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REVIEW

Update on proteomic studies of formalin-fixed paraffin-embedded tissues

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ABSTRACT

Introduction: This review is an update on recent progress in proteomic studies of formalin-fixed paraffin-embedded (FFPE) tissues, which open the opportunity to investigate diseases and research potential biomarkers, particularly when availability of fresh/frozen tissues is low.

Areas covered: We described improvement of existing protocols or the new ones regarding deparaffinization and protein extraction of FFPE samples published from 2014 to today. Moreover, the growing interest to use FFPE tissues for mass spectrometry imaging approach is presented together with the search of post-translational modifications.

Expert opinion: In the last few years, the number of papers using FFPE tissues in proteomic analysis is growing. The interest to apply proteomic analysis to FFPE tissues lies in the easy accessibility of a great number of samples from archives. Nevertheless, standardization in the approach among the different researchers is not achieved, making essentially incomparable the results obtained. This limit should be overcome.

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1. Introduction

In our previous review [1], we emphasized the relevance of the great availability of formalin-fixed paraffin-embedded (FFPE) tissues in the archives of pathology laboratories all over the world. Particularly, we evidenced the opportunity to study diseases and research potential biomarkers where there is low availability of fresh/frozen (fr/fr) tissues. Another important possibility is represented by relating the peculiarities of different diseases over the years in relation to lifestyle. Furthermore, the interest in the use of FFPE tissues has considerably grown as shown by the number of publications on this topic in NCBIs PubMed that has more than doubled since our review. In fact, if the results obtained on FFPE samples probably require validation on fr/fr samples, the great availability of FFPE tissues allows the initial identification of potential biomarkers, the confirmation of data obtained on fresh tissues or biopsies and the retrospective evaluation of the pathology, also allowing a not neglectable saving of sampling, particularly for rare diseases.

In this update, we underline the recent publications on the role of pre-analytical factors (fixation time, archival age and tissue processing) on proteomics studies, the new approaches to the proteomic studies of FFPE tissues, the protein and peptide extraction and recovery after deparaffinization, the microproteomics of FFPE, the increased number of studies using imaging mass spectrometry approach, the applicability of FFPE proteomics to the study of post-translational modifications, the fr/fr comparison with FFPE studies and, the researches of potential biomarkers carried out both with fresh and FFPE tissues in cancer.

2. Pre-analytic factors affecting proteomics analysis of FFPE tissues

Pre-analytic factors greatly influence the results of the proteomic analysis, and from this point of view, it is fundamental to understand how the different phases from sampling until the tissue became a FFPE tissue were carried out. As we suggested in our previous review, a standardization of the entire workflow for proteomics studies on FFPE tissues should be necessary especially with regard to pre-analytic phase. There are three steps in the pre-analytic sample preparation that can influence the proteomic studies. The first step is naturally when the sample is collected by resection or biopsy, the temperature of process, the time before fixation and the time of transport to pathology laboratory. The second step is the processing of the sample, time of fixation, type of fixative, ratio tissue/fixative, and dehydration, type of paraffin and time and type of block formation. The third step is the time of storage, temperature of storage and temperature of sectioning. Two reviews [2,3] describe in detail the pre-analytic factors affecting proteomics studies of FFPE tissues. The critical role of pre-analytic factors is emphasized also by other authors [4–6]. In a recent paper Piehowski et al. suggested [7] that the ability to identify specific peptides or phosphopeptides after an initial decline due to the formalin fixation process was unaffected by specimen age in agreement with other authors [2,8]. In our opinion, it is important to deepen the knowledge on the role of the effects of pre-analytic factors, and, in this context, the standardization of the pre-fixation processes and fixation method could promote the development of multicenter studies on rare diseases.

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Article highlights

- Pre-analytical factors affects proteomics analysis of FFPE tissues
- New approaches using non toxic solvents in deparaffinization
- Progress on protein extraction protocols of FFPE tissues
- Advantages of FFPE tissues mass spectrometry imaging
- Use of FFPE tissues in the investigation of posttranslational modifications

3. Deparaffinization

Deparaffinization is the first step in the approach with FFPE tissues and consists in the solubilization of paraffin with different apolar organic solvents or mineral oils (see [1]). Rehydration process is carried out with alcohol (methanol or ethanol) at decreasing concentration. Xylene is the apolar solvent most widely used still today, despite its toxicity, followed by rehydration with ethanol. Recently the use of efficient xylene free protocols for paraffin solubilization was proposed by three different research groups [9–11] utilizing hot water (95°, 90°, 80°C, respectively) for protein extraction or DNA extraction. The authors suggested that these protocols have the advantage to avoid toxic xylene and to perform solubilization and rehydration at the same time reducing the time of deparaffinization.

