

The mandate for a proper preservation in histopathological tissues

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Abstract

A sequence of technically reproducible procedures is mandatory to guarantee a proper preservation of tissues and to build up the basis for sound diagnoses. However, while the goal of these procedures was, until recently, to assure only structural (histological and cytological) preservation, an appropriate preservation of antigenic properties and of nucleic acid integrity is now additionally requested, in order to permit pathologists to provide the biological information necessary for the adoption of personalized therapies. The present review analyses the sequence of technical steps open to critical variations. Passages such as dehydration, paraffin embedding, sectioning and staining are relatively well standardized and allow adoption of dedicated (automatic) apparatuses, while other pre-analytical steps, i.e. time and modalities of transfer of surgical specimens from the surgical theatre to the pathology laboratory (s.c. “ischemia time”) and the type and length of fixation are not standardized and are a potential cause of discrepancies in diagnostic results. Our group is involved in European-funded projects tackling these problems with the concrete objective of implementing a model of effective tumors investigations by high performance genetic and molecular methodologies. The problem of the discrepant quality level of histopathological and cytological preparations involved five European countries and exploiting the potential of “virtual slide technology”. Concrete issues, techniques and pitfalls, as well as proposed guidelines for processing the tissues are shown in this presentation.

Keywords: histopathology, fixation, standardization.

Reasons

The essence of histopathological investigations is an analytical approach to both form and tissue content, finalized to detect nature, causes and evolution of diseases. In the early days, diagnosis was solely based on morphological features, and structural patterns, including ultrastructural details, 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 optimal sections for microscopical observation, while at the same time preserving the biological integrity, particularly of proteins and nucleic acids. A series of technical procedures involving fixation and paraffin embedding were thus devised and properly standardized, with the goal of obtaining morphological patterns both reproducible and matching the original *in vivo* situation. For this purpose, the time-honored process of paraffin embedding, as originally devised by Klebs E (1869) [1] maintains his value, since it allows the preservation of tissues for long periods (years) in a non-reactive environment. Paraffin embedding has to be preceded by fixation, which blocks and preserves the structure and the cell components as close as possible to the living condition. This is accomplished by chemical and physical

procedures, and several alternatives have been proposed.

Variations on the conditions of fixation and paraffin embedding heavily influence both structural and chemical preservation, thus bearing great impact on diagnostic accuracy and ultimately on subsequent therapy. Alcoholic fixatives, such as Carnoy fixative or mixtures of chemical reagents have been proposed, but still the most popular, dependable and cheap solution remains formalin fixation, as originally proposed by Bloom [2, 3] in 1898. The choice in favor of the use of a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4 (buffered formalin) as universal fixative, followed by dehydration and paraffin embedding, was further stressed by the serendipitous observation that a simple heating of formalin-fixed histological sections, using microwaves or alternative procedures was able to retrieve, to a great and advantageous degree, the antigenic reactivity [4]. This observation, which opened the way to a routinely use of immunohistochemistry for both diagnostic and predictive purposes, implied also a mandate for a more reliable and standardized processing of histopathological tissues. In fact, a major factor determining accuracy and reliability of the immunohistochemical results is the modality of tissue preservation, fixation and paraffin embedding.

These preliminary processes performed before microscopic examinations are unfortunately poorly standardized in pathology laboratories worldwide. The need to optimize the pre-analytical processing of tissue

specimens was stressed at the National Cancer Institutes (USA) by the Office of Biorepositories and Biospecimen Research (OBBR) whose mission is “to guide, coordinate, and develop the Institute’s biospecimen resources and capabilities and ensure that human biospecimens available for cancer research are of the highest quality” (www.biospecimens.cancer.gov).

We agree with Groenen PJ *et al.* [5] 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”.

Histopathological diagnosis is a fundamental, integrated element of the prognostic, predictive and therapeutic management of diseases. The field has been gaining even more interest in recent years with the advent of personalized therapies for various tumor entities.

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 assist conventional histopathology. This is reflected by the progressive evolution of the WHO Classification report outlined in the “Blue Books” which were initially relying only on histological features, while more recent editions use the results of genetic analysis to complement, but never substitute, the morphological characterization [6–8].

