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REVIEW ARTICLE

# Analysis of the formalin-fixed paraffin-embedded tissue proteome: pitfalls, challenges, and future prospectives

**Evelyne Maes · Valérie Broeckx · Inge Mertens · Xavier Sagaert · Hans Prenen · Bart Landuyt · Liliane Schoofs**

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**Abstract** Formalin-fixed paraffin-embedded (FFPE) tissues are a real treasure for retrospective analysis considering the amount of samples present in hospital archives, combined with pathological, clinical, and outcome information available for every sample. Although unlocking the proteome of these tissues is still a challenge, new approaches are being developed. In this review, we summarize the different mass spectrometry platforms that are used in human clinical studies to unravel the FFPE proteome. The different ways of extracting crosslinked proteins and the analytical strategies are pointed out. Also, the pitfalls and challenges concerning the quality of FFPE proteomic approaches are depicted. We also evaluated the potential of these analytical methods for future clinical FFPE proteomics applications.

**Keywords** FFPE tissue · Proteomics · Mass spectrometry · Modifications · Crosslinked proteins

E. Maes (✉) · V. Broeckx · B. Landuyt · L. Schoofs
Research Group of Functional Genomics and Proteomics, University of Leuven, Naamsestraat 59, Leuven, Belgium
e-mail: evelyne.maes@bio.kuleuven.be

E. Maes · I. Mertens
Flemish Institute for Technological Research (VITO), Mol, Belgium

I. Mertens
CFP-CeProMa, University of Antwerp, Antwerp, Belgium

X. Sagaert
Centre for Translational Cell and Tissue Research, Leuven, Belgium

H. Prenen
Department of Gastro-enterology, Digestive Oncology Unit, University Hospital Gasthuisberg, Leuven, Belgium

## Introduction

In the post-genomic era, it has become clear that protein profile changes are important reflectors of biological and clinical phenomena. Furthermore, proteins are key effector molecules influencing pathological conditions. Mass spectrometry-based proteomics has, therefore, become an attractive technology to study disease-related protein differences. In disease-related proteomics, the use of tissues is favorable over biological fluids, as investigating direct at the pathology site has several advantages, e.g., higher concentrations of disease-specific proteins. Although fresh or fresh frozen clinical specimens are ideal for proteomic analysis, the limited availability of these samples is a serious drawback. In recent years, it was suggested that formalin-fixed paraffin-embedded (FFPE) tissue might be a good alternative for frozen tissues (Nirmalan et al. 2008; Reimel et al. 2009; Klopfleisch et al. 2011).

Formalin fixation followed by paraffin embedding is the most common procedure for long-term preservation of clinical samples. Mostly, hundreds of formalin-fixed paraffin-embedded diseased and paired healthy tissues are collected in hospital archives, as they are routinely prepared for pathological analysis. So far most interesting issue of these FFPE specimens is that they hold pathological, clinical, and outcome information inherently linked to every clinical sample. Also, large cohorts of samples might be available in a short period of time. Therefore, these samples are a real treasure for retrospective proteomic analysis, to elucidate pathological pathways or retrieve disease-associated biomarkers. However, the extensive formaldehyde-induced protein/DNA/RNA crosslinking is a barrier for many analytical platforms and successful removal of these crosslinks is complicated.

Today, several research groups try to unlock the proteome of FFPE tissues. In this review, we outline the use of FFPE samples in proteome analysis. We also describe the challenges and pitfalls concerning FFPE-centric proteomics approaches and conclude with future perspectives.

