milestone srl (Bergamo, Italy—http://www.milestonemedsrl.com/histopathology/index.php) a joint (public–private) project entitled "Standardizing tissue handling in pre-analytical procedures: technological, environmentally-safe innovation in solid tumour processing" (Project Code: RF-2010-2310674). The project proposes novel approaches to the neglected issue of tissue handling in pre-analytical procedures, taking into account both public health problems and practical innovation in solid tumour processing.

The aims of the project were:

1. *To standardize and validate the use of Under Vacuum Sealing and Cooling (UVSC) procedure for handling and preserving fresh surgical specimens before fixation.*
   To overcome the concern related to the use of formalin in the surgery room we have proposed the use of an innovative vacuum biospecimen transfer system (tissueSAFE-Milestone, Italy) [2]. Our aim was to restrict the use of formalin only in pathology labs, where this toxic agent is carefully handled with hoods and gloves in safe environmental conditions. Tissue transfer in UVSC conditions meets the requests of both health authorities and workers by reducing the exposure to formaldehyde; furthermore, the cost related to the UVSC are lower than those related to the traditional transport in formalin immersion.
   Standardising the temperature of transport and storage is another important issue. In transplantation pathology it has been shown that a rapid induction of hypothermia at 0–4 °C by perfusion of organ with specific solutions better preserves organ viability [3, 4]. It seems that it is the prompt cooling that principally influences preservation. For instance, Kristensen and colleagues [5] have shown that storage at 4 °C preserved tissues to a higher degree than storage at room temperature, independently of whether the tissue was subjected to vacuum sealing or not. We have proposed the transfer of surgical specimens vacuum-sealed using a chilled plastic box at 4 °C [6]. Our results suggest the avoidance of insulating air around tissues with the UVSC system allows faster cooling at 4 °C. We are working on the rapid reduction of temperature to preserve as much as possible the tissue viability, and the integrity of phosphorylated proteins and of nucleic acids.
   The other problem is to monitor the cold ischemia time (which corresponds to the UVSC time) that starts when the specimen is excised and ends with

placement in a suitable tissue fixative. When dealing with cell cultures and xenograft implantations researchers may ideally wish to collect the sample for experiments directly in the surgery room in order to keep the cold ischemia time as short as possible. However, any such sampling may lead to problems for pathologists in terms of correct gross evaluation of tumour samples (status of surgical margins, staging etc.). UVSC may therefore represent a valuable strategy to allow tissue sampling for research purposes and proper preservation of tissue specimens for gross evaluation. However, the results of our project show that although UVSC maintains cell viability even after 70 h, the percentage of cell death increases for longer UVSC times [7].

2. *To standardize and validate the use of cold-fixation of tissues for histological diagnosis and long-term preservation of nucleic acids and proteins after paraffin embedding.*
   Preliminary data using cold-formalin fixation show that this procedure preserves RNA segments up to 660 bp and that both morphological and immunophenotypical features are fully comparable with those obtained with standard fixation [8]. Gene expression profiling analysis showed that RNAs from cold-fixed samples are significantly less fragmented than those from standard Formalin-Fixed Paraffin-Embedded (FFPE) samples: their performance was comparable to that shown by fresh-frozen samples, which are, at present, the sole specimens considered suitable for microarray gene expression analysis.
   For the project purposes, the cold-FFPE samples were re-profiled after 24 months to assess the stability of morphology, antigens and nucleic acids. The preliminary results confirmed that cold fixation is a robust procedure to maintain the integrity of these molecules.

4 Conclusion

Pathologists have to move a step forward to face innovation of their procedures taking into account the ethical, legal and social issues. In addition, we need both the support of companies/SMEs for the development of new technologies for tissue preservation and of government to facilitate professionals in rapidly translating the results of new projects into practice.

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Preanalytics in Lung Cancer

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Abstract

Preanalytic sampling techniques and preparation of tissue specimens strongly influence analytical results in lung tissue diagnostics both on the morphological but also on the molecular level. However, in contrast to analytics where tremendous achievements in the last decade have led to a whole new portfolio of test methods, developments in preanalytics have been minimal. This is specifically unfortunate in lung cancer, where usually only small amounts of tissue are at hand and optimization in all processing steps is mandatory in order to increase the diagnostic yield. In the following, we provide a comprehensive overview on some aspects of preanalytics in lung cancer from the method of sampling over tissue processing to its impact on analytical test results. We specifically discuss the role of preanalytics in novel technologies like next-generation sequencing and in the state-of the-art cytology preparations. In addition, we point out specific problems in preanalytics which hamper further developments in the field of lung tissue diagnostics.

Contents

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1 Introduction

1.1 Preanalytics

Preanalytic processing of tissue probes and cytology specimens comprises the choice of the appropriate method for tissue retrieval, optimal fixation algorithms, and subsequent careful tissue workup for morphologic and molecular analysis. Without an optimized preanalytic workflow, results of any morphological and/or molecular analytic procedure become unreliable. Today, the main focus of research and almost all our developmental efforts are centered on the analytic steps; whereas some processes and procedures in preanalytics hardly match the requirements of modern medicine. Several important aspects of lung preanalytics will be reviewed in the following chapters.

1.2 Lung Cancer

Lung cancer is the leading cause of cancer-related mortality with nonsmall cell lung cancer (NSCLC) accounting for about 80 % of all cases [1]. Since clinical symptoms are usually mild during the early course of the disease, most diagnoses (about 75 %) are made at a time point where the tumor is not resectable anymore. Therefore, in the diagnostic context pulmonary pathologists usually receive only small biopsy specimens or even only cytological specimens.

About a decade ago, the separation between small cell lung cancer (SCLC) and NSCLC was sufficient for clinical decision making in terms of therapy selection. SCLC patients received cisplatin/etoposide and NSCLC patients were either resected in cases where surgery was still possible or received platinum-based chemotherapeutic regimens and/or radiation therapy. This has changed since today a refined morphological, immunohistochemical, and molecular classification specifically of NSCLC is required for a stratification of patients for different therapeutic approaches. Due to these novel requirements, a rational usage of the usually sparse tumor tissue is essential.

2 Aspects of Tissue Retrieval

For the increasingly complex sequence of morphological diagnosis, tumor subtyping by immunohistochemistry and molecular analysis, the amount and quality of tissue at hand is of utmost importance. One of the most important variables influencing these factors is the mode of tissue retrieval in the clinical diagnostic context.

In those cases where a resection of the tumor is performed and usually ample tumor tissue is at hand, issues of fixation, and workup become the limiting factors for the extraction of clinically meaningful analytical results (see below). However, only the minority of lung cancer patients is actually treated by tumor resection and dissection of corresponding lymph nodes, since in most of the palliative cases a surgical approach has no impact on overall survival times. Therefore, preanalytical and analytical methods primarily must be optimized for the biopsy setting.

Several methods for bioptic tissue retrieval are at hand (Table 1). The selection of the appropriate method and the subsequently applied tissue workup procedures are essential to guarantee high-quality results. The choice of the biopsy method, however, usually depends on the state of the patient, tumor localization, techniques at hand, and experience of the clinician applying the respective procedures.

Bronchial as well as transbronchial biopsies have a high diagnostic yield as far as a sufficiently large biopsy forceps is used (open forceps diameter $\sim$2 mm) and at least four tissue fragments are being taken. If the forceps is too small this will lead to artifacts, which hamper the morphological assessment and diagnosis. With a larger forceps, naturally, complication rates increase [2, 3], however, the respective

Table 1 Tissue retrieval methods in lung cancer

Tumor location	Method	Material
Central tumor with bronchial contact	Bronchoscopy	Conventional bronchial biopsy
		Bronchial cryobiopsy
		Brush cytology
		Bronchoalveolar lavage
Peripheral tumor	Bronchoscopy	Transbronchial biopsy
		Transbronchial cryobiopsy
		Transbronchial needle aspiration
		Brush cytology
		Bronchoalveolar lavage
	CT-guided percutaneous puncture	Transthoracic needle biopsy
		Transthoracic needle aspiration
Peribronchial/mediastinal tumor	Bronchoscopy	Endobronchial ultrasound with TBNA
		Classic TBNA

TBNA Transbronchial needle aspiration

complications, if they occur, are usually manageable if the appropriate clinical experience and equipment is at hand. Especially, the novel method of taking cryobiopsies, in which the tissue is frozen by a cryodevice at the tip of the endoscope in vivo and subsequently chunked out, is very effective and a reasonably secure method for the retrieval of large tissue fragments by means of endoscopy [4]. However, when applied in the transbronchial setting, this method is not very widely distributed and only available in specialized centers.

Percutaneous transthoracic needle biopsies, usually guided by computed tomography, complement the endobronchial methods for the diagnostic workup of peripheral lung lesions. The success rate of this method has been reported to range between 80–95 % [5, 6]. The negative predictive value of this diagnostic procedure is given with 84–96 %, false negative results can be expected in 2–4 % of the cases [7]. For this method, however, complication rates are somewhat higher than for endobronchial biopsy approaches and reach approximately 20–50 % [8–10]. However, complications (in most cases the occurrence of a pneumothorax) are usually manageable.

Apart from these methods, which usually guarantee that enough tissue material is at hand even when complex molecular analysis are required, techniques for obtaining cytology specimens also have their established place in the diagnostic workup in patients with lung neoplasms. Although cytology specimens are somewhat restricted with respect to tumor cell content and cellularity, optimized cytology workup procedures (see below) nowadays also allow for a broad array of diagnostic procedures (including morphology, immunohistochemistry, and molecular methods).

For those lesions which could not be reached by a direct biopsy approach (e.g., intrapulmonary tumors without bronchial contact, peribronchial nodal metastases) transbronchial needle aspiration techniques (TBNA) can be used either with or without ultrasound guidance [11, 12]. Larger needle diameters (e.g., 19G, inner diameter 0.69 mm) produce better results since more cellular cytology specimens and even small tissue fragments might be obtained. Endobronchial ultrasound (EBUS) guidance further improves the diagnostic yield, specifically when hilar and upper mediastinal lymph nodes are targeted (stations 2L, 2R, 10–12) [13]. The combination of EBUS with endosonographically-guided transesophageal fine-needle aspiration (EUS-FNA) increases the diagnostic accuracy even stronger in tumor patients, with both methods combined reaching a sensitivity of up to 96 % and a specificity of up to 100 % [14].

Bronchial brush cytology specimens can be taken prior or after the retrieval of bronchial biopsy specimens, the admixture of blood in the latter scenario does not compromise the quality of the probe [15, 16]. This method is specifically useful for tumors visually detectable by bronchoscopy. Bronchial lavage fluid can also be obtained prior or after biopsy retrieval, the use of 10–20 ml isotonic saline fluid has been recommended [15–17]. The obtained material is processed to produce smears and/or a cell block (see below), however, the diagnostic yield is worse than for brush cytology specimens. Bronchoalveolar lavage (BAL) techniques in which 3 × 50 ml isotonic saline fluid is administered and recovered from the peripheral airways are mainly used for the diagnosis of infectious and interstitial lung disease [18], in rare cases, however, adenocarcinomas can be diagnosed with this

technique, as well. Finally, sputum specimens can, in principal, be screened for the presence of tumor cells. The likelihood for a positive tumor diagnosis and the yield of tumor cells, however, is lowest for sputum material, followed by BAL, brush cytology specimens, and fine-needle material [19]. Sputum as diagnostic specimen, therefore, cannot be recommended [20].

For an optimal yield of material it is recommendable to combine different technical approaches, e.g., brush cytology and biopsy for centrally located tumors [21, 22]. In addition, several needle passages (at least 3–4 per lesion) do increase the likelihood to obtain diagnostically adequate material [23].

Taken together, clearly the type of method applied for specimen retrieval not only influences the ability to render a precise morphological diagnosis, but also impacts on the ability to perform immunohistochemistry and in situ hybridizations (FISH, CISH) on the material and also severely impacts on the yield of RNA and DNA from the respective specimens (see below).

3 Aspects of Tissue Fixation

Regardless whether the specimens submitted to a pathology lab consist of cytology, biopsy, or resection material, one must be aware that besides histomorphology, which requires an immediate and thorough fixation of the specimens, application of additional diagnostic methods is potentially required to obtain a final diagnosis. Since almost all methodological approaches established in routine diagnostics can be performed using formalin-fixed paraffin-embedded tissue (FFPE), separate biobanking of fresh or cryopreserved tissue is usually not required in the majority of cases, but must be considered in specific clinical constellations (e.g., when bacterial cultures are needed) as well as in rare cases where an exploratory scientific approach (e.g., exome sequencing) is intended. Such approaches, however, might become increasingly popular especially when established treatment methods fail and exploratory targeted therapies are a last option.

FFPE tissue is suitable for immunohistochemistry (IHC), DNA extraction, RNA extraction, and FISH/CISH [24]; furthermore, it might be used for electron microscopy after re-embedding. However, when electron microscopy is required in the first place, initial fixation of parts of the specimens with glutaraldehyde is recommended.

Pathological specimens are usually transferred directly into 4 % neutrally buffered formalin and stored until further processing. Time to fixation is critical for the preservation of architecture, antigenicity, and specifically for the integrity of DNA and RNA. Since most of the cases in lung tumor diagnostics consist of biopsy specimens, this is usually not critical, because tiny tissue fragments can directly be transferred to formalin and the penetration of formalin into the probe is almost immediate. However, with larger resection specimens these issues become increasingly critical; this is discussed in more detail in other chapters of this book. Another critical issue might be the time the probe is retained in the fixative solution, before further processing is possible. However, again this applies mainly for

resection specimens, since biopsy probes are usually processed more rapidly. Subsequent dehydration and paraffin embedding is usually done with fully automated systems in a highly standardized manner.

Apart from the conventional way of formalin fixation and paraffin embedding, several other ways of tissue preservation do exist. However, none of these approaches has found its way into a broad application in routine diagnostics yet. These methods include cryopreservation by immediate shock freezing of probes in liquid nitrogen but also other novel fixation techniques like HOPE [25], PAXgene® tissue system [26, 27], or RNA*later* [28, 29] fixation. All these procedures, however, require either specialized sampling equipment (liquid nitrogen) or tissue handling techniques which are currently not automated and therefore hardly introducible into a routine workflow. Furthermore, all of these methods are more expensive compared to standard FFPE processing.

4 Impact of Preanalytical Variables on Diagnostic Results in Biopsy Specimens

As outlined above, several aspects of preanalytics may strongly influence the outcome of a broad variety of diagnostically necessary analytical methods. This will be discussed in the following in a structured manner for the specific analytical methods applied in the diagnostic setting.