The consequence of this improvement is that cancer diagnosis for individual patients has become more complex and molecular tests have become routine in some laboratories, for example, in identifying gene mutations responsible for familiar hereditary tumors of endocrine organs (MEN 1 and MEN 2 syndromes) [9] or the assessment of microsatellite instability for the identification of carriers with increased risk for Lynch syndrome [10–12]. Nowadays, morphological diagnoses are not sufficient for planning personalized therapies that require, in some instances, the detection of chromosomal translocations and aberrations in sarcomas and brain tumors [13–17], the evaluation of mutations for EGFR and K-RAS genes in lung and colorectal adenocarcinomas [18–21], cKIT and PDGFRA genes in cases of gastro-intestinal stromal tumors (GISTs) [22–24] and BRAF and NRAS genes in melanomas [25, 26] and, last but not least, the evaluation of the status of HER2 for breast cancer [27, 28].

These tests require a proper preservation of tissues destined to be analyzed in parallel for tissue structural arrangement, protein (antigen) distribution and gene sequencing.

The first, as well as the most crucial step of tissue processing in histopathology is the proper harvesting and sampling of the biological material.

The present review deals with improvements to the procedures of pre-analytical tissue processing, including pre-fixation, fixation and embedding steps, which are

mandatory to reach a proper standardization and a better preservation of tissue components.

☞ Procedure

Procedures for tissue handling basically involve a series of step-wise passages, leading from the biopsy removal up to the paraffin block and involve: (1) transfer from the surgical theatre to the Pathology laboratory; (2) optimization of formalin fixation in order to improve preservation of antigenic components and of nucleic acids in tissue specimens; (3) paraffin embedding.

Variations, thus lack of standardization, in the practice of these steps are common in pathology laboratories worldwide and depend on type of tissue, local habits and requests, by clinicians and patients, for specific investigations and for shortening the turn-around time (TAT).

In common practice, diagnostic and TAT requirements, thus processing, are different for “small” biopsies (<2 cm in size) and surgical specimens. A fast and reliable pre-surgical diagnosis is requested for the former. 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 which in recent times attracted much attention, such as the “ischemia” time (see below) and the preservation of antigens and nucleic acids. In fact, the analysis of these specimens is mainly focused on anatomical data (classification, staging, grading) and on the acquisition of prognostic and therapeutic parameters requiring preservation of antigens and of nucleic acids.

The present review will thus focus on the processing of these “large” specimens.

Histopathological processing of specimens larger than 2 cm requires gross examination 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, tissue specimens that are to be processed in “biocassettes” should not be thicker than 3–4 mm. The time interval between surgical removal of the specimen and proper fixation is defined as “ischemia time” and is crucial, since it allows activation of tissue enzymes, autolysis and degradation of proteins, as well as of DNA and RNA [29–31]. Interestingly, Chung JY *et al.* [32] have demonstrated that substantial RNA degradation may occur during this “warm ischemia” time, but the RNA degradation due to warm ischemia may be slowed down by cooling the specimen. During this “pre-fixation” time interval it is mandatory to avoid autolysis and drying of the surface, which might heavily affect tissue structure and components.

Transfer from the surgical theatre to the pathology laboratory

Methods of transfer of surgical specimens vary according to the architectural layout and distance between surgical theatres 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 two hours in order to achieve a proper processing of breast cancer specimens, permitting a correct evaluation of both morphological and therapeutic-prognostic parameters [33, 34]. Problems arise when this is not feasible for reasons determined by the internal organization within the Hospital or dislocation of surgical theatres and Pathology laboratories. It is a common practice in many hospitals to immerse large specimens and organs into large formalin filled containers, which are then transferred to the pathology laboratory in due time, usually once every day. This practice carries problems, since:

- Large plastic containers are large and relatively heavy, and therefore spilling of formalin can occur.
- Immersion of the whole specimen into formalin prevents the collection of fresh material for tissue banking. Also, fixation does begin, but only at the periphery. A delay in the transfer to pathology is somehow justified by the fact that “the tissue is already in formalin”.
- Surgical nurses are becoming increasingly concerned about potential toxicity and carcinogenicity, since the fluid has to be handled outside the hood.
- When the container does arrive at the pathology lab, the opening, extraction and handling of the specimen is a major cause of the diffusion of formaldehyde fumes.

