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FACTORS INFLUENCING THE QUALITY OF FORMALDEHYDE FIXED PARAFFIN EMBEDDED TISSUE SAMPLES –REVIEW

FACTORI CARE INFLUENŢEAZĂ CALITATEA PROBELOR DE ŢESUT FIXATE ÎN FORMALINĂ ȘI INCLUSE ÎN PARAFINĂ - REVIEW

Daniela GOLOGAN[1),4)], Alina Elena ȘTEFAN[2),4)], Raluca STAN[1)], Manuella MILITARU[2)], S. MUȘAT[3),4),*)]

ABSTRACT | REZUMAT

Variability in the handling, histological processing and storage of tissue samples has been shown to affect their molecular integrity and influence the final histopathology diagnostic/therapeutic intervention, increasing the need for standardization of pre-analytical protocols. In recent years it became widely recognized that standardization of tissue processing is critical: removal and handling of tissue samples (warm ischemia), time before fixation (cold ischemia), time and type of fixation, method and time of processing, etc. The purpose of this review is to summarize the literature which describes different pre-analytical factors associated with dehydration, clarifying agents, impregnation and embedding with a support medium with a focus on the processing methods and their influence on the result.

Keywords: pre-analytical variables, tissue dehydration, tissue artifacts

S-a demonstrat că variabilitatea în manipularea, prelucrarea histologică și depozitarea probelor de ţesut afectează integritatea lor moleculară și influenţează diagnosticul histopatologic/ intervenţia terapeutică, ceea ce crește nevoia de standardizare a procedurilor preanalitice. În ultimii ani s-a recunoscut faptul că standardizarea procesării ţesuturilor trebuie să ţină cont de: procedurile de recoltare și manipulare a probelor de ţesut (ischemia la cald), timpul înainte de fixare (ischemia rece), timpul și tipul de fixare, metoda și timpul de procesare etc. Scopul acestui studiu este de a sumariza literatura de specialitate care descrie diferiţi factori preanalitici asociaţi cu protocolul histologic (deshidratare, clarificare, impregnare și încorporare în mediu suport), metoda de procesare și influenţa acestor factori asupra rezultatelor finale.

Cuvinte cheie: variabile pre-analitici, deshidratarea ţesutului, artefacte tisulare

In pathological anatomy it is necessary that all the stages prior to diagnosis are done correctly, safely and adapted to the type of tissue samples.

The most common method used to study tissues is the preparation of histological sections that can be examined with a visible light microscope. For histological study, the tissues (whether biopsies, larger specimens removed at surgery: explants, or tissue samples harvested during autopsy), are prepared in such a way as to faithfully reveal the structure and molecular composition of the organ /structure they derive from. The stages of tissue processing include fixation, dehydration, clearing, impregnation and embedding (35).

In the age of molecular medicine, new tests are being developed for assessing which patients are likely to benefit from specific therapeutics agents ("personalized medicine"), and as a result the use of immunohistochemical methods is increasing. Making therapeutic decisions based on immunohistochemical assays brings new responsibilities and challenges to the pathology laboratory, particularly with regards to standardization of pre-analytical and analytical factors (28).

1) Politehnica University of Bucharest, Faculty of Applied Chemistry and Materials Science, Department of Organic Chemistry, Bucharest, Romania
2) University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Bucharest, Romania
3) Lumea Inc., Lehi, UT, Unites States of America
4) S.C Themis Pathology S.R.L, Voluntari, Ilfov, Romania
*) *Corresponding author: sorin@lumea.net*

STAGES OF HISTOLOGICAL PROTOCOLS

Fixation is a crucial step in processing tissue specimens. Fixation helps preserve cellular architecture and chemical composition, preserve the proteins, carbohydrates, and other molecules and prevent cellular autolysis and inactivate the enzymes. To prevent autolysis, fixation should be carried out as soon as possible after removal of the biopsy. Therefore, a variety of fixatives are available for use, depending on the type of tissue and features to be demonstrated: conventional fixatives, such as formaldehyde, glutaraldehyde, ethyl alcohol, or alternative fixatives like chromic acid, mercuric chloride, mercuric-acetic acid and mercuric-formaldehyde mixtures, picric acid, Zenker, Bouin's solution, honey, etc., (11). There is no perfect fixative, but formaldehyde seems to be the best. Routinely, tissues are fixed with 3.7% formaldehyde in water, stabilized with 1% methanol, pH 7.0-7.4 (50 mM phosphate buffer), which means 10% formalin (22).