## Crosslinking and protein extraction

The fixation of clinical samples in formalin, followed by embedding in paraffin, is a worldwide known standard operating procedure used to perform histopathological examinations and to preserve clinical samples for a long time at room temperature. However, this processing of the tissue significantly modifies the proteins due to both fixation-induced crosslinking events and exposure to heat and organic solvents (Ralton and Murray 2011). Typically, tissues are fixed for 24–48 h in a 10 % v/v solution of formalin (=37–40 % w/w formaldehyde in water with 10 % methanol as stabilizer) (Nirmalan et al. 2008). As formaldehyde is the smallest aldehyde, it can quickly penetrate tissues and fix them by crosslinking proteins, RNA, and DNA. These crosslinks are preferentially formed at primary amino groups and side chains of amino acids such as arginine, tyrosine, histidine, asparagine, glutamine, and tryptophan. More detailed information about the chemistry of crosslinking can be found in the article by Klockenbusch et al. (2012). Extracting proteins out of these crosslinked tissues requires robust methods to remove the crosslinks, but should not, on the other hand, produce changes by itself. Until a decade ago, it was believed that the proteins in these samples were inaccessible for direct mass spectrometric analysis. However, since the introduction of the heat-induced antigen retrieval technique, developed to increase the reactivity in immunohistochemical assays in FFPE tissue, better extraction of proteins has been achieved (Shi et al. 1991). Based on this antigen retrieval procedure, Ikeda et al. (1998) were the first to enhance the extraction of proteins from FFPE tissue by heating the sections in a buffer containing 2 % SDS. From then on, the quality and quantity of proteins that could be extracted was improved using customized buffer compositions. Although the QProteome FFPE Tissue kit (Qiagen, Hilden, Germany) and Liquid tissue MS protein prep kit (Expression pathology Inc., Rockville, MD) are commercially available and solubilization and isolation of intact proteins is feasible, many research groups produce their own extraction buffer to achieve even better results. Importance of heat, detergent, protein denaturant, and physical agitation for efficient protein extraction are common components in all protocols. An overview of different buffer compositions can be found in Table 1.

## Proteome analysis using mass spectrometry

Mass spectrometry (MS) based applications make it possible to analyze complex protein samples and to identify and/or quantify hundreds of proteins in a high throughput manner.

Because MS allows the analysis of hundreds of proteins within a single assay and without the need of prior knowledge of potential proteins of interest, it has a major advantage over classical immunohistological assays. Also, there is no need to purchase commercially available antibodies or antibody development.

In order to successfully analyze FFPE extracted proteins by MS, reversal of these formalin-induced crosslinks is required, and knowledge about the reaction products and the resulting modifications is needed. It is already known that the formation of modifications during fixation is influenced by several factors, including the rate of the crosslink reaction, the position and local environment of the amino acids, and the pH of the reaction solvent, as well as the components that are present and their concentrations (Metz et al. 2004). Several research groups tried to elucidate the reaction of formaldehyde with proteins and peptides (Metz et al. 2006, 2004; Toews et al. 2008). The reaction starts with the formation of methylol adducts ($\Delta m = +30$) on primary amine groups. These adducts are followed by a partially dehydration which yields a labile Schiff base ($\Delta m = +12$), that will form crosslinks with other amino acids. Although expected, both modifications are not found in raw MS data. Their results do also show that the chemistry of formaldehyde crosslinking is considerably more complex, and the resulting mixture of products is far more heterogeneous than first presumed. Therefore, these authors presume that crosslinked products might be difficult to assess by MS.

However, besides the fact that some of these modifications are not yet elucidated, FFPE tissues are already used to perform proteomic analysis. Several research groups made the comparison between the proteome of fresh frozen material versus FFPE tissue, to evaluate the use of FFPE tissue as suitable sample source for proteomic analysis. These findings are nicely reviewed elsewhere (Tanca et al. 2012a). In short, an overlap of about 40–90 % was found between the proteome profiles of fresh frozen versus FFPE tissue, depending on the analytical platform used. Also, the equivalence of the FFPE phosphoproteome and FFPE *N*-glycome compared to fresh frozen tissue could be demonstrated using IMAC phosphopeptide enrichment in combination with LC–MS/MS (Ostasiewicz et al. 2010; Gamez-Pozo et al. 2011).