Formalin, a 4% solution of formaldehyde in water, is extensively used worldwide as a fixative for histopathological specimens. Since its discovery at the end of 19th century [3], this aldehyde has been universally appreciated as a simple reagent that is a good antiseptic, penetrates tissues quickly (at a diffusion rate of 1 cm in 24 hours) and is easy to handle. In tissues that are formalin-fixed, the morphology is well preserved, as is tissue antigenicity. Immunohistochemical procedures of diagnostic interest have routinely been adapted for use on formalin-fixed tissues [35].

The medical use of formalin as a tissue preserver and fixative is extensive, especially in pathology laboratories and its substitution with alternative fixatives does not currently seem likely [35, 36].

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

Despite its advantages, formaldehyde has some drawbacks that demand caution: it is a skin allergen and produces irritating vapors that can cause asthma. The International Agency for Cancer Research [37] has classified formaldehyde as a Class 1 carcinogenic agent, and statistical evidence has been presented for a possible link between formaldehyde exposure and lymphohematopoietic malignancies [38], an observation that might explain data reporting an excess of deaths due to cancer of the lymphatic and hematopoietic systems amongst British pathologists [39]. Still, the major concern for formaldehyde use is linked to the production of toxic, irritating and allergenic vapors. A positive relationship between formalin and respiratory symptoms has been

reported not only in workers in match factories [40] but also in hospital staff members professionally exposed to this substance [41].

Several attempts have been made to find a substitute for formalin, but so far all of the proposed alternatives have failed, being either ineffective or unreliable [42]. A more practical approach would be to limit the use of formalin to pathology laboratories, where this toxic agent is carefully handled with hoods and gloves in safe conditions, and to avoid its use in other less-protected areas of the hospital, such as in surgical theatres, where surgical tissues are commonly placed in boxes full of formalin until transfer to the pathology labs.

To overcome these problems, an alternative procedure is the sealing of tissues under-vacuum in plastic bags immediately after removal, and to keep them cooled at 4°C until transfer to the pathology labs, where they are routinely processed [43]. Sealing of tissues in plastic bags is a quick procedure, taking approximately 15 seconds and is easily performed by nurses in the surgical theatres [44].

Under-vacuum sealing (UVS), *per se*, does not guarantee preservation, as experienced by Kristensen *et al.* [45]. Vacuum sealing, by removing air, prevents dehydration and favors cooling, the latter being the main preserving factor by blocking enzymatic autolysis. 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 immunophenotyping of the specimen, an issue that is presently regarded as mandatory for breast cancer processing. A bonus of the novel UVS procedure is the preservation of RNA, which is enhanced by the storage at 4°C [46], thus permitting tissue banking and gene expression profiling.

The San Giovanni Hospital in Torino, Italy is a large regional “pavilion” hospital where the distance between surgical theatres and pathology laboratories prevents an immediate transfer of fresh specimens. In this hospital, it was a time-honored habit to transfer specimens in large formalin-filled boxes, but, in 2009, the procedure of under-vacuum sealing and cooling has been tested for the transfer of all surgical specimens (larger than 2 cm). The experience accrued has been duly analyzed and reported [44]. The survey on the feasibility, compliance and quality assurance of a new procedure for transferring surgical specimens was definitely positive. Dedicated apparatuses (TissueSafeR, Milestone srl, Sorisole, BG, Italy) were located in the premises of each of the six surgical theatres of the Hospital. The Under Vacuum Sealing and Cooling (UVSC) procedure, which was favorably accepted by the staff and did not present special problems of practical or diagnostic interest, has been 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 restricted to dedicated areas in the pathology laboratory, and transfer of large boxes filled with fixative throughout the hospital

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

Optimization of fixation protocols in order to improve preservation of antigenic components and of nucleic acids in tissue specimens