4.1 Morphology

Conventional histomorphology is still the backbone of pathological diagnoses. First and foremost, it is used to confirm the presence of a neoplastic process. In addition, recent data indicate that especially for pulmonary adenocarcinomas (ADC) a precise histomorphological subtyping is of high prognostic and maybe even predictive relevance [30]. Furthermore, it is of utmost importance to (at least) separate tumors with a squamous and a non-squamous phenotype [31], since major druggable driver mutations and amplifications involving, among others, *KRAS*, *EGFR*, *ALK*, *BRAF*, *ROS1*, *FGFR1*, *MET*, and thus the selection of targeted therapies, are significantly affected by these features and are currently only tested in the specific subentities. Morphology is easily assessed in FFPE material, this is the gold standard preanalytical method in this regard. However, novel fixation techniques like PAXgene® tissue system and HOPE also achieve high morphological standards comparable to that of FFPE procedures (Fig. 1). Cryosections from shock frozen tissue as well as RNA*later* fixed tissue show considerably lower quality and require a specialized cutting technique; routine diagnostics cannot be done on these specimens. Therefore, if molecular methods which require frozen tissue are necessary, extra

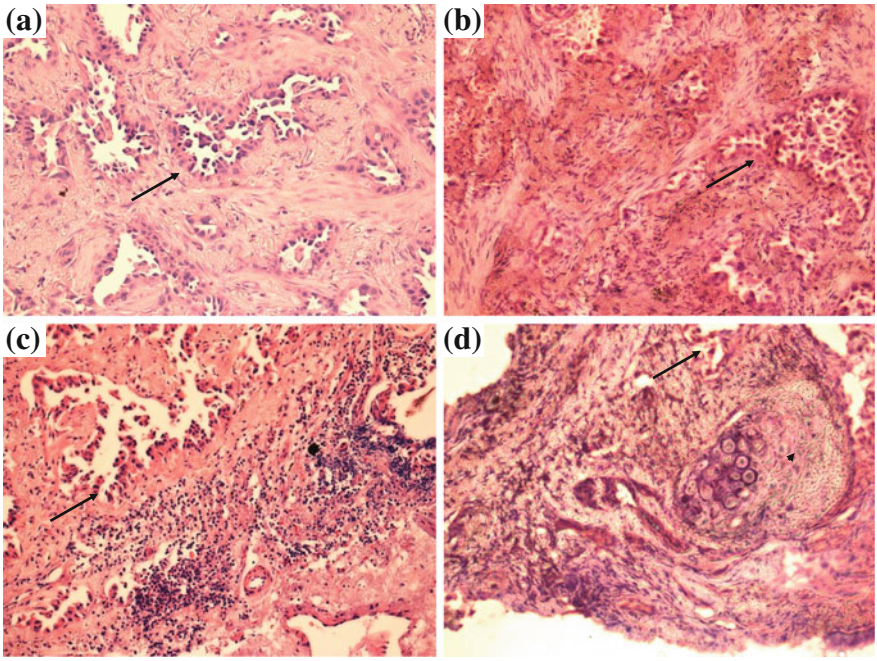

Fig. 1 Morphologic tissue quality in dependence of fixation procedure, **a** FFPE tissue with well-preserved morphology. **b** In cryosections morphology is slightly worse; however, the respective structures are still discernable. **c** HOPE fixation preserves morphology comparable to FFPE procedures. **d** Tissue fixed in RNA*later* shows a somewhat compromised morphology. *Arrows* neoplastic glands, *Arrowhead* cartilage

cryoconserved material must be taken in addition to the material necessary for conventional diagnostic purposes. In Fig. 1 morphology examples for differing tissue preserving methods are shown.

4.2 Immunohistochemistry and FISH

Since in up to 30 % of the NSCLC biopsies a precise subtyping requires additional IHC using specific markers for a squamous (p40, p63, CK5/6), an adenocarcinomatous (TTF-1, napsin, CK7) or even a neuroendocrine differentiation (chromogranin A, synaptophysin, CD56, Ki67) immunohistochemistry is critical for reliable diagnoses [31]. In addition, several IHC-based biomarkers which predict response to targeted agents are currently under development, this specifically includes MET and ALK [32]. For almost all diagnostically relevant antibodies, FFPE tissue is suitable. Since IHC staining is always interpreted in the context of morphology, optimal tissue fixation with high preservation of structural details is

important. Poor tissue fixation further results in a loss of immunoreactivity. Tissue fixation depends on time to fixation and several other variables which are not that critical in the lung biopsy setting (see above) but play an important role in the context of resection specimens in general (see other chapters in this book). Other tissue embedding methods including PAXgene® tissue system and HOPE do preserve both morphology and antigenicity; therefore, IHC is easily possible in this context. However, protocols and antibodies must be adjusted and cannot be transferred directly from the FFPE situation.

FISH analytics are necessary for amplification or translocation detection of predictive biomarkers (*ROS1*, *ALK*, *RET*). For FISH analyses, the requirements are comparable to IHC. Besides careful processing of the tissue, the most important thing is proper fixation to maintain the cellular structure and to avoid DNA degradation. From the technical viewpoint, FISH can be theoretically done with most types of fixatives (frozen, FFPE, PAXgene® tissue system, HOPE); however, data on this for some procedures are very sparse and protocols vary considerably.

4.3 Nucleic Acid Extraction

Quality and quantity of nucleic acid extracts are of utmost importance for all subsequent nucleic acid based molecular methods applied in routine diagnostics and translational research. First and foremost, tumor tissue must be marked on stained tissue slides and macro- or microdissected from the very same slide or subsequent unstained slides. Tumor cell content of the microdissected area should be documented, since it influences many factors of the analytics results like, e.g., allele frequencies in sequencing. Prior to tumor dissection the specimens need to be analyzed for a potential tumor heterogeneity including the amount of vital tumor, stroma, necrosis, or potentially contaminating normal lung or inflammatory cells in order to obtain tumor-specific, high-quality nucleic acids. Tumor cell content may vary considerably between biopsies.

Yield and quality of RNA and DNA strongly depends on the type of material used, fixation method, and extraction technology. RNA quality is frequently measured as RNA integrity number (RIN) by using capillary electrophoresis techniques (Agilent bioanalyzer) [33]. Results usually range between 2 (low quality) and 10 (optimum). RIN numbers better than 8 are widely considered as good quality. This RNA quality might serve as a template for most applications including microarray analyses.

The influence of biopsy techniques on RNA/DNA quality is low, when tissue specimens are processed/fixed quickly (1 min. range). Best results are obtained with cryobiopsies followed by forceps biopsies and core needle biopsies. DNA/RNA quality is excellent in most cases of cryobiopsies and suitable for all kinds of molecular analyses including next-generation sequencing.

Nucleic acid preservation is best for shock frozen tissue and RNA*later* material (Fig. 2). The quality of RNA isolates from RNA*later* stabilized tissue is equal to

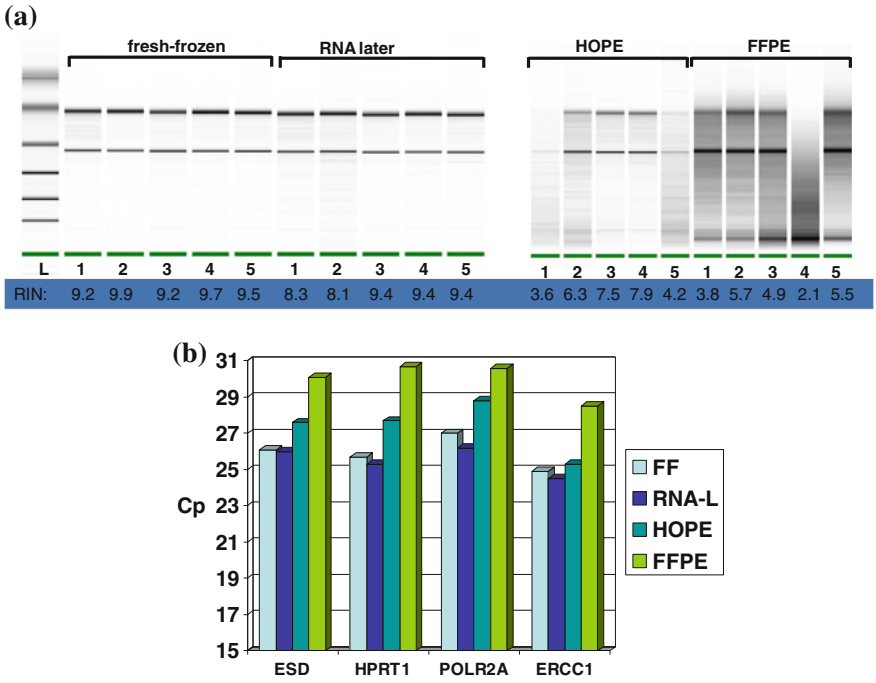

Fig. 2 RNA quality in dependence of tissue fixation method. **a** Gel electrophoresis and RIN numbers are given for representative tumor tissue samples fixed with different methods. **b** Reverse transcribed (RT) total RNA amplifiability of representative genes (*ESD* esterase D, *HPRT1* hypoxanthine phosphoribosyltransferase 1, *POLR2A* polymerase (RNA) II (DNA directed) polypeptide A, *ERCC1* excision repair cross-complementing rodent repair deficiency, complementation group 1) from samples with different fixation procedures (*FF* fresh frozen, *RNA-L* RNA*later*, *HOPE* HOPE fixans, *FFPE* formalin-fixed paraffin embedded) as measured by RT qPCR. Mean Cp values of 5 samples are shown

cryopreservation as measured by the RIN number [33]. From our experience, there is no major difference in DNA/RNA quality for nucleic acids isolated from FFPE and PAXgene® tissue system fixed probes [34]. This is exemplarily shown for RNA in Fig. 2, where the template quality for RT-PCR is compared.

High-quality DNA and RNA are usually isolated from cryopreserved or RNA-*later* stabilized tissues by hand using commercially available kits (e.g., Qiagen, Ambion, other), the respective workflow for frozen material is exemplarily shown in Fig. 3. DNA and RNA isolation from FFPE tissue might be a bit more challenging, isolation can be done by commercially available manual kits (e.g., Qiagen, others) but also by automated systems (Siemens, Qiagen, Promega). Time and method (bead mill, rotor-stator homogenizer) of tissue homogenization of small biopsies may have a significant influence on the size of isolated genomic DNA. This is important if the DNA is to be used for advanced molecular approaches such as next-generation sequencing techniques.

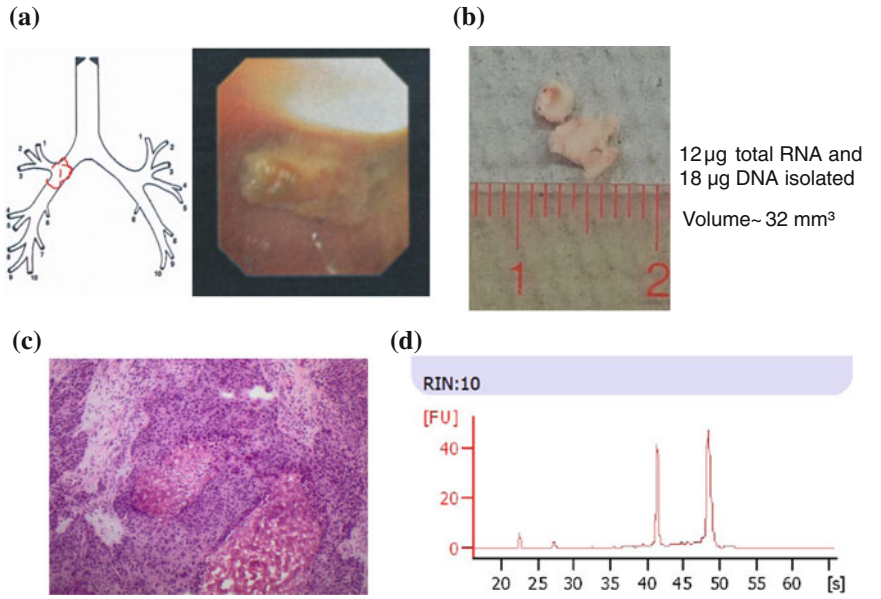

Fig. 3 Workflow of frozen cryobiopsy processing. **a** Localization of endobronchial tumor; **b** cryobiopsy; **c** frozen tissue section evaluation; **d** result of RNA analysis using an Agilent bioanalyzer

4.4 Sequencing

For conventional Sanger sequencing, a tumor cell content of about 30 % has to be considered as a lower limit for the reliable detection of point mutations. However, complex mutations like the typical exon 19 deletions can reliably be detected even in samples with lower tumor cell content [35]. DNA quality is not that critical for this method, however, major degradation may lead to compromised amplification by the presequencing PCR. For next-generation panel sequencing approaches 50 ng of DNA are usually sufficient [36]. Here, DNA quality is somewhat more important than for Sanger sequencing and must be rigorously controlled prior to sequencing.

5 Cytology Aspects

In general, morphological and molecular analyses of cytological specimens can be reliably performed using smears, cytospins, or cell blocks; there is no ultimate need for histological specimens. However, the sparseness of cellular material in some instances might hamper the sole use of cytology material. The diagnostic workup of cytological probes should be done as carefully as possible to spare material for subsequent molecular analyses. Nowadays, minimally invasive techniques are often used in a first attempt to obtain a definite diagnosis in lung cancer [37]. Therefore,

EBUS- and EUS-guided transbronchial needle aspiration (TBNA), and especially the combination of both, become increasingly popular as the initially applied diagnostic methods [11, 12, 14, 38]. As a consequence, the available material in lung pathology is increasingly shifting from histological to cytological specimens.

In order to increase the diagnostic yield of cytology specimens, the preparation of cell blocks is highly recommended. A cell block consists of fixed and embedded cell preparations that can be processed and cut like tissue material [39, 40]. The cell block method is a reliable, complimentary approach to conventional cytological or biopsy procedures.

Major advantages of conventional cytological smear or cytospin specimens over cell blocks are the intactness of the cell nuclei and the high DNA quality, usually allowing for good morphological and especially molecular analyses. However, in cell smears the amount of tumor cells is usually limited. Furthermore, destruction (from the morphological viewpoint) of the original specimen by, e.g., workup for molecular analyses may pose a problem considering the archiving requirements for original slides on which a malignant diagnosis was based. In those cases, image documentation of the slides, preferably by whole slide scanning, is mandatory prior to the dissection of tumor cells.

The advantages of cell blocks are the availability of established and validated protocols [39, 41] and the opportunity to have serial sections from the same specimens. Thus, changes to the original slides used for morphology review for molecular analyses can be avoided and molecular analyses can even be repeated or complemented by other methods using the available serial sections.

Preconditions for reliable molecular analyses using cytological specimens are experience, high expertise in cytology, sufficient technical equipment as well as a direct communication/close cooperation between histopathologists and cytologists involved in the case and those who perform the molecular analyses in order to ultimately correlate all respective results. It must be emphasized that cell blocks do not replace conventional cytological procedures (smears, cytospins) but are especially helpful as a complementary approach to fulfill the requirements for reliable and high-quality biomarker analysis [39, 41]. After sampling, cytological probes should be kept within a temperature of 2–8 °C (not frozen) or suitable fixatives (e.g., neutrally buffered formalin). The following points need to be considered for molecular analyses using cell block material:

- At least 50–100 tumor cells are usually required for molecular analysis; contamination with non-neoplastic cells should be as low as possible.
- Specimens should be fixed with buffered formalin (defined pH and buffer capacity). Bouin's solution should be avoided because the use of picric acid results in DNA damage.