The use of FFPE tissue as sample source in retrospective clinical studies is widely applied today. Many different analytical proteomic platforms were used to achieve the same goal: find protein profiles which differ between

**Table 1** Compositions of different protein extraction buffers

| No. | Type | Treatment | Buffer composition | Temperature (°C) | Time (min) | Agitation | Reference |
|---|---|---|---|---|---|---|---|
| 1 | 4 × 8 µm slices | Sonication | 2 % SDS, 100 mM ammonium bicarbonate, 20 mM DTT, pH 8,5 | 70 | 60 | No | Palmer-Toy et al. (2005) |
| 2 | Needle microdissection | Paraffin removal by SubX | Liquid tissue MS protein prep kit | 95 | 90 | Yes | Prieto et al. (2005) |
| 3 | 1 slice | NA | NDME-PE (non-destructive molecule extraction) | 100 | 30 | No | Chu et al. (2005) |
| 4 | 1 tissue ffpe block | NA | RIPA buffer | – | – | No | Crockett et al. (2005) |
| 5 | Slices | Sonication | 40 mM Tris–HCl, 6 M guanidine-HCl, 65 mM DTT, pH 8,2 | 100 | 30 | No | Jiang et al. (2007) |
| 6 | Laser microdissection | NA | 8 M urea,20 mM Tris–HCl at pH 8.0 | – | – | No | Guo et al. (2007) |
| 7 | Needle microdissection | NA | Qproteome FFPE kit | 100 + 80 | 20 + 120 | No | Becker et al. (2008) |
| 8 | 5 µm slices | NA | 30 % ACN, 100 mM $NH_4HCO_3$ | 100 | 10 | NA | Bagnato et al. (2007) |
| 9 | Macrodissection | NA | 30 % ACN, 100 mM $NH_4HCO_3$ | 95 + 65 | 30 + 180 | No | Nazarian et al. (2008) |
| 10 | 3 × 10µlm slices | NA | 100 mM Tris–HCl pH 6,8, 20 % v/v glycerol, 2 % w/v SDS, 4 % v/v beta-mercaptoethanol | 105 | 20 | No | Nirmalan et al. (2008) |
| 11 | 1 × 60 µm slices | Sonication | 50 % 100 mM ammonium bicarbonate, 50 % Trifluoroethanol | 80 + 60 | 120 + 60 | No | Sprung et al. (2009) |
| 12 | 50 mg tissue, sliced in 1-3 mm thick | Sonication | 50 % phosphate buffer, 50 % Trifluoroethanol | 60 | 120 | No | Tian et al. (2009) |
| 13 | 5 µm slices | Blender + sonication | 100 mM DTT, 100 mM Tris–HCl pH 8,0, 4 % SDS | 99 | 60 | Yes | Ostasiewicz et al. (2010) |
| 14 | Macrodissection | NA | 2 % SDS, 200 mM DTT, 20 mM Tris–HCl, 1 % octylglucoside, 200 mM glycine | 100 + 80 | 20 + 120 | Yes | Azimzadeh et al. (2010) |
| 15 | 4 × 7 µm slices | NA | 20 mM Tris–HCl pH 7, 2 % SDS, | 100 + 60 | 20 + 120 | No | Xiao et al. (2010) |
| 16 | 6 × 10 µm slices | Elevanted pressure | 50 mM Tris–HCl pH 8, 2 % SDS | 100 + 80 | 30 + 120 | No | Fowler et al. (2010) |
| 17 | 3 × 10 µm slices | NA | 0,2 % rapigest in 50 mM ammonium bicarbonate | 105 + 70 | 20 + 120 | No | Nirmalan et al. (2011) |
| 18 | 10 µm slices | NA | 2 % SDS, 200 mM DTT, 20 mM Tris–HCl | 100 + 80 | 20 + 120 | No | Tanca et al. (2011a) |
| 19 | Laser capture microdissection | NA | 0,1 M Tris–HCl, pH 8,0; 0,1 M DTT, 0,5 % (w/v) polyethylene glycol, 4 % SDS | 99 | 60 | Yes | Wisniewski et al. (2011) |
| 20 | 3 × 5 µm slices | Homogenized using mixer | High pH AgR buffer, pH 9.9; 1 % NaN, 1 % SDS, 10 % glycerol and protease inhibitor | 115 | 15 | No | Xie et al. (2011) |
| 21 | Needle microdissection | Sonication | 62.5 mM Tris–HCl pH 6.8, 4 % w/v SDS, 10 % v/v glycerol, 100 mM DTT | 105 | 45 | No | Craven et al. (2012) |

diverse clinical conditions. These analytical platforms can be subdivided in four major categories: Shotgun (LC–MS/MS) proteomics, MALDI imaging, gel-based platforms, and targeted analysis of proteins. An overview of clinical proteome studies using human FFPE tissue is summarized according to analytical platform in Table 2 (Fig. 1).