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

Several fixatives have been proposed, but those presently used are either of the aldehyde cross-linking category or alcohol-based, producing coagulation by water subtraction. The alcohol-based fixatives [47] have the advantage of lacking toxicity and of a good preservation of nucleic acids, but have a poor penetration and result in an unsatisfactory conservation of morphological details. Moreover, in our experience [48] and that of others [47, 49, 50], the performance of some immunohistochemical tests is impaired when using fixatives alternative to formalin and a wide variability in reactivity has been observed [51–53]. Mercury-based fixatives, such as B5 and Zenckers's are fixatives used particularly for hemato-lymphoproliferative pathology since they provide excellent nuclear morphology [53]. Similar to formalin, mercury-based fixatives induce molecular cross-linking, but to a larger extent than aldehyde solutions, and as a result they may hamper the reactivity of a number of important antigens, for example CD4, CD5, CD10, CD23 and sometimes CD30 [55–57]. On the contrary, antibodies for kappa and lambda light chains perform better in B5 than in formalin-fixed tissues [57]. In addition, when using these fixatives, it is necessary to remove precipitated pigment and it has been argued that denaturation of the antigens can be caused by reagents employed for such purpose, i.e. by Lugol's iodine or similar solutions [58].

As already stated, 4% formaldehyde solution in water (formalin) has been adopted as the fixative of choice in histopathology as it is relatively cheap, easy to use. It is also reliable because it does not over-fix and it guarantees, in appropriate conditions, an optimal morphological preservation so that we have to conclude that substitution of formalin with alternative fixatives cannot be foreseen at present [35, 36].

The length of formalin fixation can affect the results of the immunostaining [59–61] since underfixation 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) [58–60].

Moreover, formaldehyde fixation modifies the conformation of macromolecules, altering tertiary and quaternary organizations of proteins, whereas the primary and secondary structures are scantily affected [59, 62, 63]. Such conformational changes may hamper

the link to the antibody [52, 64, 65], but the use of antigen retrieval procedure can return immunoreactivity in formaldehyde-fixed specimens [52].

This issue is particularly relevant in onco-pathology for the evaluation of factors predicting responsiveness to therapeutic treatments, and thus, fixation in phosphate buffered formalin (PBF) of breast cancer tissue blocks for no less than six and no more than 48 hours is now required in order to guarantee an optimal evaluation of Estrogen (ER) and Progesterone Receptors (PgR) and HER2 expression by immunohistochemistry [33, 34] (Figure 1).

Estrogen and progesterone receptors were among the first prognostic tissue factors identified by immunohistochemistry, a high percentage of labeled tumor nuclei for these receptors being associated with a better outcome in the same anatomical-clinical group. Endocrine treatment of breast tumors is also guided by the immunostaining results, being indicated in most patients with hormone-positive tumors because of increased efficacy and acceptable side effects. An incorrect immunohistochemical testing may lead to misdiagnosis and initiation of inappropriate therapies or, on the other hand, withholding of appropriate therapies. Thus, consistent and reliable results of the immunohistochemical analysis are highly important in clinical therapeutic decisions of the breast carcinomas.

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 [10, 11, 66, 67]. Studies conducted on the preservation status of nucleic acids in Formaldehyde Fixed-Paraffin Embedded (FFPE) tissues generally agree on the relatively good (though not optimal) preservation of DNA [69]. 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 [32, 50, 68–71].

Innovative protocols have however been proposed for permitting gene expression profiling on FFPE tissues from cancer patients [46]. The complementary DNA-mediated Annealing, extension, Selection and Ligation (DASL[®]) assay (Illumina, San Diego, CA) [67] is a gene expression profiling system suitable for use with degraded RNAs such as those derived from FFPE tumor samples. DASL assay provides a reliable approach to gene expression profiling in FFPE tumors [46, 68].

A variation in formalin fixation resulting in improved preservation of RNA was recently proposed [72]. This is based on a fixation process in formalin at 4°C, followed by dehydration in cold ethanol and paraffin embedding (Cold Formaldehyde Fixed-Paraffin Embedded – 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.