Based on the various aspects outlined above in detail, the following points are recommended in order to improve the quality of molecular analyses using cytological specimens:

- Independently from the method used as much tumor material as possible should be obtained.
- Sputum and bronchoalveolar lavage specimens should always be combined with another method to gain sufficient amounts of tumor cells (TBNA or biopsy approaches).
- If a transport of the specimens is required this should be done in liquid form using suitable fixatives or at 2–8 °C. Freezing of the specimens should be avoided.
- Besides smears or cytospins, a preparation of a cell block from each cytological probe should be considered in order to optimize subsequent molecular analyses.

We recommend preparation of cell blocks immediately after a malignant diagnosis was made using the sediment of the original probe stored at 4 °C. For reasons of efficiency, we prepare cell blocks from EBUS-TBNA material and all specimens suspicious for malignancy immediately at the beginning of the diagnostic procedures.

6 Concluding Remarks

Regardless of whether cytology specimens, lung biopsies, or resection specimens have to be analyzed, preanalytic tissue processing is of utmost importance to optimize the results of the subsequent morphological, immunohistochemical, and molecular analytic process. Until today, only few efforts have been made to obtain reliable data on influences of preanalytics on diagnostic results. This is the reason why developments in preanalytics optimization are considerably lagging behind those in analytics. To change this, more efforts have to focus on this interesting field of research and development.

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RNA Quality in Fresh-Frozen Gastrointestinal Tumor Specimens—Experiences from the Tumor and Healthy Tissue Bank TU Dresden

Silke Zeugner, Thomas Mayr, Christian Zietz, Daniela E. Aust and Gustavo B. Baretton

Abstract

The term "pre-analytics" summarizes all procedures concerned with specimen collection or processing as well as logistical aspects like transport or storage of tissue specimens. All or these variables as well as tissue-specific characteristics affect sample quality. While certain parameters like warm ischemia or tissue-specific characteristics cannot be changed, other parameters can be assessed and optimized. The aim of this study was to determine RNA quality by assessing the RIN values of specimens from different organs and to assess the influence of vacuum preservation. Samples from the GI tract, in general, appear to have lower RNA quality when compared to samples from other organ sites. This may be due to the digestive enzymes or bacterial colonization. Processing time in pathology does not significantly influence RNA quality. Tissue preservation with a vacuum sealer leads to preserved RNA quality over an extended period of time and offers a feasible alternative to minimize the influence of transport time into pathology.

Contents

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1 Introduction

The term "pre-analytics" summarizes all procedures concerned with specimen collection or processing as well logistical aspects like transport or storage of specimens prior to the conduction of analyses in the respective pathological laboratories or research centers [6]. All of these variables, practical or administrative, affect sample quality and should thus be subject to quality assessment and optimization [7]. Tissue-specific characteristics may also have an impact on sample quality. The following processes affect sample quality in detail: duration of tissue undersupply during surgery (warm ischemia), duration of transfer to laboratory and transport conditions (cold ischemia), processing time in the laboratory, storage conditions, and tissue characteristics (Fig. 1).

Biorepositories, tissue, and sample collections of archived patient materials that allow to initiate and to evaluate retrospective and prospective clinical studies, to study biomarkers or to address pharmacokinetic questions [12], heavily rely on sample material of high quality. Apart from preparation and storage, sample quality is primarily ensured by processing of tissues of best possible quality [4]. Mainly used for expression studies, RNA is distinguished from other cellular components by its low stability. For scientific purposes, the preservation of native RNA in surgical specimens is of utmost importance and various approaches have been taken to minimize RNA degradation processes [10].

The introduction of RNA integrity numbers (RIN) greatly facilitated the discrimination of native versus degraded RNA [11]. Based on integrity measurement of S18 and S28 ribosomal RNAs, the determination of RIN values can be utilized as a quality standard for fresh-frozen tissue at the molecular level [3].

One approach to preserve RNA integrity is fresh-freezing of tissue specimens prior to preparation. Vice versa, assessment of RNA integrity can be utilized as a quality indicator for sample preservation [13] to answer the questions: how does tissue type influence RNA quality in fresh-frozen samples and does processing time in a pathological laboratory affect RNA quality? A recent approach to ensure tissue preservation is vacuum-sealing of fresh tissues [2], here compared with fresh-frozen tissues of specimens of the gastrointestinal (GI) tract in particular with RNA integrity as the quality indicator.

The aim of this study was quality assessment of various pre-analytical steps with special focus on sample preservation of GI specimens. RNA integrity expressed as RIN values served as the quality indicator to evaluate the impact of organ sites and processing times in the pathology laboratory on sample quality.

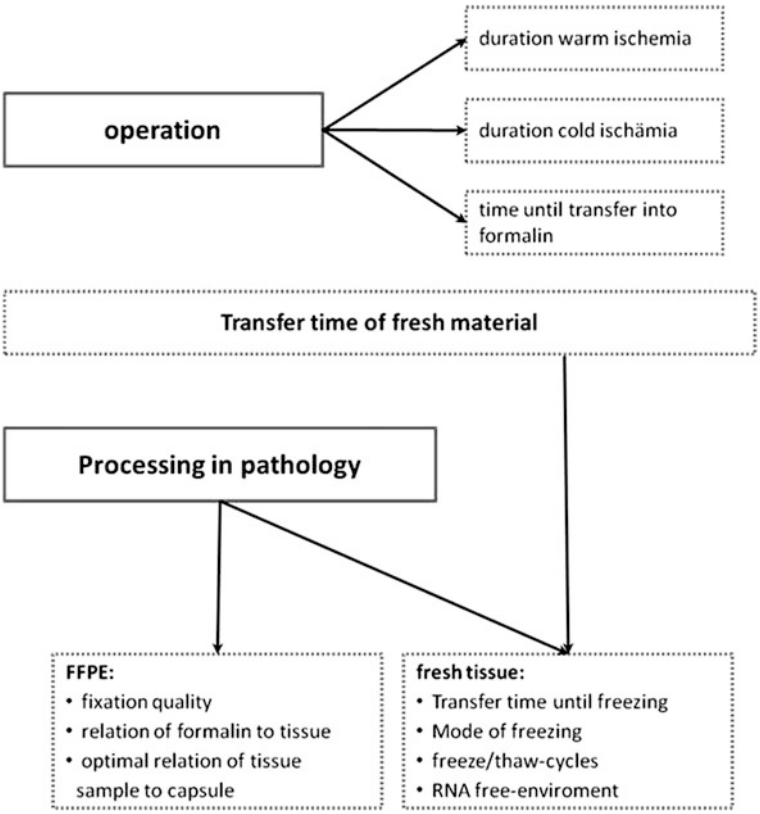

Fig. 1 Flowchart of processes in the acquisition of specimens prior to sample analysis. This study focuses on sampling of tissue specimens fresh-frozen with entry at pathology

2 Methods

Fresh-frozen tumor tissue samples, collected between 2009 and 2012 at the Institute of Pathology at the University Hospital Carl Gustav Carus, Technische Universität Dresden, and stored at −80 °C and or in the gaseous phase of liquid nitrogen, were randomly selected (n = 171) to generate a representative sample cohort.

RNA extraction was performed using the RNeasy microAmpKit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RIN (RNA integrity number) values were determined on the RNA-6000-Nano-LabChip-Kit with the Agilent-2100-Bioanalyzer (Agilent Technologies, Böblingen, Germany). Extracted RNA was used at a concentration of 25 ng/µl.

Statistical analysis was performed using the SPSS software (IBM, Ehningen, Germany). Sample size was estimated with nQuery Advisor® 7.0 (Statistical Solutions Ltd., Cork, Ireland).

3 Results

3.1 Integrity of RNA Derived from Fresh-Frozen Tissues of Different Organs Varies Substantially

Overall, 171 fresh-frozen tissue specimens stored at −80 °C or at −196 °C in liquid nitrogen were randomly collected to create a representative sample for quality assessment by RNA extraction and evaluation of integrity with the RIN algorithm. The collection is composed of specimens of lung, breast, stomach, liver, pancreas, kidney, small intestine, colon, and rectum as listed in Table 1. RIN values were classified as of sufficient quality (RIN ≥ 5) or as inadequate for further analyses (RIN < 5) according to the literature [11]. Primary analysis indicated that RNA integrity from tissues derived from the gastrointestinal tract was on average of significantly lower quality compared to other organs (Fig. 2, ***, $p < 10^{-3}$) and a smaller fraction of fresh-frozen tissue samples derived from GI specimens was suitable for subsequent studies compared to other organs. Here, about 70 % of samples derived from breast and kidney specimens were suited for further analysis (RIN ≥ 5), whereas lung specimens rarely produced samples of sufficient RNA quality (1/6 specimens). A further grading of suitability for sample derivatization is observed in the group of GI organs (highlighted in green in Table 1). In the majority of GI organs, about half of specimens appear suitable for derivatization, apart from stomach specimens with only 26 % (5/19) of samples with sufficient RNA quality.

Table 1 Tissue origin affects RNA integrity of fresh-frozen tissue samples

Tissue origin	Specimens (*n*)	RIN ≥ 5 (%)	RIN < 5 (%)
liver (Li)	32	17 (*53*)	15 (47)
Colon (C)	27	13 (*48*)	14 (52)
Rectum (R)	25	10.5 (*42*)	14.5 (58)
Breast (B)	22	16 (73)	6 (27)
Stomach (St)	19	5 (*26*)	14 (74)
Kidney (K)	19	13 (68)	6 (32)
Pancreas (Pa)	13	5.5 (*42*)	7.5 (58)
Lung (Lu)	6	1 (17)	5 (83)
Small intestine (SI)	4	3 (*75*)	1 (25)
Prostate (Pr)	4	3 (75)	1 (25)
Total	171	87 (51)	84 (49)

RNA samples from various organs were classified as of sufficient quality (RIN ≥ 5) or highly degraded (RIN < 5). Tissues of GI tract overall display reduced RNA quality and a decreased fraction of samples suitable for further analysis (*italics*)

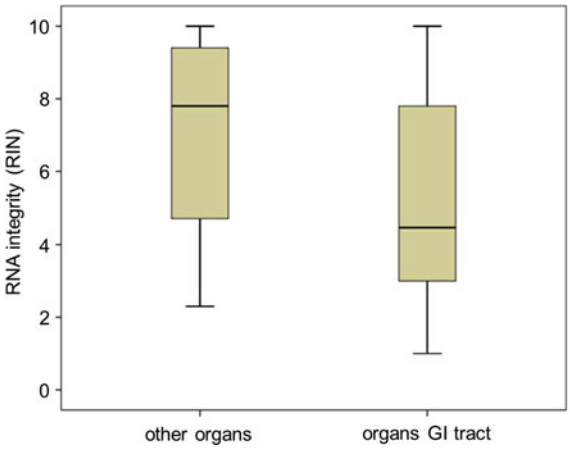

Fig. 2 Diminished RNA preservation in samples derived from organs of the gastrointestinal (GI) tract compared to other organs ($p < 10^{-3}$). Box plot analysis of RIN values of GI versus other tissues. Results of organs of the GI tract were grouped and compared to RIN values of other organs listed in Table 1

3.2 Processing of Tissue Specimens Does Not Contribute to Tissue-Specificity of Sample Quality

Ignoring intrinsic factors that affect RNA integrity like pancreatic enzymes, gastric acids, or bacterial colonization of colon and rectum, our work focused on processes suitable for optimization of tissue specimens in the molecular pathological laboratory. Sample processing times are closely linked to RNA integrity, and analysis of sample processing times after entry at pathology reveal tissue-specific differences in times to freezing of specimens (Fig. 3a). However, processing times did not differ ($p = 0.502$) between samples of sufficient RNA quality (RIN $\geq$ 5) compared to samples with lower RIN values (<5). Thus RNA integrity as indicator of sample quality is not affected by processing times (Fig. 3b) of tissue specimens at pathology. Taken together, these results show that even though organ-specific variations of processing times in pathology do occur, these variations do not contribute to sample quality as evaluated by RNA integrity as a quality indicator.

3.3 Intra- and Perioperative Factors Affect Tissue Quality

As quality assessment of tissue specimens at pathology did not reveal major options for optimization, but a wide range of differences in tissue quality before entry to the lab, our next concern were sources for intraoperative quality loss (warm ischemia) or degradation processes that might occur during transfer from the site of operation to pathology (cold ischemia). Evidently, tackling warm ischemia with the aim to

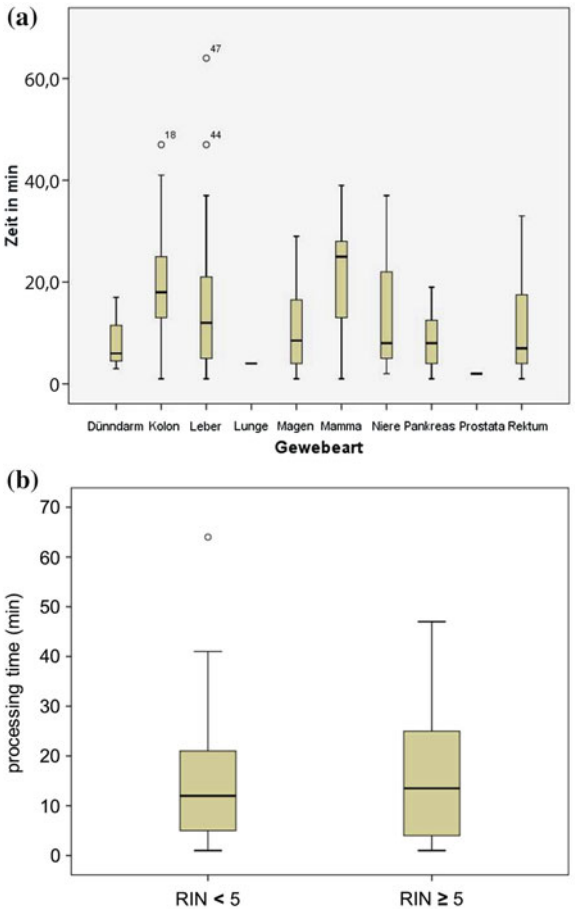

Fig. 3 **a** Sample processing times in relation to tissue type. Samples derived from various organs were evaluated according to the duration between entry of specimens in the pathology lab and sample freezing. From left to right: small intestine, colon, liver, lung, stomach, breast, kidney, pancreas, prostate, rectum. **b** Processing time at pathology does not affect the fraction of samples suited for further analyses. Duration from entry at pathology to freezing was plotted against samples of either sufficient RNA quality for suited further studies (RIN ≥ 5) or samples with low RNA integrity (RIN < 5)

optimize intraoperative processes for the sake of tissue preservation is difficult to achieve. However, tissue should be transferred to the site of tissue storage with as little damage to tissue possible. Apart from the ordinary suspects like short transport times for cooled tissue, another approach was tested: vacuum-sealing as a means for fresh tissue preservation. In a primary experiment, a range of tumor specimens were selected from duodenum and pancreas and vacuum-sealed using the Milestone Tissue-safe® with the entry at pathology. The results of this experiment are listed in Table 2. Apart from immediate freezing (fresh-frozen), vacuum-sealed tissue was kept at ambient temperature for up to twelve days (288 h). RIN values were determined as the quality indicator for extracted RNA derived from these specimens. As expected, deterioration of tissue quality was observed with increasing storage times, as reflected by decreasing RIN values, rendering samples after prolonged storage