**Table 2** Overview of human clinical studies using FFPE proteomic approaches

| | Reference | Clinical tissue | # Samples | # Proteins identified | Analytical platform | Separation | Quantification |
|---|---|---|---|---|---|---|---|
| **Shotgun** | | | | | | | |
| 1 | Craven et al. (2012) | Kidney | 16 | 2,516[§] | LC–MS/MS; LTQ | RP-C18 | Spectral counting |
| 2 | Naidoo et al. 2012) | Pancreas | 55 | 1,504 | LC–MS/MS; LTQ | SCX + RP-C18 | Spectral counting |
| 3 | Nakatani et al. (2012) | Kidney | 20 | 170 | LC–MS/MS; Q-TOF | NA | iTRAQ |
| 4 | Wisniewski et al. (2012) | Colon | NA | 9,502 | LC–MS/MS; Q exactive | MED-FASP-SAX + RP-C18 | Label-free, MaxQuant |
| 5 | Bateman et al. (2011) | Breast | 25 | 9,437* | LC–MS/MS; LTQ | RP-C18 | Spectral counting |
| 6 | Donadio et al. (2011) | Parathyroid tissue | 5 | 163 | LC–MS/MS; LTQ Orbitrap XL | RP-C18 | None |
| 7 | Tanca et al. (2011a) | Lung | 6 | 666 | GeLC–MS/MS; Q-TOF | RP-C18 | Spectral counting |
| 8 | Wisniewski et al. (2011) | Colon | NA | 4,419 | LC–MS/MS; LTQ Orbitrap XL | FASP-SAX + RP-C18 | Label-free, MaxQuant |
| 9 | Kawamura et al. (2010) | Lung | 13 | 649 | LC–MS/MS; LTQ | RP-C18 | Spectral counting |
| 10 | Rezaul et al. (2010) | Melanoma | 1 tissue block | 935 | LC–MS/MS; LTQ | NA | None |
| 11 | Xiao et al. (2010) | Pharynx | 40 | 730 | LC–MS/MS; Q-TOF | SCX + RP-C18 | iTRAQ |
| 12 | Huang et al. (2009) | Melanoma | 24 | 555 | LC–MS/MS; LTQ | RP-C18 | Chromatographic peak intensity |
| 13 | Negishi et al. (2009) | Tongue | 20 | 25,018 peaks** | LC–MS/MS; Q-TOF | 2DICAL | 2DICAL |
| 14 | Nazarian et al. (2008) | Brainstem | 2 | 188 | LC–MS/MS; LTQ | RP-C18 | Isotope labeling |
| 15 | Perroud et al. (2009) | Kidney | 50 | 777 | LC–MS/MS; LTQ | RP-C18 | Spectral counting |
| 16 | Cheung et al. (2008) | Pancreas | 1 tissue block | 523 | LC–MS/MS; LTQ | RP-C18 | None |
| 17 | Jain et al. (2008) | Oral HPV lesions | 10 | 114 | LC–MS/MS; MALDI | RP-C18 | iTRAQ |
| 18 | Patel et al. (2008) | Head and neck | 16 | 391, 866, 729, 676*** | LC–MS/MS;LTQ | RP-C18 | Spectral counting |
| 19 | Ronci et al. (2008) | Breast | NA | 70, 22, 59 **** | LC–MS/MS; Q-TOF | RP-C18 | None |
| 20 | Bagnato et al. (2007) | Coronary vessels | 35 | 710 | LC–MS/MS; LTQ | RP-C18 | Spectral counting |
| 21 | Guo et al. (2007) | Glioblastoma | 1 tissue block | 2,733 | LC–MS/MS; LTQ | RP-C18 | None |
| 22 | Shi et al. (2006) | Kidney | 4 | 1,830 | LC–MS/MS; LTQ | CIEF + RP-C18 | None |
| 23 | Crockett et al. (2005) | Lymphoma | 1 tissue block | 324 | LC–MS/MS; LCQ | RP-C18 | None |
| 24 | Hood et al. (2005) | Prostate | 2 | 1,858 | LC–MS/MS; LTQ or LIT-FT-ICR | RP-C18 | Isotope labeling |
| 25 | Palmer-Toy et al. (2005) | Ear | 1 | 123 | LC–MS/MS; LCQ Deca XP plus | RP-C18 | None |
| 26 | Prieto et al. (2005) | Colon | 1 | 350 | LC–MS/MS; LTQ | RP-C18 | None |