4. Protein and peptide extraction and recovery

Several protocols of antigen- retrieval have been suggested to obtain a high yield in terms of protein and peptide extraction and solubilization from FFPE samples [1]. Many of these use extraction buffers with sodium dodecyl sulfate (SDS) that must be removed before trypsin digestion and liquid chromatography tandem mass spectrometry LC-MS/MS analysis, with a consequent loss of sample. This is particularly true when restricted tissue material is available. To overcome this problem, direct trypsinization (DTR) protocols have been developed using ammonium bicarbonate with acetonitrile as extraction buffer or buffers with surfactants such as RapiGest [1]. Concerning the use of RapiGest two recent studies used a DTR strategy followed by LC-MS/MS for identification and quantization [12,13]. In particular, Azimi et al. [12] stained FFPE tissue slices with hematoxylin & eosin before laser microdissection to distinguish histological areas of interest. Foll et al. [14], in an interesting paper, carried out a critical comparison of the DTR protocol (using buffer with 0.1% RapiGest) with the most commonly used filter-aided sample preparation (FASP) protocol (using buffer containing 4% SDS that is removed using centrifugal filter units). In addition, the influence of histological staining was assayed. A high and comparable number of identified proteins was obtained with DTR and FASP protocols whereas the percentage of the overlap of identified proteins between protocols ranged from 61 to 68%. In spite of a quantitative reproducibility in terms of proteome coverage, nonetheless sample preparation with DTR protocol resulted more straightforward and faster with respect the FASP method, which showed long time of execution. Furthermore, a reduction of 13% in protein identifications was observed for FFPE samples stained with hematoxylin and eosin.

Broeckx et al. [15] carried out an accurate reassessment of efficiency in FFPE protein extraction of 8 different extraction buffers upgrading minor modifications to the original protocols by Addis et al. [16,17] and Crockett et al. [18]. Three buffers resulted in highest yield per unit of volume FFPE protein extracts. Two of these were a modification of Addis et al. protocol [16] with the addition of glycerol with and without CHAPS. In particular, the authors investigated the reproducibility changing the source of tissue and performing fr/fr tissues *vs*. FFPE tissues comparison. One out of eight buffers turned out to be the best regardless of the nature of FFPE tissue. The authors confirmed the efficiency of extraction with 2% SDS combined with alkaline conditions and suggested that the addition of glycerol resulted in a more consistent protein recovery and increased the reproducibility of protein extraction between samples ensuring a long-term preservation of protein extracts.

Taverna et al. [19] presented an innovative methodology that uses hydrogel extraction surface analysis (HESA) coupled with isobaric mass tags for a quantitative analysis of FFPE tissues.

Using home-made miniaturized polymeric gels acting as a carrier for trypsin, the protein digestion occurs directly onto the tissue surface within the area covered by the gel. This method assures different advantages: i) minimal manipulation of sample; ii) combination of protein identification and quantification; iii) nondestructive method, then tissue samples can be used for histology and immunohistochemistry; and iv) reduced number of workflow steps. The limit is in the capability to sample small tissue areas (7 mm^2). An interesting methodological paper is that proposed by Tanca et al. [20] which reported a comprehensively overview of technical tips, critical aspects, and drawbacks of full-length protein extraction protocol optimized for downstream gel-based proteomic application such as SDS-PAGE, western immunoblotting, GeLC-MS/MS, 2D-PAGE, and 2D-DIGE. The authors proposed an implemented protocol with extraction and analysis procedures, which reached acceptable quality levels although the FFPE patterns were still not totally comparable to those of fr/fr tissues.

Finally, as shown by our previous review [1], a valid alternative to home-made extraction buffers is the use of FFPE extraction kits [8,21,22]. To this concern the Q-proteome FFPE tissue kit and two in-house methods using Laemmli buffer were assayed to optimize protein yield from FFPE breast cancer tissues [23]. The results confirmed the higher efficiency of the commercial kit with respect to the in-house methods evaluated in terms of both quantity and quality of protein extraction as evidenced by western blotting and reverse phase protein array (RPPA) results. Table 1 shows a summary of the extraction conditions described in this paragraph.