Table 2 Tissue quality is preserved by vacuum-sealing of selected organs

Tissue	Duration (h)	Tumor size (mm^2)	RNA (ng/ml)	280/260 (nm)	RIN value	RIN ≥ 5
Pancreas	Fresh-frozen	12 × 10	1,474	2.07	7.3	Yes
Pancreas	24	2 × 5	919	2.07	4.9	Yes/no
Pancreas	48	8 × 8	528	2.05	4.4	No
Pancreas	120	5 × 5	501	2.04	4.5	No
Pancreas	288	5 × 5	959	2.08	N/A	N/A
Duodenum 1	Fresh-frozen	5 × 5	539	2.03	5.5	Yes
Duodenum 1	96	3 × 4	547	2.02	4.9	Yes/no
Duodenum 1	120	5 × 5	32	1.98	2.4	No
Duodenum 1	192	5 × 5	374	2.02	1.1	No
Duodenum II	Fresh-frozen	5 × 5	312	2.06	8.5	Yes
Duodenum II	24	3 × 4	227	2.07	6.5	Yes
Duodenum II	48	5 × 5	229	2.06	4.9	Yes/no
duodenum II	120	4 × 5	183	2.07	4.5	No
Duodenum II	288	3 × 5	178	2.06	3.9	No

Tumor specimens of either pancreas or duodenum were either fresh-frozen (0 h) or vacuum-sealed for various periods. RNA integrity of derived samples is depicted as RIN values. Samples of sufficient quality (RIN ≥ 5) with RIN values as indicator are listed (yes/no)

inappropriate for further studies. However, RNA of sufficient quality was retrieved from vacuum-sealed tissue specimens after storage for 24–96 h at ambient temperature for both tissue types selected. These preliminary results suggest that vacuum-sealing is a feasible alternative to ensure tissue preservation, unless short transport times under cooling conditions are not easy to realize. Based on these results, the Urology Department of the University Hospital Carl Gustav Carus at the TU Dresden nowadays routinely preserves prostate and bladder specimens prior to entry at pathology using Tissue-safe® vacuum-sealing.

4 Discussion

Clinical studies and medical research projects become more and more dependent on human materials of high quality collected in biorepositories [1] and great efforts were made to improve tissue quality of collected specimens [1, 4, 5]. Here we report our efforts on assessment of tissue quality from the tumor and healthy tissue bank at the Institute of Pathology, University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany. Though macro- and microscopically undamaged, tissue deterioration starts at the subcellular level, rendering part of the collected specimens unsuitable for subsequent molecular analyses. Particularly RNA, utilized mainly for gene expression studies [12], is highly susceptible to degradation. On the other hand, this instability predisposes RNA as a quality

marker, validated by the assessment of RIN values as indicators for tissue specimen quality [13].

RIN values varied significantly with tissue type as determined for a set of randomly collected fresh-frozen tumor tissues specimens of various tissue types that were kept deep-frozen from the arrival in the lab onwards. According to this molecular quality marker, around half of the specimens of the gastrointestinal tract were found to be of insufficient quality for further molecular analyses. Even though processing times within the lab until deep-frozen final storage differed with tissue type, this finding cannot explain the loss of a high fraction of GI specimens as compared to other organs. Alterations in processing times, however, did not significantly alter RNA integrity, since processing times in the pathology laboratory generally have no impact on the suitability of specimens for further molecular analyses. On average, we observed lower tissue quality of GI specimens as compared to other organs tested in this series. A variety of factors contribute to diminished tissue quality in organs of the gastrointestinal tract [14]: while stomach specimens suffer from the infiltration of gastric acids and digestive enzymes prior to storage, the presence of digestive enzymes is well known to corrupt pancreatic tissue quality [9]. On the other hand, colonization with anaerobic bacteria might contribute to deterioration of colorectal tissue specimens.

Our results indicate that processes in the pathology lab are not primarily accountable for the observed loss in tissue quality at the molecular level. Differences in tissue quality already with material receipt suggest that tissue deterioration predominantly occurs prior to entrance in the lab for final storage. Two processes appear to be accountable for this loss in tissue quality: warm and cold ischemia. Warm ischemia designates intraoperative tissue damage caused by interruption of blood supply at body temperature with few options for tissue preservation for routine surgery, but can be adapted during transplant operation [8].

Additional tissue deterioration is caused by cold ischemia [15]: tissue storage after surgery under cooling conditions in the absence of sufficient blood supply. Duration of transfer from the operating theater to the final storage location is the major source of cold ischemic conditions. Transfer times and conditions, however, open space for improvement [14]. Ideally, these periods of cold ischemia can be kept as short as possible with the installment of logistical procedures under cooling conditions [4]. When these procedures are not easily applicable, vacuum preservation of tissue specimens offers an alternative to be kept at ambient temperature or in a refrigerated state [2]. Preliminary results with the tissue-safe equipment indicate that tissue quality is maintained for molecular analyses by vacuum preservation over a period of 24 up to 48 h as compared to instant freezing of material. Consequently, this equipment is now installed at the Department of Urology, TU Dresden, for routine preservation of tissue specimens destined for transport to pathology.

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Bone Marrow Work-up: Report of a Pilot Study

Ioannis Anagnostopoulos, Dido Lenze, Michael Hummel, Manfred Dietel and Korinna Jöhrens

Abstract

n this pilot study we changed several pre-analytical variables of bone marrow trephine biopsy processing with the task to achieve not only a preservation of morphology and antigens but also of nucleic acids. The changes involved employment of a newly established decalcification solution in conjunction with a short fixation time (2 h after receiving the specimens) and performance of decalcification at 37 °C. The comparison of the obtained results from three specimens with those of our routinely established protocol unequivocally revealed that the novel decalcification solution results in a superior preservation of nucleic acids, with only slight differences in preservation of morphology and cellular antigens. These encouraging results imply that this novel decalcification solution will allow a widely accepted standardization of bone marrow trephine biopsy processing.

Contents

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1 Introduction

A bone marrow trephine (BMT) biopsy is routinely carried out to assess and classify various diseases of the hematolymphoid system. It is also important for staging of malignant lymphomas, for the identification of tumor metastases in the bone marrow and of outstanding clinical value in cases where examination of bone marrow aspirate has been unsuccessful ("dry tap"). A BMT has the advantage of the simultaneous assessment of bone marrow cellularity, the spatial distribution of various cell types, and of alterations regarding bone, marrow stroma, and blood vessels. This complex diagnostic approach can only be accomplished by the combined use of conventional histology and immunophenotyping, and if necessary, by application of molecular techniques for examination of DNA and RNA (interphase fluorescence in situ hybridization, PCR based DNA analysis, in situ hybridization for RNA detection) [1, 2].

It is conceivable that the evaluation of morphology as well as the results of immunohistochemistry and molecular biology techniques depend on pre-analytical, analytical, and post-analytical parameters. The pre-analytical steps are of particular importance due to the peculiarities of the bone marrow specimen, which contains bony trabecules and the vulnerable intertrabecular spaces and thus represents a special challenge to the chosen fixation and decalcification protocols. These pre-analytical steps in BMT processing are however not standardized. There is an enormous variation in the processing protocols among hematopathology laboratories leading to the usage of different fixatives and decalcification procedures. All these procedures have several advantages and also disadvantages, in particular regarding preservation of antigens and nucleic acids, turnaround time, and also toxicity of some components [3–6].

Also we have established a decalcification protocol for our laboratory, which allows an excellent preservation of morphology and extensive immunohistochemical studies within a reasonable turnaround time, so that a comprehensive pathology report is accomplished within two working days after receipt of the BMT biopsy. However we have been confronted with frequent negative molecular pathology results due to major degradation of nucleic acids.

In the present study we wanted to investigate whether a recently available non-toxic decalcification procedure could bypass the disadvantages of our and of most other currently used protocols and allow a BMT processing that fulfills the requirements for contemporary bone marrow diagnostics.

2 Materials and Methods

2.1 Routine Pre-analytical Steps in Our Laboratory

Bone marrow trephines are sent to us in 10 % neutral buffered formalin. The trephines are kept in this solution for at least additional 6 h at room temperature without any agitation and are placed afterwards in the decalcification solution. The decalcification solution used by our laboratory contains EDTA as well as 10 % formaldehyde adjusted at pH 7.0. The advantage of this solution is that fixation and decalcification are performed in parallel. The trephines are incubated in this solution without any agitation at 65 °C for 18 h. After decalcification the specimens are embedded in paraffin using a rapid microwave tissue processor (KOS, Milestone, Sorisole, Italy). The blocks are cut and the obtained paraffin sections are subjected in parallel to conventional stains (Haematoxylin and Eosin, Giemsa, periodic-acid Schiff (PAS), iron stain and Gomori silver impregnation) and to immunohistochemistry using varying antibody panels according to the presumed clinical diagnosis and particular requests from the hemato-oncologists. This procedure allows a comprehensive histological and immunohistochemical analysis of a BMT within 30 h after reaching our laboratory.

2.2 Modifications of Pre-analytical Steps for this Study

For the purpose of this study we introduced an instrument (BoneSTATION, Milestone, Italy), which allows standardization of fixation, decalcification time and temperature. The BoneSTATION consists of two work platforms, one featuring a heating plate with infrared sensors for automatic temperature control up to 50 °C and another with built-in magnetic stirring only. The following changes were introduced in the pre-analytical steps:

I. Shortened fixation time: the freshly obtained BMT included in this pilot study have been placed immediately in 10 % neutral buffered formalin. Once received in the laboratory the specimens were put into labelled cassettes, loaded in the rack of the BoneSTATION and remained under stirring for 1 h at room temperature. Afterwards the fixation was continued for another hour under stirring at 37 °C.
II. Decalcifier agent used: for this study, the MOLDecal Solution (Milestone, Italy) was employed. This is a 10 % EDTA solution consisting of a proprietary mixture of EDTA salts resulting in an optimized pH of 7.2–7.4.
III. Decalcification temperature and time: all BMT samples of the present study were decalcified at 37 °C in the BoneSTATION. One sample was decalcified for a shorter time period than used in our laboratory (4 h) and two others for 18 h as already established.

2.3 Bone Marrow Trephines Included in this Study

Due to the fact that the fixation/decalcification instrument has been delivered to our laboratory 1 month before, only a limited number of BMT biopsies could be analyzed. A prerequisite for inclusion in this pilot project has been the length of the trephine, which should exceed 20 mm. The trephines were then divided in two parts and processed in parallel. The following samples were investigated: one BMT of a patient presenting with anemia of unclear etiology, a further biopsy from a patient with a cutaneous marginal zone lymphoma and one biopsy from a patient with suspected multiple myeloma.

2.4 Conventional Histology and Immunohistochemistry

From the decalcified and paraffin-embedded BMT serial sections were cut at 2 μm and stained with H&E.

For immunostaining, paraffin sections were deparaffinized and subjected to a heat-induced epitope retrieval employing an EDTA-buffer prior to incubation with primary antibodies. Deparafinization as well as epitope retrieval and immunostainings were performed within the automated Leica BondMax^{TM} system (Leica Microsystems, Nussloch, Germany). For this study we selected three antibodies directed against antigens located in the cell membrane/cytoplasm i.e. against CD20 (clone L26, Dako, Denmark), CD3 (clone LN10, Leica Biosystems Novocastra, UK) and CD117 (clone MI15, Dako) as well as Ki67 (clone MIB1, Dako) located in the cell nucleus. For visualization of bound antibodies the detection kit Bond Polymer Refine Diaminobenzidine (DAB) was used, which was obtained from Leica Biosystems.

2.5 DNA Extraction and Quality Assessment

Five sections (10 μm each) were cut from the paraffin blocks of the decalcified bone marrow trephines and subjected to DNA extraction using the Maxwell 16 FFPE Plus LEV DNA Purification Kit on a Maxwell 16 instrument (both Promega, Germany) according to the manufacturer's protocol for isolation of genomic DNA from paraffin embedded tissue specimens.

DNA sample concentration was measured by spectrophotometry.

The quality of the DNA extracted from paraffin-embedded samples was assessed by employing a set of control gene PCR primers designed to amplify products of exactly 100, 200, 300, and 400 bp applied in a multiplex PCR according to the Biomed-2 protocols. PCR products were analyzed on acrylamide gels.

2.6 Detection of Clonal Immunoglobulin Heavy Chain Rearrangements

Multiplex PCRs for the amplification of the immunoglobulin heavy chain genes were performed using the Biomed-2 primers and protocols except that 50 PCR cycles were used [7]. PCR products were separated on a Genetic Analyzer 3130 (Thermo Fisher USA) to assess the clonality status of the samples. In case of a polyclonal B-cell proliferation, this analysis results in a Gaussian distribution of multiple peaks representing many different PCR products, while a monoclonal B-cell population results in a single peak from one type PCR product.

2.7 Fluorescence In Situ Hybridization (FISH)

Interphase FISH was performed on tissue sections using a break apart probe for the *BCL6* gene supplied by Abbott, Germany together with the Dako Hybridizer and the FISH Accessory Kit (all from Dako, Denmark). Signals were detected using an Axio Imager Z1 (Zeiss, Germany) and Isis software (version 5.3.1, MetaSystems, Germany).

3 Results

All variations in the pre-analytic steps were compared with our routine protocol regarding preservation of morphology, maintenance of antibody reactivity and ability to perform molecular studies (FISH and IgH clonality analysis).

3.1 Morphology

- Decalcification for 4 h at 37 °C: Two differences to the conventionally decalcified BMT could be identified: the cells appeared shrunken and the bone trabecules were not sufficiently decalcified (Fig. 1a, b). This fact did not allow sectioning of the tissue at 2 µm leading to difficulties in the precise evaluation of cellular details at higher magnification (Fig. 1c, d).
- Decalcification for 18 h at 37 °C

These specimens showed a sufficient decalcification of bony trabecula, resulting in thin sections with good preservation of cellular details. There was also a slight shrinkage of the cells detectable (Fig. 2).