**Table 2** continued

| | Reference | Clinical tissue | # Samples | # Proteins identified | Analytical platform | Separation | Quantification |
|---|---|---|---|---|---|---|---|
| **MS imaging** | | | | | | | |
| 27 | Lazova et al. (2012) | Melanoma | 114 | 5 differential peptides | Maldi-MSI | None | None |
| 28 | Morgan et al. (2013) | Kidney | 70 | Peptide signature of 7 and 12 peptides | Maldi-MSI; Autoflex Speed | None | None |
| 29 | Morita et al. (2010) | Stomach | 9 | 4 cancer specific peptides | Maldi-MSI; Qstar XL | None | None |
| 30 | Groseclose et al. (2008) | Lung | 50 + 10 | 50 | Maldi-MSI | None | None |
| **Gel-based** | | | | | | | |
| 31 | Tanca et al. (2012c) | Stomach + Lung | NA | NA | Gel-based | 2D gel | 2D DIGE |
| 32 | Ono et al. (2009) | Cervix | 21 | 9 | Gel-based + LC–MS/MS: LCQ | 2D gel | 2D DIGE |
| **Targeted** | | | | | | | |
| 33 | Sprung et al. (2012) | Kidney, breast | NA | 1982 protein groups | LC–MS/MS; LTQ XL | RP-C18 | MRM |
| 34 | Gamez-Pozo et al. (2011) | Lung + kidney | 3 + 3 | 151/49 phospho + 154/42 phospho | LC–MS/MS; LTQ-Orbitrap XL | IMAC + RP-C18 | Phospho + MRM |
| 35 | Güzel et al. (2011) | Placenta | 10 | 141 | LC–MS/MS; QTRAP | RP-C18 | MRM |
| 36 | DeSouza et al. (2010) | Endometrium | 25 | 17 targeted proteins | LC–MS/MS; QTRAP | RP-C18 | MRM (mTRAQ) |
| 37 | Nishimura et al. (2010) | Lung | 27 | NA | LC–MS/MS; QTRAP | RP-C18 | MRM |
| 38 | Hwang et al. (2007) | Prostate | 30 | 428 | LC-MS/MS; LTQ | RP-C18 | MRM (AQUA) |

§ Total number of unique proteins in all samples

* Sum of proteins after triplicate injections of 25 samples

** Only number of peaks described

*** Number of unique proteins per category

**** Number of unique proteins per protocol

## Shotgun (LC–MS/MS) proteomics

For a couple of years now, shotgun proteomics is the method of choice for the analysis of complex samples. Here, enzyme-digested proteins are separated using liquid chromatography (LC) and analyzed by tandem mass spectrometry (MS/MS). This peptide-centric gel-free analysis platform has the possibility to analyse samples in a high throughput manner and enables the identification and quantification of thousands of peptides in just a couple of hours.

In the context of FFPE proteomics, the first shotgun experiments were performed to identify as much confident peptides/proteins as possible, without any quantification purposes. Palmer-Toy et al. pioneered in 2005 in reverse phase (RP) LC–MS/MS proteomics of FFPE tissue and could identify 123 proteins in FFPE material. To identify these proteins, they prespecified the standard modifications, methionine oxidation, and cysteine carbamidomethylation, but ignored other modifications induced by the crosslinking event. However, they tried to compensate this with an increased number of trypsin miscleavages and the use of the reversed database peptide strategy, i.e., translated open reading frames in reversed ('decoy') orientation, reducing false positive identifications (Palmer-Toy et al. 2005). Also in 2005, Prieto et al. (2005) identified 350 proteins in colon FFPE tissue using RP LC–MS/MS and in house developed software. To gain insights in the pathology of prostate cancer, Hwang et al. (2007) provided a global proteomic analysis using the direct tissue proteomics method, in which the whole tissue slice is used to extract proteins, further digested with trypsin and separated applying RP