5. Microproteomics of FFPE tissues

Frequently it may happen that small FFPE tissue samples are available and therefore the number of cells is very few. Major advances have been made and several papers and methodological articles have been published to overcome these problems. The use of laser microdissection in the case of very

Table 1. Extraction conditions.

Extraction buffer	T(°C)/min	Tissue	Sample (sections)	Protein identified	Ref
RapiGest™ SF Surfactant in 50 mM triethylammonium bicarbonate (TEAB)	95/30	Cutaneous Squamous Cell Carcinoma	10 µm	1310	[12]
MQ water	95/20	Lung and colon adenocarcinomas; healthy tissue from skeletal muscle, liver, kidney, testis, myometrium, breast, prostate, and skin	1, 3, and 5 µm	Nd	[13]
RapiGest SF in 0.1 M HEPES pH 8 and 1 mM dithiothreitol (DTT)	95/240	human tonsil mouse kidney	5–10 µm	1970	[14]
20 mM Tris HCl, 2% SDS, 200 mM DTT, 20% glycerol, 1% protease pH 8.8	98/20 80/120	Murine liver/murine colon/Human colon	10 µm	1208/1040/ 1411	[15]
RIPA lysis buffer, 2% SDS, pH 8	98/20 80/120	Murine liver/murine colon/Human colon	10 µm	1060/290/1217	[15]
20 mM Tris HCl, 0.5% SDS, 1.5% CHAPS, 200 mM DTT, 10% glycerol, pH 8.8	98/20 80/120	Murine liver/murine colon/Human colon	10 µm	1047/354/1073	[15]
hydrogels activated by enzyme solution containing 125 ng μL^{-1} of trypsin, 100 µM TEAB, 2 mM DTT, and DOC 1% w/v	-	Cardiac myxoma	12 µm	1949	[19]
Q-proteome FFPE Tissue Kit plus Laemmli solution	100/20 80/120	Human breast cancer	10 µm	Nd	[23]

early cancer lesions and small samples to be handled is well described by Longuespée et al. in two papers [13,24] The methodology, the approach, and several considerations to be taken into account to avoid sample loss and the suggestions for optimal digestion of the proteins from the tissue samples are reported. Moreover, specific analytical settings more adequate to the analysis of restricted amounts of proteolytic peptides from small tissues are suggested. Another approach to microproteomics was recently described by Wisztorski et al. [25] such as spatially-resolved liquid microextraction. They described strategies to extract previously digested peptides or intact proteins from tissue section in a localized manner by liquid microjunction.

The microproteomics was recently applied to different pathologies. Laser capture microdissection was utilized by Herfs et al. [26] to study anal canal carcinoma identifying two distinct entities with different cells of origin, proteomic signatures, and survival rates. Le Rhun et al. [27] utilized microproteomics combined with mass spectrometry imaging for glioma III classification. The obtained information could allow a more accurate classification of the biopsies according to the prognosis. Cystic echinococcosis, an endemic parasitic disease, was studied by Longuespée et al. [28] by laser microdissection-based microproteomics. This study evidenced specific markers of a parasitic cyst in liver. A study on microproteomic profiling of high-grade squamous intraepithelial lesion of the cervix was carried out by Pottier et al. [29]. They used their developed laser microdissection-based microproteomic method, designed for the proteolytic digestion of small regions of FFPE tissues. They provided an extensive proteomic profiling of HSIL and suggested potential future new biomarkers to differentiate squamous intraepithelial lesions of cervix. A microproteomic analysis to characterize the type of amyloidosis was published by Reglodi et al. [30]. They detected in PACAP knockout mice an altered cytokine profile that possibly creates a pro-inflammatory condition facilitating amyloid deposition.