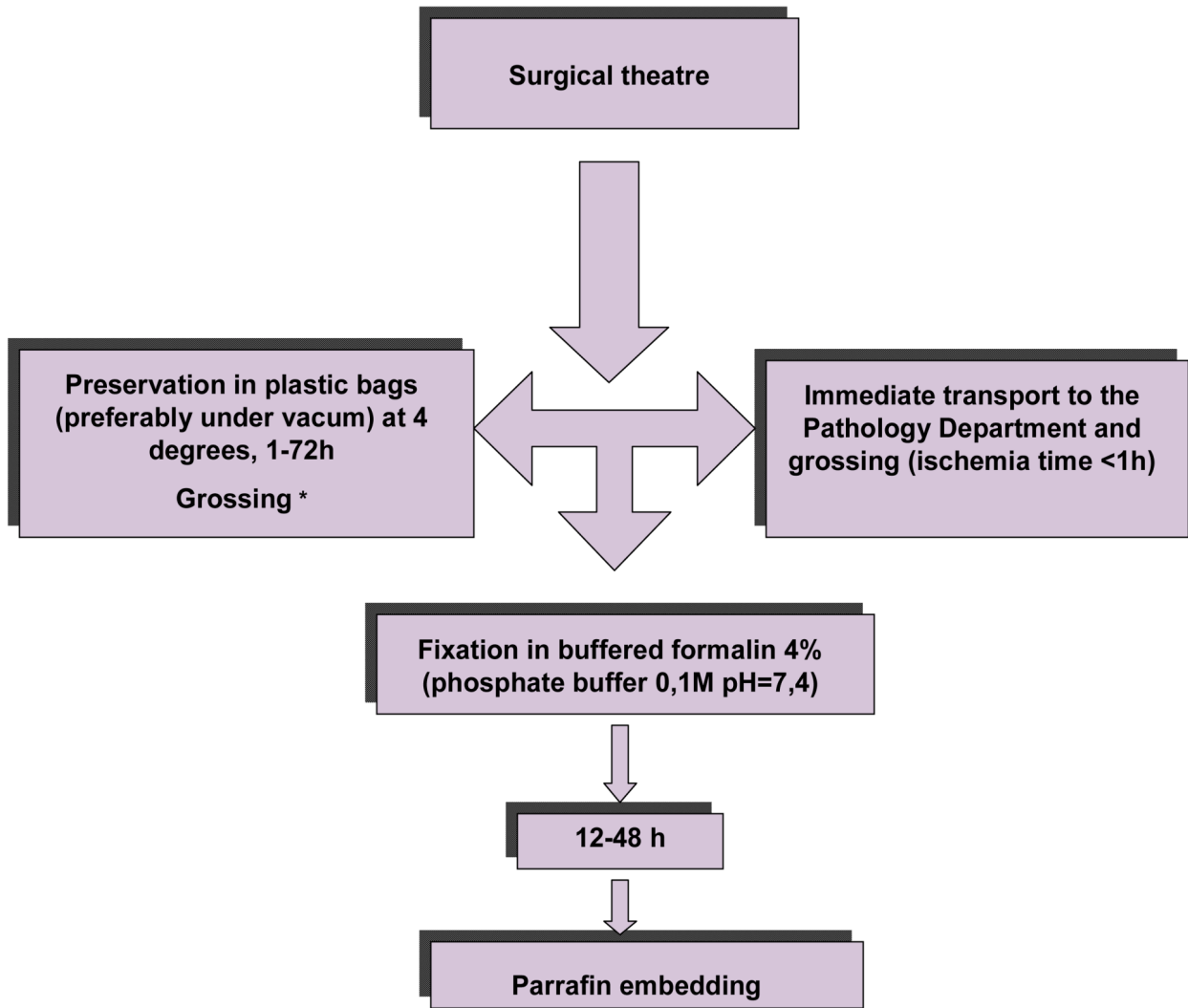


Figure 1 – Flow chart of preanalytical steps in HER2 testing.

Tissue embedding

The steps of tissues processing and embedding are well standardized and seem to have less influence than fixation on the results of immunohistochemical procedures and nucleic acid sequencing. However, sub-optimal processing cannot only affect morphological preservation, but also impact on immunohistochemical results. It is common in practice that immunohistochemical staining can be problematic in old archival tissue blocks [57] but the reason is not clear. Recently, Xie R *et al.* [73] presented experimental evidence that retention of endogenous water in paraffin-embedded tissue blocks results in a process of antigen degradation progressing with storage time, which may negatively affect diagnostic immunohistochemical reactions. These data emphasize the mandate, for present-time and future investigations, for a proper processing at all steps of histopathological processing.