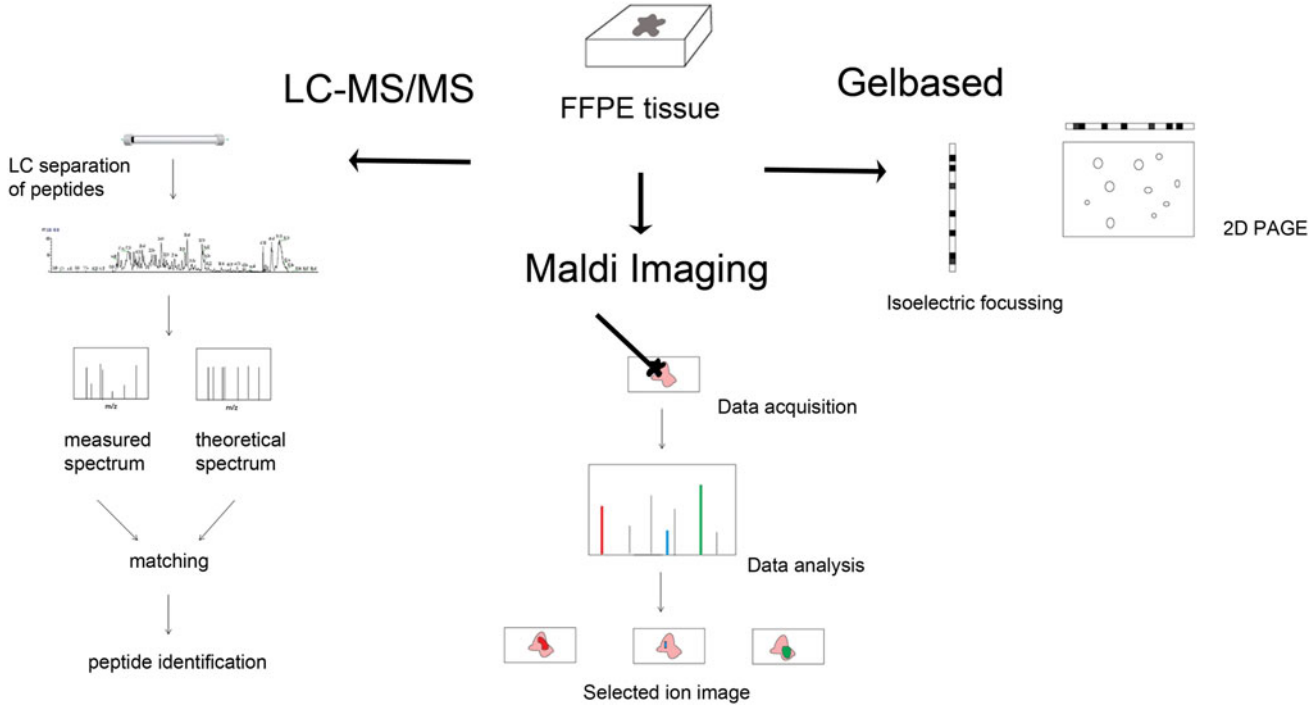


**Fig. 1** Overview of different analytical platforms used in FFPE proteomics

LC–MS/MS. For the confident identification of proteins, they prespecified methionine oxidation and phosphorylation (STY) as possible modification in combination with the decoy database searching approach to minimize false positive identification. However, again no special settings were used to handle with the crosslinked protein products. Bagnato et al. also used the direct tissue proteomics (DTP) method, but compared it with laser capture microscopy (LCM), which can isolate specific areas or cell types in a FFPE tissue. They found 710 proteins in coronary vessels FFPE tissue using the DTP strategy, but only 225 proteins were identified with more than one peptide identification. Using LCM in combination with in-solution digestion and LC–MS/MS, on the other hand, resulted in 495 multi-hit protein identifications (Bagnato et al. 2007). Again, no special settings were proposed to deal with FFPE tissue.