6. Mass spectrometry imaging approach

Mass Spectrometry Imaging (MSI) presents the advantage to analyze a tissue section directly after simple treatment and allows both the identification of proteins or peptides and their localization in the tissue. MSI might provide many results with translational characteristics. The sample preparation requires removal of paraffin with xylene washes and the use of ethanol or water washes. In the editorial published in the issue 1, vol. 13 2019 of Proteomics Clinical application (special issue Proteomics in Pathology) [31] many of the major aspects, issues and applications of the method used in pathology are presented and, in particular, the scientific advances in the field of histoproteomics, with special emphasis on translational pathology. These aspects were previously underlined in a view point [32] of the editor of the special issue. Different articles [29–37] presented the application of MSI to FFPE samples in different diseases also suggesting some technical steps. The section thickness on m/z peak intensity assumes particular importance [37]. A section thickness of 1 μm results in higher spectral intensities compared with 5 μm. An important aspect in proteomic imaging is the reproducibility between laboratories. In an article by Ly et al. [38], this aspect is discussed, and it is suggested that spatial resolution and site-to-site reproducibility can be maintained by adhering to a standardized MALDI-MSI workflow. In a recent article by Buck et al. [39] described a round robin of FFPE tissues from four research centers with similar or equal MS instruments and conclude on the feasibility of running successfully multicenter mass spectrometry imaging studies on FFPE samples.

An important aspect in the use of FFPE tissues for MALDI imaging of proteolytic peptides is the difficulty of an easy identification of peptides, due to different effects. A combination of MALDI imaging with LC-MS/MS methods for the parallel identification of peptides detected by MALDI imaging is used (see [36] and [40,41])

Intratumor heterogeneity is the result of the evolution of cancerous cells within a tumor, strictly related to cancer progression, resistance to therapy, and recurrences. As evidenced in [41,42] the combination of MALDI imaging and microproteomics may represent an adequate tool for future investigations and approach to intratumoral heterogeneity. An important study on the inter- and intra heterogeneity of tumors is represented by the publication of Buczak et al. [43] in which they describe a strategy that can be universally applied to FFPE samples to study heterogeneous cancer cell populations in solid tumors with excellent spatial resolution.

Spatial proteomics using imaging provides a technology for the visualization of proteins in their cellular environment avoiding cell lysis and separation of compartments or organelles.

The study of spatial proteome is therefore essential for a complete understanding of cell biology. A recent elegant review on spatial proteomics has been published by Lundberg [44].

In Table 2, the different techniques of FFPE tissues treatment for MSI are reported. Vos et al. [45] recently suggested strategies for managing 3D mass MSI.

7. Post-translational modifications (PTMs) in FFPE tissues

The study of PTMs is a powerful approach to evidence differences between normal tissues and cancer tissues. Indeed many proteins associated to different tumors are characterized by PTM e.g. phosphorylation, glycosylation, acetylation, ubiquitination, SUMOylation, nitrosylation, methylation, lipidation, and proteolysis.

Ostasiewicz et al. [47], with their methodology of analysis to FFPE tissues by Filtration Aided Sample Preparation/(FASP), demonstrated that phosphoproteoma in FFPE samples is not different from fresh tissues indicating that is conserved during preservation.

Similarly, Wakabayashi et al. [48] suggested that their protocol could be useful for phosphoproteome analysis of FFPE samples, especially scarce from patients with rare diseases. However, as reported in the introduction, Piehowski et al. reported [7] that the ability to identify specific peptides or phosphopeptides after an initial decline due to the formalin fixation process was unaffected by specimen age. The effect of time storage and freezing mode before fixation may be very important. Therefore, caution is recommended when using different protocols of sample collection and conservation for proteomic and phosphosproteomic research.

In their readable article, Gustafsson et al. [49] highlighted how the quantitative use of bottom up proteomics can be a very important tool for the study of PTMs in tissues.

More recently Noberini et al. [50,51] reported the applicability of MS-based analysis to study the PTMs of histones by using the PATH-MS approach and the use of laser microdissection for more accurate results [52]. Wang et al. [53] studied the glycosylation, a very important PTM, and confirmed the feasibility on FFPE samples in lung adenocarcinoma and the utility for clinical diagnosis and discovery of potential biomarkers. In conclusion, these publications show that FFPE tissue archives are ideal to identify and understand PTMs that are critical in the comprehension of cell biology and to help disease prevention and therapy.

Table 2. FFPE tissue treatment for MSI analysis.