Fixation is the variable that can affect most significantly immunohistochemical staining (17).

The most used fixatives for immunohistochemical staining are formaldehyde, ethyl alcohol and acetone.

Formaldehyde reacts with amino groups in proteins to form methylene bridges that crosslink proteins and can mask protein epitopes from antibody binding. This might require the addition of an antigen retrieval (unmasking) step for dependable immunohistochemical staining. Formaldehyde based fixatives can also induce the movement of phosphorylation-dependent epitopes from the membrane to the cytoplasm. Fixation with ethyl alcohol obviates the need for antigen retrieval and is more suitable for the detection of membrane-bound proteins because it can cause precipitation of soluble proteins. Acetone fixation is generally slower and because of that may be completed with alcohols or formaldehyde fixation (16).

Dehydration is the process of complete removal of residual fixative and water from the tissue, and it is required because the support medium used for tissue infiltration is hydrophobic. The hydrophilic capabilities of the dehydrant combined with a gradual increase in its concentration will result in a slow and steady displacement of free water (integral tissue components such as connective tissue fibers and cellular components can become distorted or damaged by the effects of precipitous dehydration) and the removal of structural bound water (bound water molecules are normally attached to proteins by hydrogen bonds and are part of the structural configuration of the amino acids). Bound water can be removed during excessive dehydration and harsh and irreversible changes of tissue morphology can result. These powerful hydrogen binding dehydrants compete with tissue molecules for the availability of water molecules, because of their efficiency in removing interstitial and bound water (26). The dehydrating agent is replaced with a "transition or clearing" agent (aromatic or aliphatic hydrocarbons, esters or terpenes) which further facilitates the infiltration of the support medium to allow the tissue to be sectioned into 4-5 µm thick paraffin sections (usually with a rotary microtome) and affixed on a glass slide for microscopic examination. The term "clearing" is used due to the relatively high refractive index of the cleared samples wich makes the tissue clear or transparent (35, 39).

Infiltration and embedding refers to the stage when the tissue is infiltrated with support medium (i.e. paraffin, celloidin, agar, gelatin, acrylic or epoxy resin) (7). The duration of this procedure depends largely on the dehydration and clearing agent employed and on the consistency of the sample (12).

PRE-ANALYTICAL FACTORS

Bass B.P. identified in 2014 the following potential sources of pre-analytical variability: procurement, fixation, processing, and storage of human tissue biospecimens (Table 1 and Table 2). Deleterious effects on DNA, RNA and protein integrity as well as regarding microscopic anatomy (morphology) are also documented (2, 18, 21). While the impact of variability in the pre-fixation and fixation stages of tissue processing is fairly well studied, a lot less attention was paid to the undeserved lack of standardization concerning dehydration and clearing.

Table 1
Pre-analytical factors associated with procurement and fixation of tissue biospecimens

Procurement	Cold ischemia (delay of fixation) Warm ischemia (delay in removal and handling of tissue) Postmortem interval Specimen size Pathology marking ink
Fixation	Decalcification Fixation duration Pre-fixation handling Fixative buffer Tissue to fixative ratio Fixation temperature Fixative delivery method Fixative freshness Commercial versus in-house fixative Use of recycled formalin Movement during fixation Light exposure during fixation Fixation container Fixation alone or with other biospecimens Post-fixation wash solution and conditions

Table 2
Pre-analytical factors associated with processing and storage of tissue biospecimens

Dehydration and clearing	Dehydration reagents and protocols used Storage of reagents Use of recycled dehydration and clearing reagents Clearing reagents and protocols used Automated versus manual processing
Paraffin block	Duration of paraffin block storage Block size and section thickness Use of recycled paraffin Embedding conditions
Slide	Type of slide, coverslip and adhesive used Slide drying duration and temperature Storage duration of mounted slide Sections Slide pretreatment Equipment and conditions of sectioning and section transfer

DEHYDRATION REAGENTS AND CONDITIONS

Dehydration is employing water soluble agents of increasing concentration for avoiding excessive distortion. The period of immersion in dehydrating agents varies on size (the thicker the block, the longer the time) and penetrability of the tissue sampled as well as physical factors such as temperature and agitation. If the tissue is exposed for a too long period of time in a high concentration

it will shrink significantly. A too long treatment with low concentration of dehydrant will macerate the tissue. If the tissue is incompletely dehydrated the paraffin will not infiltrate properly generating blocks that will section with difficulty (if at all) resulting in holes and breaking artifacts. One sign of incomplete dehydration is the depression of the tissue block within the surrounding paraffin when exposed to air (35, 37).