☞ Projects

The achievement of a proper standardization of histopathological processing along the above-described lines requires dedicated attention and re-programming,

even sometimes a re-organization of Pathology laboratories. Accordingly, a series of Projects, at national and European level, have been launched and are in progress.

As testimony, we shall shortly describe three projects the present authors are involved in. Common to these Projects are the involvement of European and Romanian Institutions and of both Pathologists and Technical staff with the goal of improving the quality of histopathological preparations:

- (a) The PersoTHER Project;
- (b) The AnatomicoPat Project;
- (c) The TASTE project.

(a) The research project “Implementing tissue molecular assays for cancer in Romania. A high-level research oriented to personalized oncology – PERSOTHER – POS CCE nr 549/12024/2010” has as main objective the setting up and implementing of a model of effective investigations of tumors by high performance genetic and molecular methodologies types in order to decrease the disparities in the implementation of EU strategy on cancer.

PERSOTHER (www.ivb.ro/persother) aims to increase research capacity by training a core of highly

qualified specialists in molecular biology, competence established under the coordination of a foreign specialist (Prof. G. Bussolati), so as to form a national pool of excellence in the field. The information obtained in the diagnosis and prognosis of breast, lung, and digestive tumors will strengthen the supply of knowledge in all areas of pathology, with direct application in clinical oncology. The Project is specifically focused on tumors of the breast, lung, gastro-intestinal tract and hemolymphatic system. In the project's aim, the dissemination of research results will form the basis of technology transfer to the pathology laboratories in Romania.

(b) The project "Pathology Laboratory – professional and organizational formation by management quality implementation (ANATOMOPAT) – POSDRU/81/3.2/S/59842" addresses both technicians and specialists from the pathology departments all over Romania. The overall objective is the training of employees of these health facilities to improve performance on implementation of new technologies and quality management in the field of pathology, in order to increase efficiency and competitiveness of healthcare. It promotes specific vocational training of employees in the Laboratory of Pathology, acquisition of new working practices, staff training for the implementation of new diagnostic technologies, changing professional attitudes by increasing the number of hospitals accredited by the Romanian Accreditation Association ISO 15189. This comprehensive training program is conducted over three years and consists of nine courses (216 hours) and 11 workshops (88 hours), which deal with state-of-the-art issues of pathology, biostatistics, quality management, and project management. The target group will also participate in experience exchanges at both national and international locations. In fact, the program involves six stages of one week for groups of 20 pathologists and Technicians from different Romanian laboratories to the Pathology Department of the University of Turin, Italy. Stages have already been run and will continue until the end of the project (June 2013). The ultimate goal of the project is the harmonization and improvement of practices in the Pathology Departments in Romania (Figure 2).

(c) A project focused on the use of Telepathology for the Assessment of Histopathological Techniques at European level (TASTE) was recently approved and financed by a European grant from – ICT (Information and communications technology) – Multilateral projects, project number: 519108 – LLP-2011-IT-KA3-KA3MP, Grant Agreement number: 2011-4018/001-001 KA3.

The Project (www.tasteproject.eu) tackles these problems by building-up an ICT environment TASTE System whereby technicians and students from different countries (Italy, Romania, Portugal, Belgium and Sweden) will submit *via* the WorldWideWeb, using Tele-pathology procedures featuring "virtual slides", microscopic images of their own preparations to a panel of internationally-recognized experts who will give

them comments and suggestions. Practicing pathologists as well as residents in Anatomic Pathology will be involved. Assessment with real users will be organized in order to smooth-down the major problems encountered. A step-wise approach for the assessment (and hopefully the improvement) of the various histopathological and cytopathological preparations will be conducted, so that the exchange of images will start with basic routine stains (Hematoxylin–Eosin stained slides, Papanicolaou-stained smears) in order to check quality and reproducibility of fixation, processing and staining procedures. The program will then proceed with more sophisticated techniques such as special stains immunohistochemical and FISH preparations.

The ultimate goal of this approach (unprecedented at world level) is to fuel a comprehensive Web-based community of students and staff personnel, aimed to a harmonization and improvement of histopathological and cytopathological preparations, thus leading to an innovative training and more reproducible diagnoses, a basic requisite for disease treatment (Figure 3).