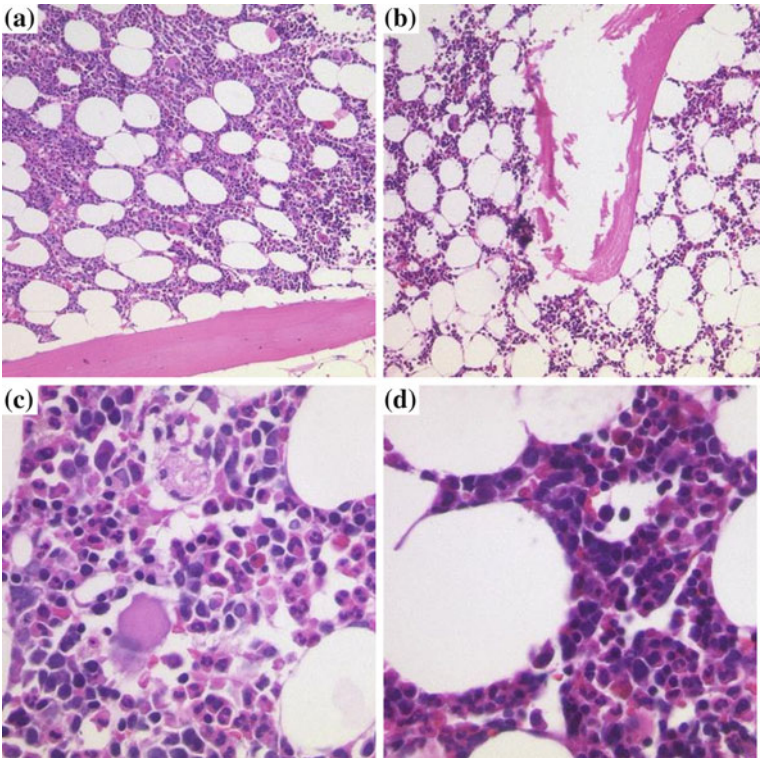

Fig. 1 Morphological evaluation of two differently processed parts of a bone marrow trephine biopsy (H&E). **a**, **c** Fixation in 10 % neutral buffered formalin (NBF) at room temperature (RT) for at least 6 h followed by decalcification for 18 h at 65 °C in a solution containing formaldehyde and EDTA at pH 7.0 leads to sufficient bone decalcification and preservation of cellular details. **b**, **d** Fixation in 10 % NBF for 1 h at RT and 1 h at 37 °C, followed by decalcification in MOLDecal for 4 h at 37 °C leads to incomplete decalcification of the bone with fragmentation of trabecules during cutting of the block. The resulting section is *thick* hampering evaluation of cellular details

3.2 Immunohistochemistry

- Decalcification for 4 h at 37 °C: The nuclear antigen detected by Ki67 was equally good preserved in sections from both protocols. Also the expression patterns of CD3 and CD138 were identical independent of fixation/decalcification protocol. Only the immunostains for CD20 and CD117 were weaker after decalcification with MOLDecal and the reaction product was also more granular. This weaker intensity resulted also in an incorrect estimation of the number of positive cells.

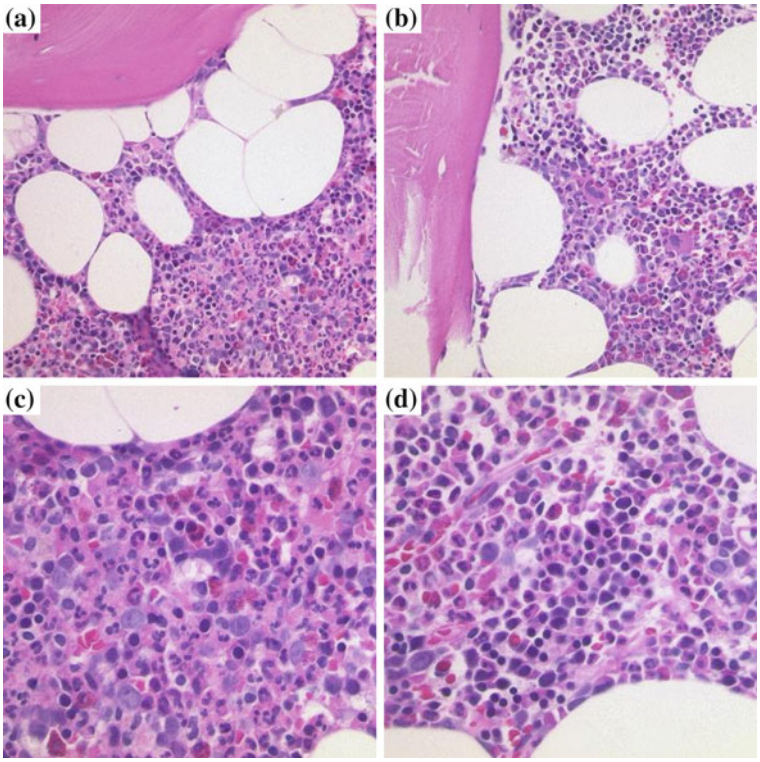

Fig. 2 Morphological evaluation of two differently processed parts of a bone marrow trephine biopsy (H&E). **a**, **c** Fixation in 10 % neutral buffered formalin (NBF) at room temperature (RT) for at least 6 h followed by decalcification for 18 h at 65 °C in a solution containing formaldehyde and EDTA at pH 7.0 leading to sufficient bone decalcification and preservation of cellular details. **b**, **d** Fixation in 10 % NBF for 1 h at RT and 1 h at 37 °C, followed by decalcification in MOLDecal also for 18 h at 37 °C. There is only a slight shrinkage of the cells detectable, while bone trabecules are sufficiently decalcified and the cellular details easily evaluable

- Decalcification for 18 h at 37 °C: Both specimens showed weaker and more granular immunostains for CD20 and CD138 (Fig. 3) after decalcification with MOLDecal, while the results for CD3, CD117, and Ki67 (Fig. 3) were largely identical in both protocols.

3.3 DNA Quality Assessment

The multiplex control PCR amplification according to the Biomed-2 protocol resulted in a ladder of four fragments (100, 200, 300 and 400 bp) only in the specimens decalcified in the MOLDecal solution after acrylamide gel electrophoresis. In contrast, all samples decalcified with our current protocol showed only 100 bp fragments (Fig. 4).

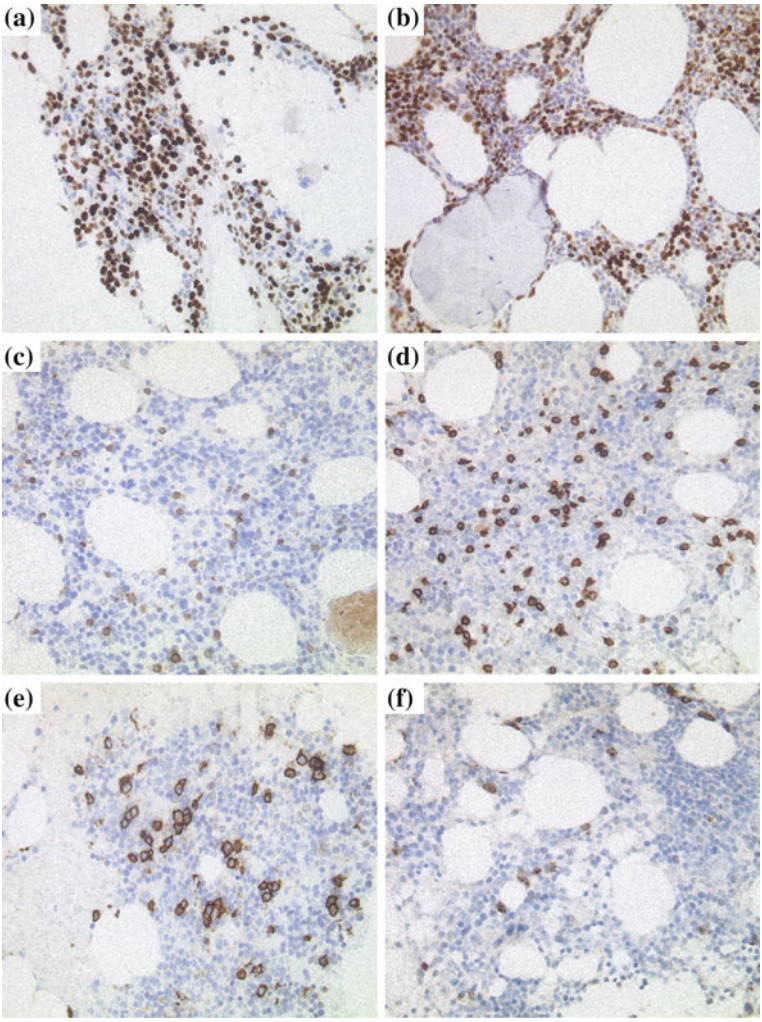

Fig. 3 Immunohistological evaluation of two differently processed parts of a bone marrow trephine biopsy. Following antigens have been detected: Ki67 (**a**, **b**), CD3 (**c**, **d**) and CD138 (**e**, **f**). Our current protocol (fixation at RT for at least 6 h followed by decalcification for 18 h at 65 °C in a solution containing formaldehyde and EDTA at pH 7.0) leads to good preservation of the various antigens (**a**, **c**, **e**). The novel protocol with shorter fixation (1 h at RT and 1 h at 37 °C) and decalcification in MOLDecal for 18 h at 37 °C leads also to similar results with the exception of CD138 detection where the labelling is weaker and more granular (**b**, **d**, **f**)

3.4 Clonality Analysis

The highly degraded DNA after decalcification according to our routine protocol did not deliver evaluable PCR products with the primer sets for the framework regions (FR) 1 and 2 of the VH gene segments, while decalcification with

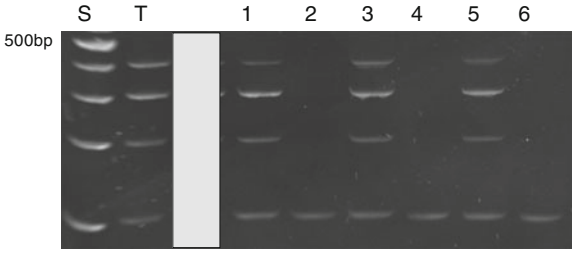

Fig. 4 Quality control of the extracted DNA by acrylamide gel electrophoresis: *S* corresponds to the control gene PCR products forming a ladder of 400, 300, 200, and 100 bp, *T* corresponds to tonsillar tissue, *lanes* 1, 3, 5 represent those parts of biopsy trephines processed with short fixation time and decalcifcation in MOLDecal revealing an intact DNA containing amplificates all four fragments, *lanes* 2, 4, 6 represent those parts of biopsies processed according to our routine protocol that leads only to 100 bp amplificates

MOLDecal led to sufficient PCR products with all 3 FR primer sets. The following results could be obtained:

- Biopsy 1 (decalcification for 4 h at 37 °C): the evaluation of the PCR amplificates obtained from the routinely processed sample pointed out to an oligoclonal B-cell population, while the new protocol revealed the presence of a B-cell clone embedded in a polyclonal background.
- Biopsies 2 and 3 (decalcification for 18 h at 37 °C): only the PCR with primers of the FR3 region could be evaluated from the routinely processed samples leading to the assessment of a polyclonal B-cell population. Decalcification with MOLDecal revealed the presence of a monoclonal B-cell population in both instances (Fig. 5).

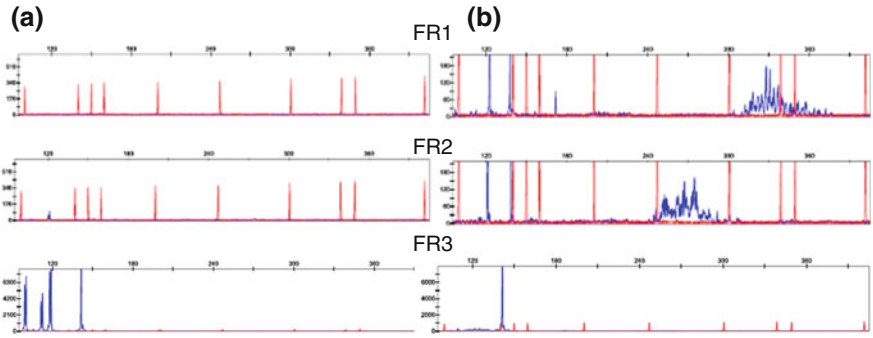

Fig. 5 Evaluation of the immunoglobulin heavy chain gene (IgH) rearrangement results in a case with suspected manifestation of multiple myeloma. The *left panel* (**a**) corresponds to the results obtained from the trephine biopsy part processed according to our current protocol which indicate the presence of an oligoclonal B-cell population. The *right panel* (**b**) represents the results obtained after shorter formalin fixation and decalcification in MOLDecal at 37 °C for 18 h, which reveal the presence of a clear-cut monoclonal B-cell population

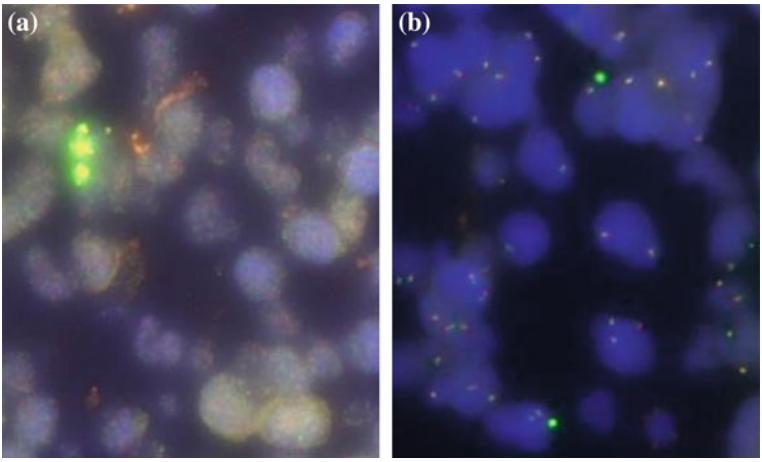

Fig. 6 Comparison of the FISH results using a break-apart probe for the BCL6 gene. Our current fixation and decalcification protocol (**a**) delivers a non-evaluable result with high background and no discernible *BCL*-6 specific signals, while shorter formalin fixation combined with MOLDecal at 37 °C for 18 h leads to clear specific signals without any background (**b**)

3.5 FISH

None of the samples decalcified with our routine protocol delivered evaluable FISH results. All samples exhibited high background staining with blurred *BCL6*-specific signals. In contrast, all specimens processed with the MOLDecal solution were characterized by an absent background staining and clear-cut *BCL6*-specific signals (Fig. 6).

4 Conclusion and Outlook

The results of this small pilot study, demonstrate that introduction of modifications in pre-analytic steps can greatly influence the results of morphological, immunohistochemical and molecular evaluation of BMT biopsies. The novel decalcification solution tested leads to an excellent preservation of nucleic acids. A larger scale study is already planned to establish a protocol that will allow a universally applicable standardized BMT biopsy processing.

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Two-Temperature Formalin Fixation Preserves Activation States Efficiently

David Chafin

Abstract

Modern pathology is built around the principle of preserving tissues such that the in vivo molecular status is maintained at levels representative of the disease state. Tissues are immersed in a solution of fixative which slowly inactivates biological activities, thus preserving the sample. Further processing ultimately allows the tissue to be embedded into wax for thin sectioning and staining for interpretation microscopically. Every year, around 7 billion tissue samples are submitted for processing in the United States alone. With this huge workload, histology laboratories are looking for faster methods of performing fixation, which currently require from several hours to days to complete. Ideally, this procedure could be standardized and would be quicker with better preservation over a wide range of biologically relevant molecules.