In choosing the appropriate dehydrating agent we need to take into consideration the nature of the task, the processing method, the embedding medium, costs, etc. Some solvents can be used both as dehydrant and clearing reagents, due to their miscibility with both water and paraffin. Exemplary solvents in this category are butyl alcohol, dioxan, tetrahydrofuran (39).

Commonly used dehydration reagents are: **ethyl alcohol:** rapid, efficient and the most widely used dehydrant; it has a powerful penetrability into the tissue and under microwave processing conditions is also a good lipid solvent (7, 12, 39); **isopropyl alcohol:** a superior lipid solvent than ethyl alcohol (39); **methyl alcohol:** a poor lipid solvent (7, 39); **acetone:** has the capacity to disolve fat and preserve the imunologic characteristics of tissue (7, 13); **n-butanol** and **isobutanol:** slow in action (39); **tert-butanol:** has the disadvantage that freezes at 26°C (39); **dioxane:** gentle in action but very toxic (39); , **tetrahydrofuran:** dehydrates rapidly but can be toxic after long-term exposure (7); **acetic acid:** freezes at 16.6°C (22); **phenol:** caustic and toxic (39); **acetonitrile:** poisonous (39); **ethylene glycol, glycerol** and **propylene glycol:** miscible with water and dehydrating agents, but not miscible with clarifying agents (39).

CLEARING REAGENTS AND CONDITIONS

When choosing a clearing solvent, one has to take into consideration the type and size of the tissue, processor type, processing conditions, safety factors, cost and convenience. Most widely used clearing agent is **xylene** which displaces rapidly the alcohol and leaves the tissue transparent. Tissues fixed in non-protein coagulant fixatives and cleared with xylene become hard, while tissues fixed with protein coagulant fixative are less affected (39). Xylene main drawback as a clarifying agent resides in its neurotoxicity after long-term exposure (6). Moreover, xylene passses through the placenta barrier and is also present in the maternal milk (20); **toluene** clears rapidly but is expensive (1, 7, 39); **benzene** acts more rapidly and gently than both xylene and toluene but it is carcinogenic (39); **n-butanol** is slow in action (12); **carbon tetrachloride, carbon bisulfide and gasoline** are very toxic and flammable (7); **chloroform** is a slowly penetrating solvent and is usually mixed with benzene (7, 22, 39); **1,1,1 trichloroethane** is very volatile (38, 39); **methyl benzoate** (39) and **amyl acetate** (22) are used especially as nitrocellulose solvents; **methyl salicylate** has gentle and rapid clearing properties (19, 39); **benzyl benzoate** gives transparency to the tissue (22); **amylic alcohols** show toxicity and carcinogenic activity in animal studies (30).Safer alternative clearing agents include vegetable and essential oils but presently are used rarely because of high cost. They are volatile but not enough to be replaced by paraffin during infiltration. For this reason the essential oils have to be removed by xylene or toluene prior to infiltration. Tissue cleared with essential oils harden less then after other clearing agents (7).

Vegetables and essential oils used for clearing: **cedar oil** does not cause skrinkage and hardening of the tissues giving good microscopic preparations in terms of staining quality, but is expensive (7, 12, 22); **olive oil** has equivalent properties to xylene but it is displaced by molten paraffin with difficulty due to its viscosity (4, 36); **coconut oil** is a good substitute for xylene but has the tendency to solidify at a lower temperature (33); **clove oil** gives a strong and slightly sweet odor (1); **palm kernel oil** is less expensive, non-toxic and non-hazardous (1); **bleached palm oil** gives good tissue sections (32); **orange oil** is excellent for preserving fine tissue structures but is not stable and can produce compounds that will affect slide staining (1); **mineral oil** after alcohol dehydration results sometimes in very poor infiltration with paraffin, because ethanol is not completely eliminated from the tissue. This problem can be solved by clearing the tissue after dehydration at 50°C with a mixture of isopropanol and mineral oil and increasing its proportions from 5:1 to 2:1, followed by undiluted mineral oil) (4). A list with alternative clearing agents used in histology was compiled by Buesa in 2009 (4).

INFILTRATION AND EMBEDDING

Various substances have been used to infiltrate and embed tissues for microtomy, such as various natural or synthetic waxes which are fluid when heated and solid when cold, or a solution of nitrocellulose in alcohol-ether, which solidifies on evaporation of the solvent. The most widely used infiltration medium is paraffin wax. Paraffin is a mixture of hydrocarbons produced by petroleum cracking which contains additives to improve its properties for histological applications such as: hardness, stickiness, and brittleness (39).