Crockett et al. downscaled the LC-system and used nano-RP-LC–MS/MS to achieve higher sensitivity. In their proof-of-principle experiment, they used the SUDHL-4 cell line, derived from human transformed B cell lymphomas, pelleted the cells, fixed and embedded them, and performed a shotgun proteomic analysis. To digest the extracted FFPE proteins, they used both trypsin and glutamic C endopeptidase, ensuring that some crosslinked proteins, inaccessible for trypsin digestion, could be digested and thus identified. A result of 324 unique protein identifications from 10 replicate runs was achieved by combining the identified proteins from trypsin digestion, glutamic C digestion, and a combination of both enzymes. However, the authors did not specify any modifications using database search, probably explaining why the number of protein identifications is still limited (Crockett et al. 2005). Cheung et al. (2008) identified 523 unique proteins in pancreatic cancer using tryptic digestion in combination with triplicate nano-RP-LC–MS/MS runs. Also here, no modifications were specified. Donadio et al. optimized extraction buffers containing high amounts of SDS for both gel-based and gel-free purposes, and furthermore tried to use it for LC–MS/MS by combining ‘SDS out kit’ and TCA/Acetone precipitation. With this gel-free protocol, they were able to identify 163 confident proteins from adenoma parathyroid tissues using RP LC–MS/MS settings (Donadio et al. 2011). However, despite the effort, they put in the efficient extraction of proteins, no solutions were proposed to deal with the protein crosslink problem for MS data analysis.

Since the development of the multidimensional protein identification technology (MudPIT) in 1999 (Link et al. 1999; Washburn et al. 2001), where complex samples were first separated using strong cation exchange (SCX) and all the separate fractions are then analyzed using online RP chromatography, the use of 2D-LC to elucidate complex proteomes has emerged exponentially. Also in the FFPE proteomics, 2D-LC was used in several different setups. The use of an integrated capillary isoelectric focusing (CIEF) and nano-RP-LC–MS/MS system was used to

identify over 1,800 confident proteins in renal FFPE samples (Shi et al. 2006). Although the authors did not mention any prespecified modifications, the increased number of identification compared to prior proteome experiments is stunning and results from the combination of 2D separation and nano-LC systems. This 2D-LC combination was also used by Guo et al., who splitted the FFPE protein sample into 19 CIEF fractions and resolved them further by nano-RP-LC and nano-ESI-LTQ-MS/MS. Using this approach, they could identify 2,733 proteins in glioblastoma multiforme FFPE tissues (Guo et al. 2007). Concerning prespecified modifications, the authors made following settings: alkylated cysteine as fixed modifications and acetylation (N-terminus + lysine) and oxidized methionine as variable modifications. They also included the decoy strategy to out rule false positive identifications.

The combination of 1D gel electrophoresis and LC–MS/MS is another 2D experimental setup which is used by Tanca et al. (2011a) to reduce the complexity of the proteome of neuroendocrine archival tissue. They compared two different fractionation methods: excision of 13 visible protein bands vs slicing of the whole gel lane into 38 fractions. Whole gel lane fractionation yielded more protein identifications (153) compared to visible band excision (92). Again, crosslinked protein products are ignored in the database searching methods, although they mention the fact that the position on 1D gel gives an indication of the proteins or their crosslinked complexes.

In clinical FFPE proteomics, however, most of the research groups perform proteome studies to find protein profiles which differ in abundance between diverse conditions. For quantification purposes in FFPE proteomics research, label-free approaches have become popular. In these label-free approaches, each sample is individually prepared and analyzed in an 1D or 2D LC–MS/MS setup. Furthermore, quantification can be achieved by protein abundance correlation through either mass spectrometric ion intensities of MS or MS/MS signals (peak intensity) or the number of MS/MS spectra per peptide and protein (spectral counting) (Li et al. 2012). This last method is more frequently been used in FFPE proteomics, as it suffers less from the peptide overlap of different fractions. Bateman et al. (2011) for example, used spectral counting to find differential protein profiles in breast cancer samples to indicate early-to-late stage progression as well as recurrence of the disease. Also, other research groups used FFPE proteomics in combination with spectral counting to elucidate protein expression changes involved in tumor progression. Patel et al. (2008) profiled the progression of head and neck cell squamous carcinoma at proteome level. In total, 391 proteins were identified in normal squamous epithelium, 866 in well differentiated epithelium, 729 in moderate differentiated, and 676 in poorly differentiated epithelium. Also in renal cancer, spectral counting was used to characterize different stages using LC–MS/MS (Perroud et al. 2009). In the search for stage-related protein candidates in lung cancer, Kawamura used spectral counting and found more than 500 identified proteins, of which 81 were correlated with stage Ia or IIIa (Kawamura et al. 2010). To understand the proteomic changes in melanoma progression and metastasis, Huang et al. (2009) used the label-free peak intensity approach and did find 120 proteins that were differentially expressed in metastasis compared to primary melanomas.