Contents

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Recent Results in Cancer Research 199, DOI 10.1007/978-3-319-13957-9_11

1 Introduction

Modern pathology is built around the principle of preserving tissues such that the in vivo molecular status is maintained at levels representative of the disease state. Tissues are immersed in a solution of fixative which slowly inactivates biological activities, thus preserving the sample. Further processing ultimately allows the tissue to be embedded into wax for thin sectioning and staining for interpretation microscopically. Every year, around 7 billion tissue samples are submitted for processing in the United States alone. With this huge workload, histology laboratories are looking for faster methods of performing fixation, which currently require from several hours to days to complete. Ideally, this procedure could be standardized and would be quicker with better preservation over a wide range of biologically relevant molecules.

Formaldehyde-based chemicals are the overwhelming choice of preservatives. Formalin is an aqueous formaldehyde solution in a simple buffer, usually to 3.7 % (W/V), and also termed 10 % neutral buffered formalin (NBF). Formaldehyde-based fixatives work by a two-step mechanism of diffusion and crosslinking. Since formaldehyde is one of the smallest organic molecules, its diffusion into most tissues is relatively rapid. While the penetration rates have remained somewhat controversial, many believe formalin penetration to be on the order of 1 mm/h [1]. Once the fixative has penetrated the tissue, the formaldehyde molecule forms crosslinks between proteins and nucleic acids to halt biological activity. In addition, crosslinking the cellular constituents forms a lattice structure that allows the tissue to withstand subsequent dehydrating reagents that would otherwise distort the morphology. Unfortunately, this crosslinking step is rather slow for several reasons. The formaldehyde in solution is in equilibrium with its nonreactive methylene glycol molecule. The reaction is also a trimolecular reaction mechanism between two free binding sites (presumably on different molecules) and the formaldehyde molecule. And, there needs to be sufficient concentration of fixative to drive the unfavorable crosslinking reaction in an aqueous environment.

Tissue fixation is a nonstandardized process allowing the choice of many different types of fixatives, various times to get the tissue to the fixative (Cold Ischemia), various time in the fixative, thickness of tissues, as well as other pre-analytical variables, all of which could have an effect on downstream assay results. One driver of fixation variability in most histology laboratories stems from workflow considerations, where fixation time is selected to fit within a standard workday. Current preservation methods result in a wide range of tissue quality. It is clear that new technologies will be required to shift from a workflow centric process to one in which tissue preservation is optimized for medical content.

A rapid advancement in the understanding of cancer biology pathways has shifted the pathology field into an era of “personalized medicine” [2–5]. As the number of relevant cancer biomarkers increases and new and specialized drugs are developed, there will be a need for new fixation methods capable of capturing medically relevant levels of all molecules. Many cancer pathways involve a series of phosphorylation cascades that implicate one of numerous pathways for growth of

tumors (so called driver pathways). Unfortunately, there are not many reliable tests available for biological molecules containing phosphate modifications due to extremely active enzymes that remove or add phosphate groups to proteins. It is well documented that phosphorylation status changes rapidly upon removal of tissues from the body and current fixation methods are not efficient enough to preserve relevant posttranslational modification levels [6–9]. It has been recently documented that some of the most labile phosphomarkers are simply lost due to the use of room temperature 10 % NBF [10].

An attempt has been made to reduce the preanalytical variation for some biomarkers such as Her2 in breast samples where the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for HER2 immunohistochemistry (IHC) call for fixation in neutral buffered formalin for at least 6 h and no more than 48 h [11]. This still allows for an 8-fold variation in fixation time. Faced with this problem, we sought to identify a standardized formalin fixation protocol that was applicable to a broad range of tissue types and optimized both for speed and preservation of medically relevant biomarkers. In an attempt to standardize fixation and increase the medical utility of tissue samples, we studied basic formalin chemistry to find a protocol that was more efficient, would retain excellent tissue staining properties, and preserve signal/protein activation states. Optimal protocols were validated in clinical testing using a wide range of downstream molecular assays to include protein and nucleic acids.

2 Alternative Fixation Methods Involving Heat

The overwhelming majority of histology laboratories use 10 % NBF, a standard off-the-shelf fixative that is inexpensive and has been in use for over a century [1]. Most procedures involving formalin use the fixative at room temperature as a transportation and holding liquid between the operating room and histology laboratory. Use of NBF at room temperature is a time-consuming process taking several hours to days depending on the thickness of the tissue. With ever more pressure being placed on histology laboratories to decrease turnaround times, there has been an interest to explore the use of rapid alternative technologies.

One such technology already being employed is to simply raise the temperature of the fixative to increase the crosslinking rate [12–15]. While this is practically effective, the use of increasing temperatures has led to many reports of unsatisfactory tissue morphology (H&E) and variability in other molecular analysis, including routine IHC stains [15]. Tissues heated in the presence of fixative, which has not had a chance to sufficiently diffuse throughout the tissue, causes two major problems. The first is that formaldehyde crosslinking kinetics increase dramatically and form a crosslinked shell that may prevent or slow further diffusion of the fixative. Second, the tissue is heated in the absence of fixative (especially in the center) which causes cellular enzymes to become more active with resulting cellular degradation. While the outer layers of tissue look properly fixed, the centers of these tissues remain largely unfixed and damaged (Fig. 1).

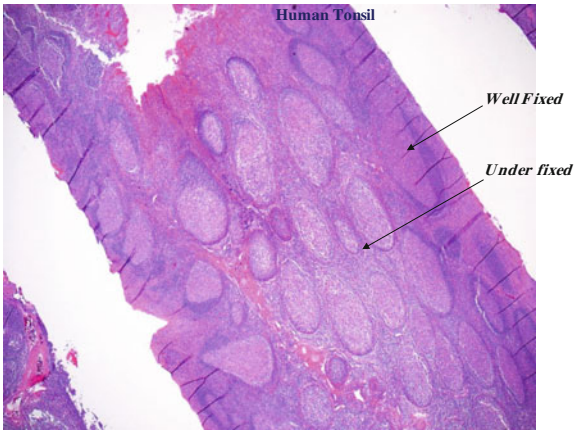

Fig. 1 ×200 image of a 4-mm-thick human tonsil section placed directly into 40 °C 10 % neutral buffered formalin for 1 h. Sample is stained with hematoxylin and eosin to reveal tissue morphology

In order to make a broadly adaptable fixation protocol it was first necessary to think about the chemical principles that make formaldehyde a good fixative. Since fixation is a two-step process driven by diffusion and crosslinking, we focused on the optimization of these two major chemical functions. Diffusion is largely driven by concentration gradient. This scenario exists when the tissue is first placed in the fixative, where there is an overwhelming excess of NBF on the outside and none in the middle. As time passes, a gradient forms where formalin penetrates the tissue, until sufficient amounts of formaldehyde have accumulated to drive the crosslinking reaction. The diffusion of formaldehyde can be accomplished under a wide range of temperatures as this molecule is very small. However, the second step of crosslinking is largely driven by concentration and temperature. As in any other chemical reaction, crosslinking needs a minimal concentration of reactant (formaldehyde) to drive the reaction to completion (crosslinked biomolecule). Increasing the temperature shifts the chemical reaction to favor crosslinking and if sufficient concentrations of formaldehyde are present fixation happens rapidly. In order to alleviate the fixation gradient that forms with heated reactions, we reasoned that it would be necessary to allow sufficient diffusion of formaldehyde before increasing the temperature to initiate crosslinking.

This was accomplished by employing a two-temperature fixation protocol with standard 10 % NBF (Fig. 2). In this scenario, tissues are placed in cold formalin (optimally 4 °C). Under these conditions, little crosslinking occurs (data not shown) but diffusion still proceeds. By cooling the tissues, cellular enzymes are largely inactivated and the cold environment helps preserve biomolecules, especially posttranslational modifications. After a period of cool time, depending on tissue thickness, samples are placed directly into warm formalin (optimally 45 °C) favoring formation of crosslinks. Since formalin is already present throughout the tissue sample, crosslinking occurs evenly throughout the tissue and superior cellular structure is realized (Fig. 3, H&E). In addition, biomolecules are better preserved due to the almost immediate decrease of cellular enzyme activity (Fig. 3, IHC/bcl-

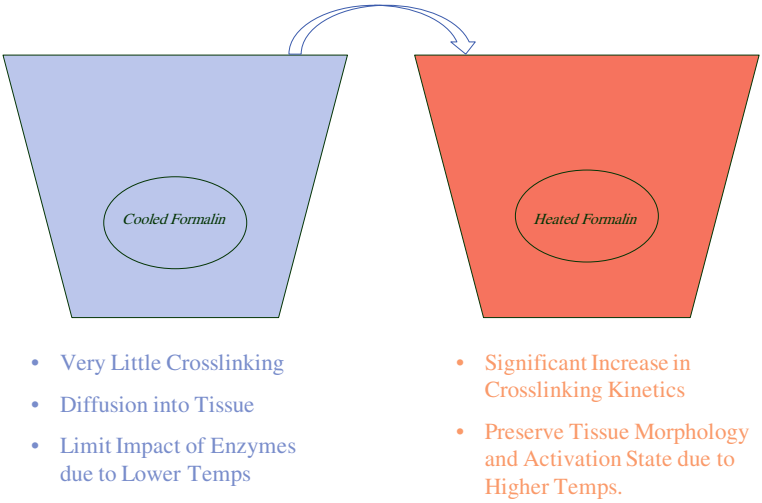

Fig. 2 Sequence of events to carry out cold + warm tissue fixation. Tissue is first placed into cooled formalin at 4 °C for 2 h followed by immediate transfer and immersion into formalin heated at 45 °C for 2 h

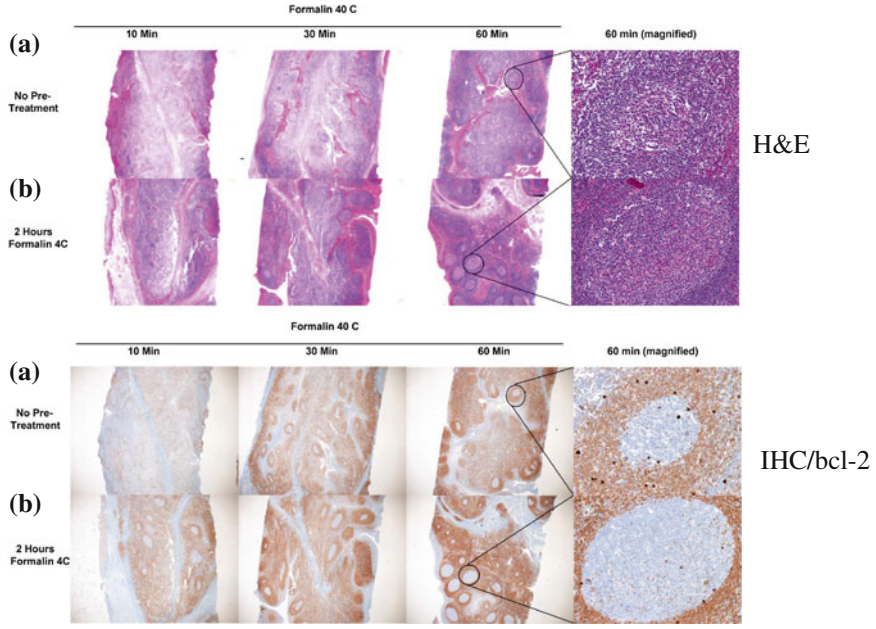

Fig. 3 *Top two panels* show a time course of formalin fixation of 4-mm-thick human tonsil sample with hematoxylin and eosin stains. **a** Warm only formalin fixation, **b** cold + warm formalin fixation. *Right side panels* show ×400 magnification of center germinal centers. *Bottom two panels* show the same set of samples stained with an antibody that recognizes bcl-2 protein and developed with DAB (*Brown*) on a Ventana Medical Systems Discovery XT Automated Staining platform

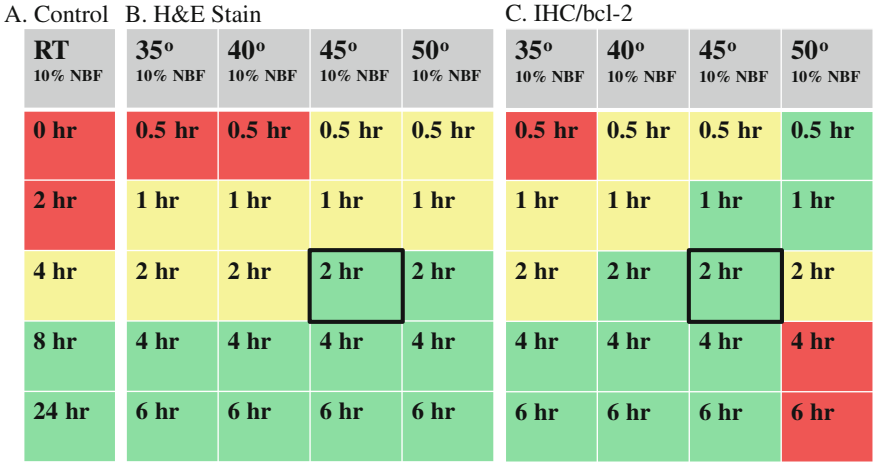

Fig. 4 Heat maps of tissue fixation quality. *Left panel* shows 4-mm-thick human tonsil samples fixed with room temperature 10 % NBF (Control). Tissues were immersed in 10 % NBF for indicated hours (0–24 h). *Colors* indicate quality of resulting cellular morphology. *Green* excellent, *Yellow* Good, *Red* poor. For all samples, tissues were deemed excellent after 8 h in RT formalin (*Green shading*). Middle panel shows hematoxylin and eosin stains of 4 mm human tonsil samples placed into heated formalin after a cold soak pretreatment (2 h in 4 °C 10 % NBF). Samples were then placed into 10 % NBF at indicated temperatures (*Gray shaded boxes*) for indicated times (0–6 h). 4 mm human tonsil samples placed into heated formalin after a cold soak pretreatment (2 h in 4 °C 10 % NBF). Samples were then placed into 10 % NBF at indicated temperatures (*Gray shaded boxes*) for indicated times (0–6 h). *Bold black boxes* indicate the area of the heat map for the 2 + 2 method

2). For tissues up to 4 mm in thickness, 2 h of cold followed by 2 h of warm formalin fixation works best (Fig. 4b, c, see bold boxes). We therefore named this the "2 + 2" fixation protocol.