Paraffins with a melting range of 55°C-58°C are used for routine work to provide good support for most tissues and have to be typically used during processing at 2-4° C above the melting point for good infiltration. Too much heat and/or extended stay in liquid (hot) paraffin may harden excesivelly the tissues and create problems during microtomy (7). Low melting temperature waxes (45 °C) have been reported to produce better staining results for immunohistochemistry, particularly in T-lymphocyte staining (15). However, some authors argue that hardening appears if the tissue is not well dehydrated and cleared prior to paraffin infiltration, and not necessarily because of exposure to hot paraffin (22). Alternative infiltration media like epoxydic resins and methyl-metacrylate (embedding media for electron microscopy and undecalcified bone), agar (used after fixation as a cohesive agent for small, friable pieces of tissue and followed

by regular paraffin embedding, since alone, agar does not provide sufficient support during sectioning), gelatin and celloidin (for dense and hard tissue) can be employed when paraffin wax is an unsuitable medium (35).

After tissue infiltration, it is necessary to place the tissue in a mould filled with molten embedding medium, for creating after cooling and solidification "paraffin tissue blocks" ready for microtome sectioning. Usually, the paraffin used for infiltration is also used for embedding, but occasionally lower melting point paraffins are used for infiltration while for embedding a harder (higher melting point) paraffin is preferred (e.g., for sectioning of very dense tissues such as tendon, cartilage, bone, etc.). For special applications some other embedding media can be used, such as celloidin, gelatin, carbowax and synthetic resins (12,35).

PROCESSING METHODS

All or some of the stages required for tissue processing can be done either manually or automated. Manual processing is slower, requires personal safety equipment for avoiding toxic fumes and is labor-intensive since all solution changes are done by hand and usually performed at room temperature. Its advantage is that it is not an expensive procedure. Automated processing includes the same steps as manual processing, is faster, usually results in more consistent outcomes, can operate with both positive pressure and/or vacuum, can be accelerated by controlled heating and most importantly can operate without human supervision (5, 31).

Automated processing is done by 3 main types of tissue processors (35):

1. Specimen-transfer or "dip and dunk": carousel or linear (cassettes with tissue samples are transferred from station to station). The disadvantage of using this type of tissue processor is that the open reagent containers not only allow release of toxic fumes in the laboratory but can also cause a fairly quick deterioration of the dehydrating solvents because of absorption of water vapors. They are also lacking the options of pressure, vacuum, and temperature control.

2. Fluid transfer or "enclosed" processors (reagents are pumped in and out from a central retort where cassettes are hold). Each solvent station can be tailored in terms of duration, temperature and cycles of pressure / vacuum.

3. Microwave assisted processing allows the fastest tissue dehydration, clarification and paraffin infiltration but requires very careful optimization for each tissue-type and thickness of specimens.

As we move into 21st century, the clinical demands in pathological anatomy are increasing faster than they ever did (mainly because of the advent of molecular techniques employed for personalized medicine). The need for faster tournaround times in conjunction with the new interest in switching to nontoxic, non-petroleum based and recyclable chemicals is resulting in a new impetus in developing new equipment and protocols, in a medical laboratory discipline which was notoriously very conservative. Numerous commercial conventional tissue processors, microwave or hybrid are available.

Buesa (2007), made a brief analysis of the impact of the type of tissue processor on the histology workflow and on costs, concluding that the selection of microwave (or hybrid) tissue processors coupled with an optimized workflow (by proven automated technologies for as many tasks as possible), can reduce turnaround time for any laboratory (5).

Compared with conventional methods, microwave-assisted tissue processing is cost effective and has no deleterious effects on the quality of staining and cellular details regardless of the brand of the microwave oven employed: domestic (IFB India 20PG 1S, LG Model no. MS-285SD, India), manual microwave vacuum histoprocessors (Milestone RHS-I) or advanced automated microwave processors (Tissue-Tek Xpress Rapid Tissue Processor, Sakura) (25, 27, 29, 34). The main limitation of microwave tissue processing is the formation of "hot spots" (areas within the tissue sample with a higher temperature than others), generally in samples thicker than 9 mm, resulting in inadequate histological quality of microscope slides (31).