As a supplement to what we said before about the need for standardization in pathology, we have initiated a new program, linked to the ANATOMOPAT – "Quality Control on breast cancer testing-pilot program" that involves pathologists and technicians from Romania and Italy. We consider the extra laboratory quality control system as mandatory for each prognostic and predictive test, this program providing an external validation-test results from one laboratory verified by another, independent external laboratory. These proposed guidelines are suggested to be further analyzed, commented and eventually applied at the level of any pathology laboratory involved in processing tumor samples in order to have standardized, reliable diagnostic results.

Acknowledgments

Study conducted with the support of the following projects:

- Project PERSOTHER – SMIS-CSNR: 549/12.024; with the support of Sectoral Operational Programme "Increase of Economic Competitiveness" Priority Axis 2: Research, Technological Development and Innovation for Competitiveness;

- Project ANATOMOPAT – POSDRU/81/3.2/S/59842; with the support of Sectoral Operational Programme Human Resources Development 20071013 Priority Axis 3: Increasing adaptability of workers and enterprises;

- Project TASTE – Multilateral projects, project number: 519108 – LLP-2011-IT-KA3-KA3MP, Grant Agreement number: 2011-4018/001-001 KA3 – with the support of the Lifelong Learning Programme of the European Union. This project has been funded with support from the European Commission.

This publication reflects the views only of the authors, and the Commission cannot be held responsible for any use, which may be made of the information contained therein.

Project title: Pathology Laboratory - Professional and organizational formation by management quality implementation" (ANATOMOPAT)