The use of heat as a means of decreasing fixation time is proven to be useful and can alleviate some of the workflow bottlenecks in standard histology operations. However, the use of excessive heat was detrimental to downstream assays that depend on epitope recognition. After a 2 h incubation in cold formalin, crosslinking in temperatures higher than 45 °C resulted in a severe decrease in immunohistochemical signaling (Fig. 4c, see 2–6 h at 50 °C). Since the fixation of tissues needs to be universal for all downstream assays, 2 h at 45 °C provides the maximum staining for both tissue morphology (H&E) as well as antigen recognition (IHC) and nucleic acid-based assays (Fig. 4 and data not shown).

3 Clinical Validation

The 2 + 2 fixation method was tested under clinical conditions to assess if this protocol would be truly useful for clinical practices. This study utilized tissue collection of unfixed surgical samples of >20 tissue types that was considered waste

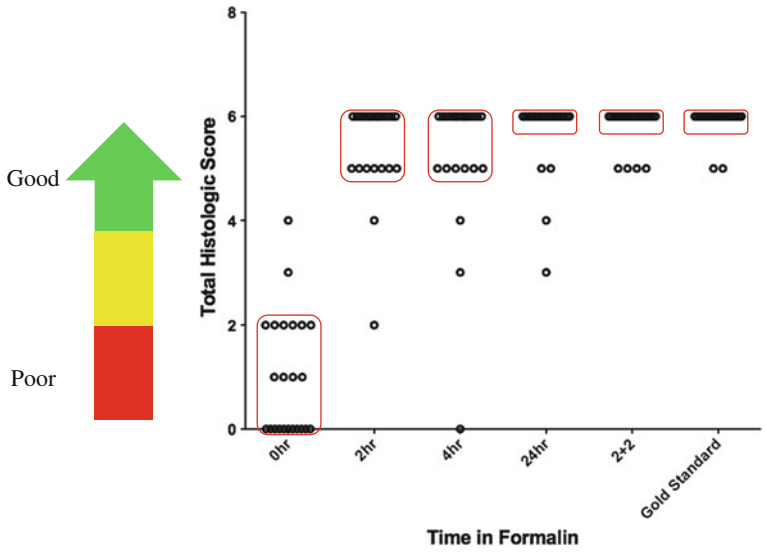

Fig. 5 Summary of all samples comparing the 2 + 2 protocol with standard room temperature fixation protocol. Over 30 different tissue types were fixed and stained with hematoxylin and eosin and scored for morphological details. 0–2 points were given for nuclear detail, cytoplasmic detail and general morphology. A score of 6 signals excellence, see calibration *arrow* at *left* of graph. Samples were fixed either with 2 + 2 or 0, 2, 4, and 24 h with room temperature formalin as indicated. All fixation methods were compared to a gold standard sample consisting of a cut and stain of the actual clinical specimen used for pathological review

material in a clinical laboratory setting [10]. Surgical tissues were fixed with the 2 + 2 protocol, and conventionally fixed tissue with increasing times of room temperature 10 % NBF (0, 2, 4 and 24 h) as comparison. Hematoxylin and eosin stains were performed on both sets of tissue to examine cellular morphology. Slides were scored by a board-certified pathologist by blinding the samples and examining nuclear, cytoplasmic, and general morphology of the tissues. Each category was given a score up to 2 and added together to make up a "histologic score," a total of 6 indicating excellence. A summary of all of the data points in the experiment are shown in Fig. 5 where increasing numbers on the Y-axis represent better morphology. As can be seen, samples that received no fixation on average score very poorly, with many scores at or below 2. However, as fixation time is increased, data points migrate up the graph to reveal better morphology until at 24 h, most of the samples have histologic scores of 5–6. In comparison, the 2 + 2 method compared favorably with the 24 h fixation times and with a gold standard sample that consisted of a stain of the actual clinical sample used for diagnosis. These samples were also compared in several antibody and nucleic acid-based assays. In each case, the 2 + 2 method compared favorably with 24 h standard fixed samples (data not shown).

4 Phosphomarker Preservation and Personalized Medicine

Many cancer pathways utilize a variety of protein activation cascades to carry growth signals from the outside of the cell to internal cellular compartments. While there are many types of activation signals, the most prominent is the addition of phosphate groups to serine, threonine, or tyrosine positions on the protein backbone. Addition of phosphate groups alters the tertiary structure of the protein target and often times "activates" an enzymatic activity that is normally masked. This new activity can then act on the next protein target in the pathway until the last protein in the pathway acts on a cellular function causing either an increase in cellular growth, lack of cell death, or some other vital function. The addition of phosphates is carried out by kinases, and their removal by enzymes called phosphatases. These two enzymes are a tightly regulated pair used for the control of cell growth and regulation of cellular functions such as DNA replication, RNA transcription, and protein translation. Study of these pathways of activation is of high importance and gaining traction in the age of personalized medicine [16–21]. Many proteins that were once unavailable as new drug candidates are becoming targets for the next generation of pharmaceuticals. Hindering these efforts are the unstandardized fixation methods that simply cannot preserve activation states at levels necessary for assay development. One such marker, pAKT (also known as protein kinase B (PKB)), resides in the heavily studied PI3 K pathway, but researchers have not been able to fully preserve the protein in its activated state [22–24]. This molecule is phosphorylated at the cellular membrane and migrates into the cytoplasm where it acts on proteins in various signaling pathways. Due to the transient nature of the activated form, no reliable assays utilizing this important biomarker have been developed for tissue diagnostics.

Tissues fixed with the 2 + 2 method were tested for preservation of the phosphorylated form of AKT. Mouse xenograft tumors made from a human lung adenocarcinoma cell line that expresses pAKT at high levels (Calu-3, ATCC HTB-55), were found to have extremely high levels of DAB staining using an automated slide stainer and an antibody specifically recognizing the phosphate group at serine 473 (Fig. 6a, 2 + 2 vs. 24). Attempts to stain for the molecule from room temperature methods were unsuccessful, even if fixed a full 24 h. Attempts to place the tumors directly into warm formalin, skipping the cold diffusion, also produced negative staining (data not shown). High levels of pAKT preservation were also observed from a clinical cohort of 23 human colorectal carcinoma (CRC) samples fixed with the 2 + 2 method (Fig. 6b, 2 + 2). Interestingly, moderate levels of pAKT staining were observed when CRC samples were placed into room temperature formalin with short cold ischemia times, of less than 15 min (Fig. 6b, 24). When the samples had longer cold ischemia times, as might be observed in routine medical practice, all pAKT staining was lost (Fig. 6b, 1 h ischemia + 24). This indicates that the cold step indeed lowers cellular enzyme activity and allows for maximum preservation. Phosphorylated forms of several other cancer pathway proteins were also observed indicating that the 2 + 2 method will have positive effects on cellular proteins in general.

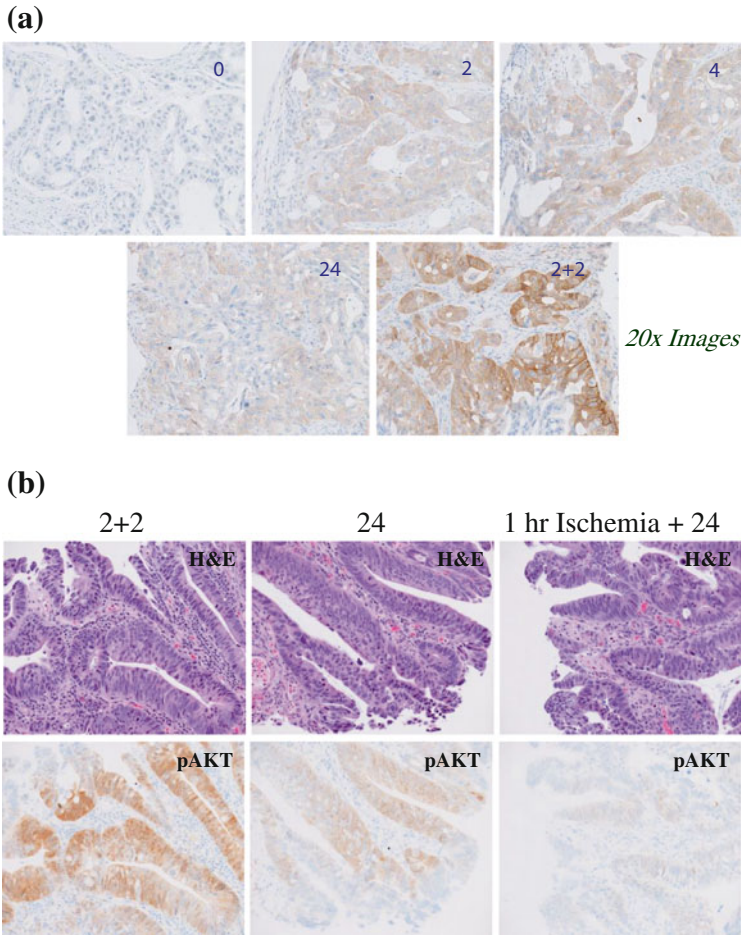

Fig. 6 **a** Calu-3 xenograft tumor samples fixed in room temperature formalin for 0, 2, 4, and 24 h (as indicated) and with the 2 + 2 protocol. Tissues were stained with an antibody recognizing the phosphorylated form of the protein AKT at Ser473 (CST #4060). **b**. Human Colon Carcinoma tissues with short cold ischemia (17 min). Tissues were fixed with room temperature formalin for 24 h [24] with the 2 + 2 protocol (2 + 2) or with room temperature formalin for 24 h after a purposeful 1 h additional cold ischemia (1 h ischemia + 24). For purposeful cold ischemia, samples were wrapped in saline-soaked gauze for 1 h at room temperature

5 Discussion

Histology is largely built on years of empirical observations and over time has drifted away from a centralized methodology. Unlike clinical chemistry that has standardized collection of blood samples with color-coded caps and tamper-proof containers, many different tissue preservation and fixation techniques exist. These

so called pre-analytical factors are present from the surgical suite to the grossing rooms to the histology laboratory. Taken together, there are staggering combinations of events that can take place with tissue samples even within a single institution. When multiple medical facilities are considered, the possibilities are endless and make comparison of similar diseases difficult. Just about every pharmaceutical company utilizes tissue samples obtained from biobanks around the world. However, the large number of preanalytical variables makes each tissue block unique. This makes it extremely difficult to achieve predictable results from comparison drug trials.

Even worse, variable fixation practices often limit the medical utility of resected material. One aspect of personalized oncology will be diagnosing each individual based on measuring the levels of activated proteins in driver pathways. It is clear that to get to the next advanced assays for this level of detailed diagnosis, fixation practices will need to advance such that stable and measurable biomolecules are preserved. With this in mind, we have shown evidence that the gold standard fixative, 10 % NBF, can be used effectively with a slight protocol modification. The key is to allow the fixative to diffuse into the tissue in an environment that preserves biomolecules such as lowering the temperature. Once there is sufficient fixative in the tissue, biomolecules can be rapidly locked into inactive states by increasing the temperature rapidly.

One important detail for tissue preservation is decreasing the time taken for the sample to get from the operating theater into fixative, so called cold ischemia [25]. Lowering cold ischemia time has been important for proper HER2 protein testing in invasive breast cancer [26–34]. ASCO/CAP guidelines mandate that these samples are placed into fixative in less than 1 h after surgery. However, activation state preservation will require even more strict guidelines to be implemented as many of these moieties are lost or change within 10–60 min post-surgery [35]. The good news is that lowering cold ischemia can help preserve posttranslational modifications although some still change with room temperature fixation. We have shown an additional benefit when tissues have low cold ischemia in combination with the 2 + 2 fixation protocol.

As medicine transitions from the one-size-fits-all approach to the ability to treat each patient's disease as a unique medical event, better assays targeting a wider variety of proteins will be required. One untapped area is being able to accurately utilize activated proteins. Currently, there are not many stabile assays built on these molecules due to their rapid turnover and instability. It is only natural that a good signaling molecule is unstable and able to rapidly change its concentration depending on extracellular events. We have presented evidence that with proper care, these next generation targets can be stabilized rapidly and with high fidelity.

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Towards the Lean lab: The Industry Challenge

Franco Visinoni

Abstract

In anatomic pathology, the current state encompassing the pre-analytic processes of tissue collection, handling, examination, preparation, processing, and storage are largely uncontrolled, inconsistently performed, and/or not standardized according to the sound scientific data. Pre-analytic defects result in nearly three-quarters of the problems in laboratory diagnostics. This is evident in quality surveys from well-respected institutions that document high miss rates in the required basics of information related to patient and tissue identity, let alone parameters documenting quality aspects related to the surgical specimen and its preservation. This talk will describe the historical approach to tissue processing and identify gaps from worldwide observations in current laboratory practices. It will also offer potential methodological and technological solutions and process improvements that laboratories may consider in serving the ultimate users of pathology information: the clinician and the patient. It illustrates the need for scientifically validated specimen guidelines and a performance based, standardized and documented "chain of custody" of the pre-analytical steps from the patient's body through fixation. For thought leaders and professional standard setters, opportunities for optimizing molecular studies exist in specimen collection, transfer, grossing, fixation, and decalcification protocols. In this evolving era of molecular profiling and personalized therapeutic decision-making, a well-reasoned and coordinated focus on pre-analytic processes that optimizes specimens for subsequent testing will result in:

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- Improved specimen quality for molecular testing
- Improved accuracy of diagnostic and molecular test results
- Reduced Turnaroundtimes for same-day diagnosis
- Enhanced satisfaction of clinicians and patients.

Contents

1 Introduction

The study of cells and tissues of pathological lesions, of their structural features and of related chemical components, still play a pivotal role in diagnosis, management, and treatment of patients. With the advent of modern personalized therapies, the role to be played by surgical pathology has even become more critical, since proper tissue preservation and handling according to standardized protocols should be mandatory and I agree with Groenen et al. [16] 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."

Indeed, standardization in tissue processing represents a major challenge for both, research and industry and the need to optimize the pre-analytical processing of tissue specimens was stressed by the National Cancer Institutes Office of Biorepositories and Biospecimen Research (USA), stating that billions of dollars have been wasted in the past because researchers developing biomarkers supposed to be predictive of cancer and responses to therapies relied on tissue samples that were utterly useless: tissue had been subject to careless handling and storage and sampling procedure were missing, so that results were not reproducible.

We have to realize that, within the diagnostic domain, while chemical Pathology laboratories and imaging departments have in the latest times witnessed a technological revolution requiring major investments, Pathological Anatomy is still basically bound to procedures introduced in the nineteenth century, when a series of technical procedures involving fixation and paraffin embedding were devised with the goal of obtaining morphological patterns both, reproducible and as close as possible to the original "in vivo" situation. For this purpose, the process of paraffin embedding, as originally devised by Klebs [24] had a few changes and the main technical improvement introduced by the industry was the semiautomatic processor. This apparatus, originally devised in 1909, had minor improvements in a century and its principle is still at the base of present-day histoprocessors.

The same lack of technical improvements applies to various different steps along the histopathological processing. Tissue fixation is still conducted manually and with the use of formaldehyde originally proposed by Blum [2, 14]. Moreover, grossing and decalcification are still carried out by manual, traditional processes. Not only, lack of technical improvements is paralleled by lack of proper standardization, so that concentration of the reagents time and temperature of use vary in different laboratories worldwide according to local habits.