Tissue (bibliography)	Deparaffination	Antigen retrieval	Trypsin
Brain [39]	XEM	CAR	Try1
Pancreas [39 33]	XEM	CAR	Try1Try2
	XEM1	TAR	
Colon [39]	XEM	CAR	Try1
Heart [39]	XEM	CAR	Try1
Muscle [39]	XEM	CAR	Try1
Lung [39]	XEM	CAR	Try1
Head-Neck [36]	TXE	CAR1	Try3
Thyroid [46]	XEW	CAR2	Try4
Duodenum [56]	XEW	CAR2	Try5
Kidney			
Cerebellum and cortex			
Bladder cancer [45]	XEM	CAR3	Try6

XEM = Xylene 2 washes 5,10 min; 2 washes with ethanol 100% 2 min;2 washes with water MilliQ 5 min
XEM1 = Xylene, ethanol 100%. 96%, 70%
XEW = 1 h at 65°C; 3 washes 2 min, xylene (HPLC);2 washes 3 min 100% EtOH,1 wash 3 min 70% EtOH,2 washes 2 min, H_2O (HPLC)
TXE = 1 h at 60°C;3 washes 220s xylene; ethanol 100%, 95%, and 70% for 220 s each; 2 washes 220 s 10 mM ammonium bicarbonate CAR = 10 mM Citric acid pH 6 Antigen Retrieval 2100 (Aptum Biologics)
TAR = 10 mM Tris pH 9 20 min 95°C CAR1 = 10mM ammonium bicarbonate twice for 220 s each, 10 mM citric acid pH 6 30 min at 95°C
CAR2 = 10 mM citric acid buffer at 97°C for 30 min H_2O (HPLC) 2 min
CAR3 = citric acid at 10 mM pH 6.0 121 °C for 20 m Antigen Retriever 2100
Try1 = 0.02 µg/µl 18 h 37°C
Try2 = ,1 µg/µl 40 mM ammonium bicarbonate 10% acetonitrile 2 h 37°C
Try3 = 2 $\mu g/\mu L^{-1}$ 50 mM ammonium bicarbonate 10% acetonitrile 2 h 50°C
Try4 = 0.1 µg/µl overnight at 40 °C
Try5 = 0.1 µg/µl for 2 h at 45°C
Try6 = 0.02 µg/µl 17 h 37 °C

8. Comparison between fr/fr and FFPE tissues

In our previous review [1] we stressed the importance to compare fr/fr and FFPE tissues in relation to the age of storage and protein recovery capacity in order to increase the use of FFPE archives. With respect to the number of proteins identified, most of the publications until our previous review reported a superiority of fr/fr tissues over FFPE tissues with a variable percentage of common proteins. The influence of pre-analytical factors and FFPE block age is evidenced. Gaffney et al. [54] showed that phosphorylated proteins were found over-expressed, or under-expressed in relation to the delays before formalin fixation. On the other hand, Gustaffson et al. [49] reported a favorable comparison of the protein extraction results from frozen and FFPE tissues using a FASP protocol with a 91% of protein overlap. In a recent article, Taverna et al. [19] proposed the hydrogel extraction surface analysis coupled with isobaric mass tags to detect and quantify protein changes within distinct areas of FFPE tissues of cardiac myxoma specimens. In the comparison of the same biopsy on both FFPE and frozen sections 1949 (FFPE) and 2491 (frozen) proteins were identified, with a total overlap of 56%.

The identification of potential biomarkers both in fr/fr and FFPE head and neck cancer tissues was carried out by Hoffman et al. [36]. They identified vimentin, keratin type II, nucleolin, heat shock protein 90, prelamin-A/C, junction plakoglobin, and PGAM1 as common biomarkers and underlined the importance of using both kinds of samples for biomarker discovery. Patel [55] reports the protocols for analysis of both fr/fr and FFPE tissues for Mass Spectrometry Imaging. Liquid Tissue MS Kit (Expression Pathology) cited by [56] and Qproteome FFPE Tissue Kit (Qiagen, Valencia, CA) are commercial kits for the isolation of proteins from FFPE tissues after deparaffinization. Qiagen states that using the Qproteome FFPE Tissue Kit, protein yields are comparable to frozen material, enabling, for example, comparison between different tissues for identification of disease-specific markers. However, Bayer et al. [57] observed that many protein modifications

and cross-links due to formalin fixation persist after the different proteins recovery procedures and concluded that the proteomics of FFPE-tissues is very important and useful, but these limitations must be evaluated and taken into account.