Recent studies are showing that processing the tissues with microwaves or ultrasounds may reduce the time required for sufficient tissue processing. Chu et al. (2005, 2006) applied continuously ultrasounds to the tissue samples via a transducer immersed in the solutions holding the tissue samples (fixative, alcohols, xylene, and paraffin), during a 1-hour total time protocol. This technique resulted in superior morphology (evaluated by hematoxylin–eosin staining), improved protein antigenicity (evaluated by immunohistochemistry), and better DNA and RNA preservation (8, 9).

Bleuel et al., (2005) developed and patented a fast, solvent-free tissue processing method using supercritical carbon dioxide (Tispa Tissue Processor from Tispa Medical B.V, the Netherlands) that can be operated with continuous flow. The results obtained with Tispa Tissue processing are almost similar with the results obtained with a routine tissue processor (Shandon Excelsior ES A78410 106) but with results ready in only 4 hours, instead of 14 hours for 4 mm thick samples (3).

It is very important that the tissues are adequately fixed and processed. When the tissue is not completely fixed and/or dehydrated and/or cleared, artifacts can occur. Artifacts exemplified by Taqi et al. in 2018 can be greatly reduced by the standardization of processing protocols (37).

Despite numerous observations suggesting that incomplete fixation and dehydration of tissues as well as improper storage of the resulting paraffin blocks or tissue sections will lead to hydrolysis of protein biomarkers (and nucleic acids) (40) resulting in antigen loss (2,14), there is a lack of systematic studies aimed at quantifying the residual water in tissue samples processed for histology.

A more detailed study discussing residual water percentage at various stages of tissue processing was per-

formed by Bleuel et al., 2012 demonstrating that the residual water in tissue samples varies across the three main stages of histological processing (after ethanol, after xylene and after infiltration with paraffin) and is heavily influenced by the freshness of the reagents used in the process. After routine dehydration in a graded ethanol series with fresh solvents the samples contain up to 6% water. After clarification with xylene up to 5% residual water (for fresh solvents) and 5.5% (for reused solvents) is still left within the tissue while even after the completion of the processing protocol (i.e. after infiltration with paraffin) up to 1.5-3% water can be detected in the resulting paraffin blocks. Interestingly, while fresh solvents resulted in slightly less residual water in the paraffin blocks versus older solvents, shrinking and brittleness of the processed tissue increased (undesirable outcomes since sectionability is becoming difficult and the diagnostic quality can be also hampered by the resulting artefacts) (3). The quantification of the diffusion rate of reagents into the sample to be processed, probably the most critical parameter for a successfully processed tissue was tackled only very recently by Lerch et al., in 2017 who provided a quantifiable quality metric for tissue fixation with an ultrasound time-of-flight (TOF) instrument, capable of monitoring and imaging the critical step of formaldehyde fixation (23).

Lerch et al. expanded their investigations on tissue processing in 2019 by monitoring dehydration and clearing. TOF detected this difference in solvent diffusion correlated well with histomorphology artifacts, (improperly processed tissue resulted in folded tissue sections, tears, etc.). This technique has the potential to provide an important preanalytical quality metric that might ascertain that immunohistochemistry and/or other downstream bioassays can deliver accurate diagnosis to patients (24).

Unfortunately, tissue processing protocols are still not standardized, each histology laboratory empirically trying to adjust them for the type and size of tissue being processed, number of cassettes, required turnaround time, type of processor and reagents used. The only metrics used are the perceived (by the microscopist) quality ("beauty") of the resulting slides, consistent, specific and reproducible tinctorial affinities, etc.

With regards to the ideal processing strategy, there is a general consensus that in routine processing protocols the time ratios are 40% dehydration, 30% clearing and 30% infiltration. For xylene free processing, ratios are 35% dehydration, 30 % clearing (isopropanol) and 35% infiltration. The tissue processing protocol is the most common variable with modern tissue processors. Due to this lack of standardization, at present total processing time varies between 1-2 hours (especially with microwave tissue processors) to 24 (or more) hours (10, 35).

CONCLUSIONS

Formalin-fixed paraffin-embedded tissue is a powerful tool in anatomical pathology. However, despite the constant pressure for faster turnaround times the basic principles for accurate pre-analytical protocols (proper fixation, gentle but complete dehydration, proper clarification, complete paraffin infiltration and careful orientation during embedding) should never be overlooked. The unsatisfactory diffusion of solvents into the tissue and the presence of residual water in the paraffin blocks could lead not only to unsightly artifacts but also to equivocal or even erroneous diagnostics.

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