This status of affairs is perhaps understandable, since until recently the sole criterium of validity of histological preparations was morphological preservation and structural patterns, comprehensive of ultrastructural images, were the main basis for disease classification.

In more recent times, the use of histochemical and immunohistochemical techniques has allowed pathologists to develop more precise, reliable, and reproducible disease classifications and to complement morphology with information regarding protein (antigen) expression and distribution. It has thus become mandatory to rely

on technical preparation providing sections optimal for microscopical observation, but at the same time preserving the biological integrity, particularly of the proteins and nucleic acids.

Since 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.

Lately, 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 [3, 13, 39].

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) [23, 45] or the assessment of microsatellite instability for the identification of carriers with increased risk for Lynch Syndrome [32, 35]. 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 [4, 21, 22, 34, 44] the evaluation of mutations of EGFR and K-RAS genes in lung and colorectal adenocarcinomas [25, 33, 37, 38], of cKIT and PDGFRA genes in cases of gastrointestinal stromal tumors (GISTs) [1, 19, 26] and of BRAF and NRAS genes in melanomas [17, 41], and last but not least the evaluation of the status of the HER2 for breast cancer [10, 28].

These tests require a proper preservation of tissues, to be analyzed in parallel for tissue structural arrangement, protein (antigen) distribution, and gene sequencing. Variations on the conditions of fixation and paraffin embedding heavily influence both structural and chemical preservation, thus bearing great impact on diagnostic definition and, ultimately on therapeutic treatment.

We are in conclusion witnessing a severe gap between the request for proper, selected, and standardized processing of pathological tissues and lack of novelty in the offer by the industry of dedicated apparatuses. This gap is, in our opinion, critical in the areas of (1) Tissue transfer, (2) Grossing, (3) Fixation, (4) Decalcification, and (5) Fast Embedding in Paraffin.

2 Tissue transfer

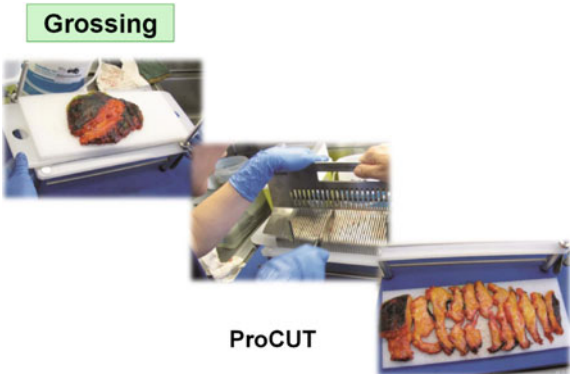
Once surgically removed from the body, the specimen has to be transferred to the pathology lab for histopathological examination. Time and conditions of transfer are critical, since fresh tissues are susceptible to autolytic process, which are bound

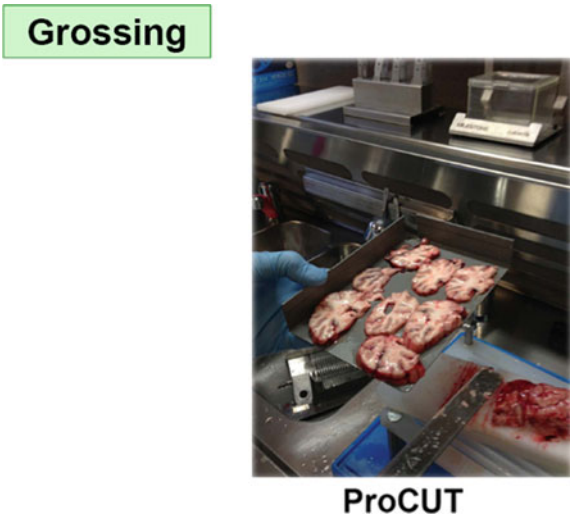
to affect structure and biochemical components. Ways of transfer of surgical specimens vary according to the architectural layout and distance between surgical theaters and pathology laboratories. The ideal situation is when physical location and hospital protocols allow for the immediate transfer of "fresh" tissue specimens for prompt grossing and fixation. Accordingly, it has been recommended the "ischemia time" to remain below 1 h to achieve a proper processing of breast cancer specimens, permitting a correct evaluation of both morphological and therapeutic-prognostic parameters [15, 43]. Problems arise when this is not feasible being the pathology laboratory located far away from the surgical theater. 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 usually once every day. This practice carries problems, since autolysis will continue in central areas of the specimen. Protection of fresh tissues from drying is essential, and this can be achieved by sealing tissues under-vacuum in a plastic bag [6]. This offers the additional advantage of removing air, thus oxygen with the double advantage of blocking aerobiotic processes and of facilitating cooling of specimens because of the lack of insulating air. Our company has been patenting an dedicated apparatus (Tissue Safe, patent EP 2070410 B1) which meets these demands and encountered the approval of several laboratories.

3 Grossing

Histopathological processing of specimens larger than 2 cm requires grossing and selection of sections from significant areas, a process which can only be performed in pathology laboratories by qualified personnel 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.

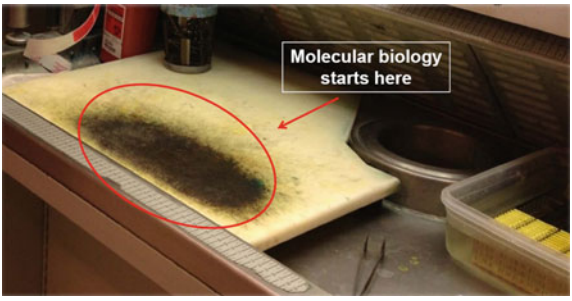
Still, the grossing of fresh and soft tissues is not easy. Our company, in order to facilitate the grossing at proper thickness has been devising and producing special forceps (CUT-Mate forceps).

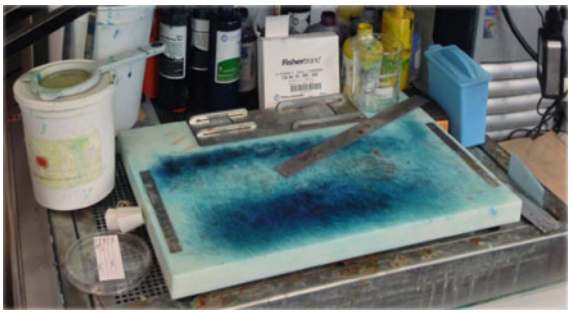




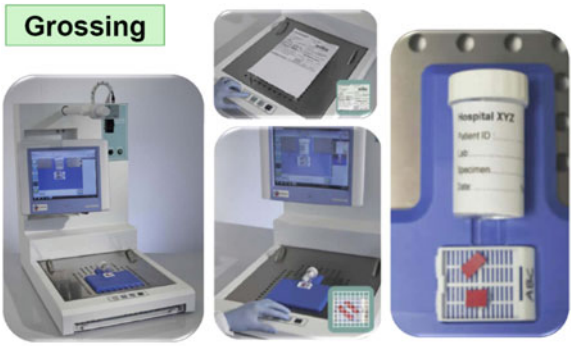
CutMATE Forceps (2-3-4 mm)

The grossing time is at risk of incorrectly mixing specimens from different cases (and patients). Until recently, the consequences of this risk were ultimately minor and did not influence the final diagnosis, since minute residues of "external" source could be histologically recognized and therefore dismissed. The advent of molecular analyses applied to tissue blocks has changed the situation, because of the lack of histological control and of the sensitivity of the procedures. It has therefore become essential to avoid the risk of mixing of specimens, as could be for instance due to the use of a single table for the grossing of all specimens. Our company has developed special polypropylene sheets to be placed and held on top surface of the cutting board. A new one for each single specimen. These sheets provide a safe, simple, and effective solution of the problem of contamination of specimens. In addition, the sheets can be washed in the cab washer during the night to be reused the next day.





Once grossed, the surgical specimens are not recognizable any more, while documentation of the original specimen, of the site of the grossing and a measure of the different areas is essential both, for the pathologists when reading the slides and for a proper and documented discussion with surgeons, clinicians, and radiologists. Our company has devised the Macro-Path apparatus which meets these requirements, by digitally documenting the whole grossing process, including sizing, sections, and area of disease.



Lean BX Station

Macro documentation of small biopsies

4 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.

A 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 overfix and it guarantees, in appropriate conditions, an optimal morphological preservation so that we have to conclude that substitution of formalin with alternative fixatives cannot be foreseen at present [8].

However, length, temperature, and modalities of formalin fixation are not standardized and vary extensively in different laboratories.

The length of formalin fixation can affect the results of the immunostaining [9, 18, 27] since under fixation often produces a reduced immunostaining in the central region of the tissue block with stronger immunoreaction in the marginal area of the section, while overfixation generates the opposite aspect (good staining in the inner area and poor staining outside.

Moreover, formaldehyde fixation modifies the conformation of macromolecules, altering tertiary and quaternary organizations of proteins, whereas the primary and secondary structures are scantily affected [12, 29, 31]

Such conformational changes may hamper the link to the antibody, but the use of antigen retrieval procedure can return immunoreactivity in formaldehyde-fixed specimens [40].

Finally, if formalin does not penetrate completely the tissue during the fixation process, the subsequent use of ethanol (as a dehydrating agent) during paraffin embedding may produce in the tissue blocks central areas which are predominantly alcohol-fixed, and this can procedure additional variable results [20, 42].

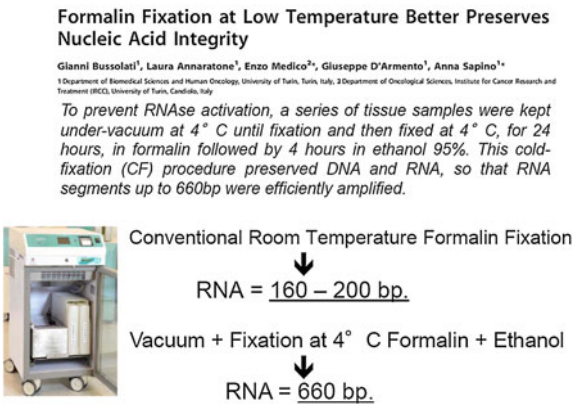
This issue is particularly relevant in oncopathology for the evaluation of factors predicting responsiveness to therapeutic treatments, and thus, fixation in phosphate buffered formalin (PBF) of breast cancer tissue blocks for no less than 6 and no

more than 48 h is now required in order to guarantee an optimal evaluation of Estrogen (ER) and Progesterone Receptors (PgR) and HER2 expression by immunohistochemistry [15, 43].

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. Studies conducted on the preservation status of nucleic acids in FFPE tissues generally agree on the relatively good (though not optimal) preservation of DNA. On the contrary, 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 [11, 30, 36].

Innovative protocols have, however, been proposed or permitting gene expression profiling on FFPE tissues from cancer patients [7].

A variation in formalin fixation resulting in improved preservation of RNA has recently been proposed by Bussolati et al. [5].

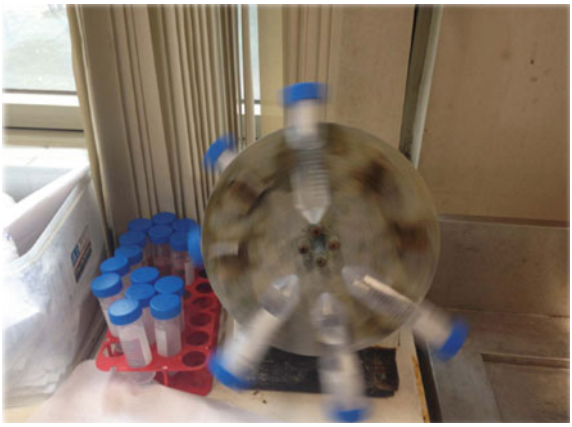


This is based on a fixation process in formalin at 4 °C, followed by dehydration in cold ethanol and paraffin embedding (CFFPE). Using this procedure, we succeeded in obtaining a substantial reduction in RNA fragmentation in FFPE tissue blocks, as assessed by RT-PCR and gene array analysis, while at the same time preserving the morphological and immunohistochemical properties which make formalin the fixative of choice in histopathology.

5 Decalcification

Decalcification is the process whereby mineralized tissues are currently examined histologically on sections from paraffin-embedded blocks. Removal of calcium salts is effected, on fixed tissues, by agents (acids or chelating agents) leaving unaltered the protein components and the structure of the tissue. This process, in histopathology, is currently used for the study of bone tumors and of bone marrow biopsies, a diagnostic procedure of great value in hematopathology. Decalcification

is carried out almost universally in a manual manner, without any standardization of time–temperature, pH, agitation, and reagents management.

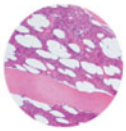


The time length and conditions of decalcification vary according to the agent used and the temperature, so that the stronger the acid solution and the temperature, the faster the decalcification. However, a fast decalcification, as for instance the one obtained by using 5 % Nitric acid, can have a detrimental effect on the tissue structure and the preservation of antigenic components.

This issue is of the upmost importance in the study of bone marrow biopsies, where an immunohistochemical study of the cellular population is of diagnostic interest.

Our company has developed an automated decalcification unit to standardize all parameters and, by using a proprietary solution of bi/tri-basic EDTA, allows the full recovery of nucleic acids for molecular studies.

Protocols for Bone Marrow



DECAL REAGENT	DECAL TEMPERATURE	FIX	DECAL	PROCESS	TOTAL
EDTA 10%	A/T (27° C)	4h	36h	1h40m	41h40m
EDTA 10%	37° C	1h30m	17h30m	1h40m	20h40m
EDTA 10%	50° C	1h30m	5h	1h40m	8h10m
Formic 10%	50° C	1h	1h30m	1h40m	4h10m

6 Conclusions

Role and significance of histopathological diagnoses are basically the detection of origin and genesis (etiopathology) of diseases and the prognostic forecast of their evolution. However, histopathology is also pivotal in the planning and conduction of proper therapies and, if possible, its importance has been enhanced in recent times with the advent of personalized and molecular therapies.

It might therefore appear surprising that the technical procedures leading to the preparations of histopathological slides (s.c. pre-analytical steps) have remained practically unchanged since over one century. We are witnessing a real need of technological advancement of the pre-analytical technical procedures, in order to guarantee a more reliable, standardized, and rapid preparation of paraffin-embedded tissue blocks. In order to fulfill the gap, the industrial companies can and should play a necessary role, not deprived of commercial interest and in collaboration with Pathologists.

Since over 20 years, our company has been proposing and distributing on the market original apparatuses for quick fixation and paraffin embedding, for grossing in a more reproducible and recorded way, for decalcification of bone biopsies, and for the safe transfer of surgical specimens. We have, in other words, been proposing innovative solutions for each of the separate and interconnected steps involved in the pre-analytical phase. The proposed solutions represent steps forward whose acceptance by the market and, in parallel, the appreciation by pathologists, are the necessary stimulus for proceeding along an ongoing process, whose ultimate goal is the care of patients.

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