9. Potential biomarkers in cancer FFPE tissues

One of the most important aspects of FFPE tissue study is the possibility of identifying potential biomarkers from the comparison of different tissues particularly in cancer. Table 3 reports the studies [58–68] carried out on FFPE cancer tissue published after our previous review [1]. In particular, Chauvin et al. [59] highlighted the possibility of identifying biomarkers for colorectal cancer, the fourth most lethal cancer in the world, using archived FFPE samples. The potential biomarkers suggested can distinguish non-responder or total responder patients who received neoadjuvant radio-chemotherapy with 5-fluorouracil. The authors identified a group of proteins over-expressed in non-responder patients and another group over-expressed in total responder patients. Drendel et al. [60] reported the particular opposing behavior of Xaa-Pro Aminopeptidases-1 and Xaa-Pro Aminopeptidases-2 in patients, which developed clear cell renal cell carcinoma using FFPE samples. Shimura [68] described a particular example of the use of FFPE tissues for the identification of biomarkers of liver metastases derived from pancreatic neuroendocrine neoplasm to be distinguished by paired metastatic liver tumor. A protocol for quantitative analysis of proteins, enabling studies of tissue biomarkers in archival FFPE tissues is described by Kennedy et al. [69] using multiple reaction monitoring (MRM)-based proteomic analysis.

10. Conclusions

The use of FFPE tissues obtained, where it is possible, from pathology archives especially for cancer research seems to be moving toward an increase in interest by researchers as observed in term of publications in the last six years. In particular, to study rare diseases FFPE pathology archival samples represent the unique possibility to reach a sufficient number of samples. The analysis of recent publications confirms the improvement in protein recovery even if it appears to lack standard protocols in the early stages, which prevents the creation of FFPE tissues databases. Furthermore, the protein modifications induced by the treatment with formaldehyde strongly limit the possibility of protein recovery. In any case, even though the use of FFPE tissue samples is of great potential in the search for biomarkers useful in the diagnosis, in the prevention and evaluation of the effects of therapies the results should be verified using fr/fr tissue samples to better understand the molecular mechanism of the diseases.

11. Expert opinion

The interest in the use of FFPE tissues for proteomics studies has considerably grown as shown by the number of published papers on this topic in the last 6 years. However, the chemical modifications associated with FFPE-treatment are complex and currently cannot be fully reversed. The possibilities of protein recovery from FFPE slices have improved, although there is no standardization in the approach by the different researchers. Moreover, the enormous amount of information that can be obtained from the large number

Table 3. Selection of potential biomarkers from cancer FFPE tissue studies.

Tissue	Proteomic Approches*	Potential Biomarkers	Ref.
Small Cell Lung Carcinoma (SCLC) vs Low-grade Pulmonary Carcinoid Tumors (PCTs)	Liquid Tissue MS Protein Prep Kit LC-MS/MS	MCM7,MCM6,,MCM2,MCM4, RCC2, PHGDH,CHD4, TYMP,MSH2,CORO1C,IPO9	[58]
Clear Cell Renal Cell Carcinoma	100 mM HEPES pH 7.5, 4 % SDS, 50 mM DTT, LC-MS/MS	XPNPEP-2 ↓; XPNPEP-1↓	[60]
Prostate Cancer	FASP, LC-MS/MS	CPT2, COPA, MSK1/2, pro-NPY	[44,61]
Colorectal Cancer (chemotherapy 5-fluorouracil)	NuPAGE gel 4–12%, LC-MS/MS	IFIT1, FASTKD2, PIP4K2B, ARID1B, SLC25A33: ↓Total responder; CALD1, CPA3, B3GALT5, CD1, RIPK1: ↓ Non responder	[59]
Bladder Urothelial Carcinoma	FASP, LC-MS/MS	AHNAK, EPPK1, MYH14, OLFM4	[62]
Muscle Invasive Urothelial Cancer	LC-MS/MS	ANXA1, BGN, IGFBP7, ISLR, MDP1, PLS3	[63]
Colorectal Cancer	LC-MS/MS	PPIA, ANXA2, ALDOA	[64]
Advanced Serous Ovarian Carcinoma	RPLC-MS/MS	PRDX1, ANXA1, CDC42,CTNNB1, PPP2R1A,	[65]
Lung Adenocarcinoma	iTRAQ, LC-MS/MS	FBN2, FTH1eIF-4A1,ANXA5, DEFA1, hAG 3, MUC5B	[66]
Wilms' kidney tumor	Heptane, RapiGest SF,LTQ-LC-MS/MS	SH3BGRL3, CLIC1	[67]
Novel liver metastasis-correlated protein of pancreatic neuroendocrine neoplasm	Liquid Tissue MS Protein Prep Kit; LC MS/MS	CNPY2;ANXA6	[68]