Identification number of the contract: POSDRU/81/3.2/S/59842

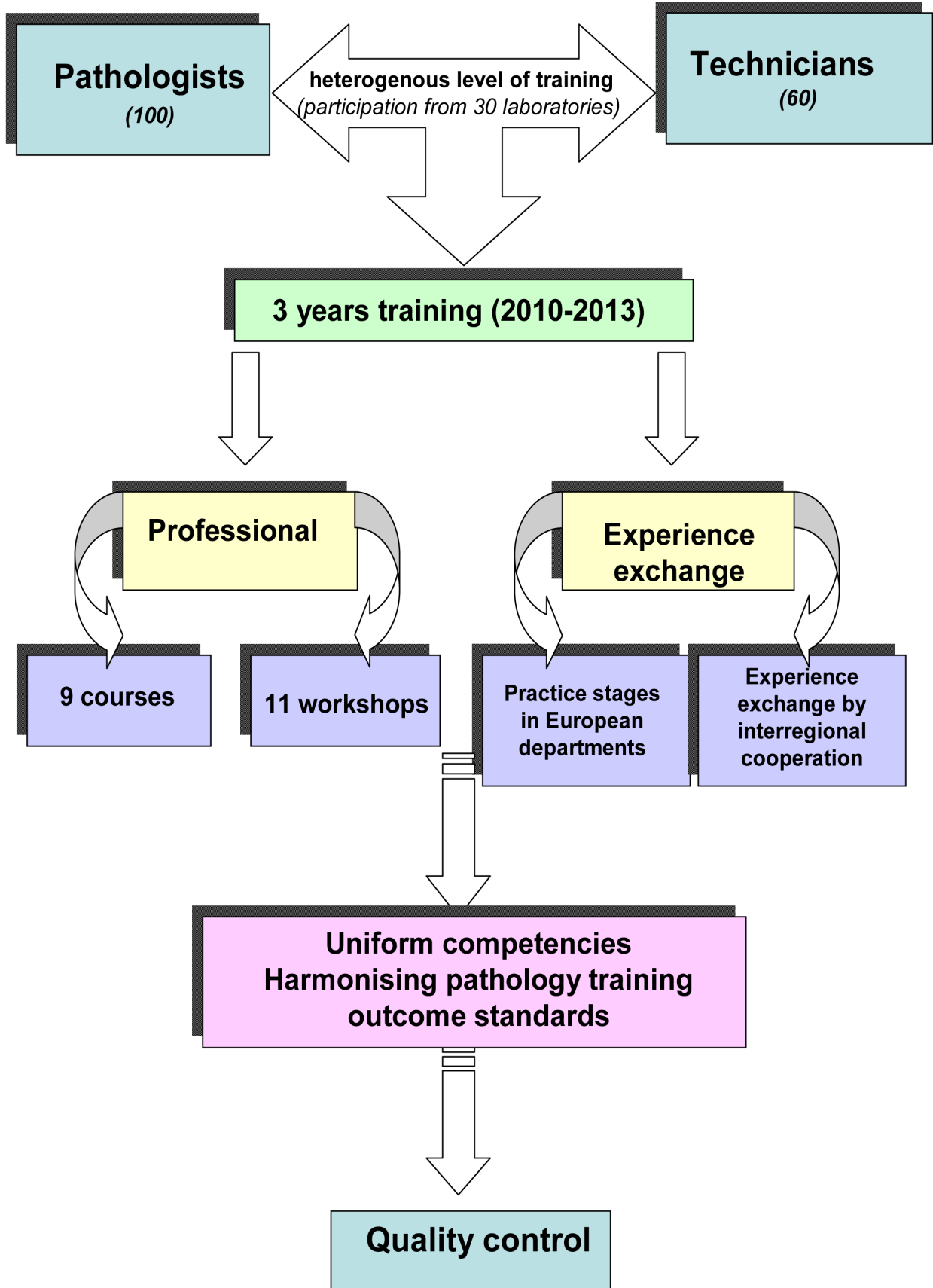
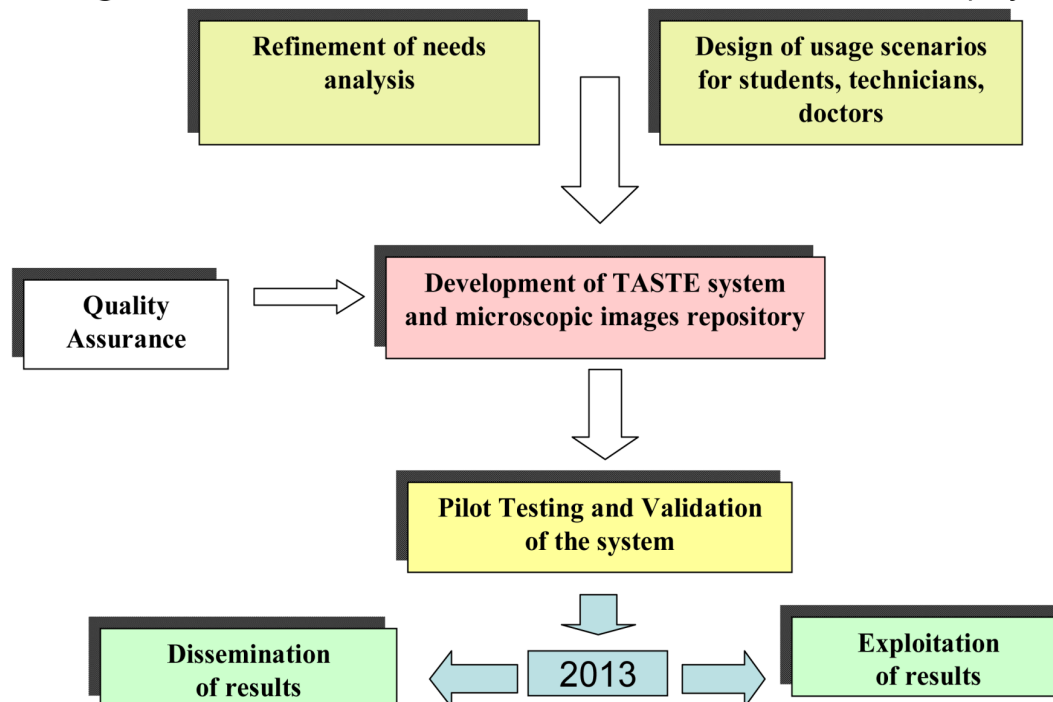


Figure 2 – Flowchart of the ANATOMOPAT project.

TASTE - Telepathological ASsessment of histopathological and cytological TEchniques
Project number : 519108 - LLP-2011-IT-KA3-KA3MP
Grant Agreement number: 2011-4018/001-001 KA3 – ICT-Multilateral projects



Final Goal: Improvement of technical preparations of Histo- and Cyto-pathology at European level.

Figure 3 – Flowchart of the TASTE project.

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Received: March 20th, 2012

Accepted: May 10th, 2012