*Where not indicated, deparaffination was carried out with xylene

Abbreviations: 1hAG 3 = anterior gradient protein 3; AHNAK = Neuroblast differentiation-associated protein AHNAK; ALDOA = Fructose-bisphosphate aldolase A; ANXA1 = Annexin A1; ANXA2 = annexin A2; ANXA5 = annexin A5; ANXA6 = annexin-A6 ARID1B = AT-rich interactive domain-containing protein 1B; BGN = Biglycan; B3GALT5 = beta-1.3-galactosyltransferase 5 CALD1 = caldesmon; CD1 = E-cadherin; CDC42 = Cell division control protein 42 homolog; CHD4 = **Chromodomain-helicase-DNA-binding protein 4;** CLIC1 = chloride intracellular channel protein 1; CNPY2 = Protein canopy homolog 2 COPA = Coatomer subunit alpha; CORO1C = Coronin; CPA3 = carboxypeptidase A3; CPT2 = Carnitine O-palmitoyltransferase 2; CTNNB1 = Catenin beta-1; DEFA1 = alpha-defensin; eIF-4A1 = eukaryotic translation initiation factor 4A1; EPPK1 = Epiplakin; FASTKD2 = FAST kinase domain-containing protein 2, mitochondrial; FBN2 = fibrillin-2; FTH1 = Ferritin heavy chain; IFIT1 = interferon-induced protein with tetratricopeptide repeats 1; IGFBP7 = Insulin Like Growth Factor Binding Protein 7; IPO9 = Importin-9; ISLR = Immunoglobulin Superfamily Containing Leucine-Rich Repeat; MCM7,6,2,4 = DNA replication licensing factor MCM7,MCM6,MCM2,MCM4; MDP1 = Magnesium-Dependent Phosphatase1; MSH2 = DNA mismatch repair protein Msh2; MSK1/2 = mitogen- and stress-activated protein kinase 1 and 2; MUC5B = mucin-5B; MYH14 = Myosin-14; OLFM4 = Olfactomedin-4; PHGDH = D-3-phosphoglycerate dehydrogenase; PIP4K2B = phosphatidylinositol 5-phosphate 4-kinase type-2 beta; PLS3 = Plastin 3; PPIA = Peptidyl-prolyl cis-trans isomerase A; PPP2R1A = Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform; PRDX1 = Peroxiredoxin-1; proNPY = proneuropeptide-Y; RCC2 = Protein RCC2; RIPK1 = receptor-interacting serine/threonine-protein kinase 1; SH3BGRL3 = SH3 domain-binding glutamic acid-rich-like protein3; SLC25A33 = solute carrier family 25 member 33; TYMP = Thymidine phosophorylase; XPNPEP-2 = Xaa-Pro aminopeptidase 2; XPNPEP-1 = Xaa-Pro aminopeptidase 1; mitochondrial;

of samples in the archived pathologic specimens makes easier to identify potential biomarkers as diagnostic tools and to follow chemotherapy efficacy or for stratification of tumor subtypes. Furthermore, it is possible to overcome the availability of fr/fr tissue collections of rare diseases. Another useful application of the proteomics to FFPE tissues is the possibility to follow transformation of the different pathology hallmarks over time, especially in the study of tumors. In recent publications FFPE and fr/fr tissue have been compared in relation to the latest protein recovery techniques. Particular interest is represented by the application of MSI to FFPE tissues where the overlap of proteomic investigations between FFPE or fresh/frozen tissues appears even more evident and MSI offers the study of intratumor heterogeneity.

The chemical modifications due to formalin treatment are complex and can be only partially reversed. In the next years, it is desirable that studies will be oriented to overcome this problem and we hope that the advent of shotgun workflows for protein identification of total proteomes of FFPE tissues will help in the identifications of peptide modifications caused by formalin. We retain that a standardization of the proteomic approach to FFPE tissues according to tissue treatment might be useful also in increasing multicenter proteomic studies by the use of FFPE archives especially for rare diseases. We also believe that an important experimental problem is the so-called antigen retrieval. New approaches as methylene hydrolysis or the use of surfactants and direct trypsinization have been proposed to overcome the problem and will be further improved to obtain results in FFPE tissues closer to those in fr/fr tissues. MSI, another proteomic approach, which allows the investigation of spatial distribution of proteins, will increase the possibility in translational medicine

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