Also for quantitation purposes, the addition of an orthogonal LC dimension will help to mine deeper into the FFPE proteome. Naidoo et al. (2012) for example, used spectral counting in combination with MudPIT separation to assess proteomic differences between primary pancreatic tumors and matched lymph node metastasis. From the 115 proteins that were differentially expressed, S100P and 14-3-3 sigma were further confirmed using immunohistochemistry (Naidoo et al. 2012). Negishi et al. used their own developed label free method called 2DICAL (2 dimensional image converted analysis of liquid chromatography) to find differentially expressed proteins in tongue cancer. They could collect 25,018 MS peaks and did find 72 peaks which were significantly differentially expressed between cancer and normal epithelia (Negishi et al. 2009). The research group of Mathias Mann used specialized MaxQuant software to perform label-free quantitation for proteins extracted from both colon and breast archival tissues, using filter aided sample preparation—Strong Anion Exchange (FASP-SAX) fractionation, followed by RP separation coupled to an Orbitrap mass spectrometer (Wisniewski et al. 2011). Using the same strategy and multi-enzyme digestion, the research group revealed that identification of 10,000 proteins from human colon FFPE tissue is possible (Wisniewski et al. 2012).

All these studies indicate that these label-free techniques have potential to elucidate biological questions regarding differential protein profiles using archival FFPE tissues. The major advantage of this label-free technique is that no additional steps for labeling are needed and thus no extra costs are involved. Certainly in FFPE context, where additional labeling chemistry might complicate the results even more, label-free methods are popular. The major disadvantage, however, is that run-to-run variation needs to be controlled to the most possible extend, which might be a challenge, even with state-of-the-art instruments. Also, although a broad dynamic range is reached for peptide identification, a high amount of spectral counts is necessary to obtain reliable quantification results. Therefore, some authors opted to use enzymatic or chemical labeling strategies.

Hood et al. pioneered in labeling-based methods on FFPE tissue using enzymatically facilitated $^{18}O$ labeling to quantify tryptic peptides after separation using nano-RP-LC–MS/MS. They could identify 1,300 unique peptides from 702 proteins selected out of 200,000 cells of benign prostatic hypotrophy tissue and found 69 differentially expressed proteins between prostate cancer and benign prostatic hypertrophy (Hood et al. 2005). Although identified and quantified, prostate specific antigen (PSA) could not make a distinction between these two prostate-specific diseases. The $^{18}O$ proteolytic labeling strategy was also employed by Nazarian et al. (2008) to profile the FFPE proteome of brainstem glioma compared to normal tissue. In total, they could identify and quantify 188 proteins and found 54 up-regulated proteins, of which several were already linked to cancer. Although incubation of peptides with trypsin in $^{18}O$ enriched water provides a straightforward way to label peptides, a major disadvantage of this technique is that the reproducibility is heavily dependent on many factors including labeling time, temperature, amount, and activity of the trypsin, and also back reaction between $^{18}O$ and $^{16}O$ is possible (Wu et al. 2012).

For that reason, chemical labeling strategies, e.g., isobaric tags for relative and absolute quantification (iTRAQ) are also used. The major advantages using isobaric tags involve the possibility to multiplex up to eight samples into one LC–MS/MS run, thereby saving instrument time and reducing the run-to-run variation seen in label-free methods. Moreover, it has been shown that isobaric chemical labeling can provide more accurate and precise quantification of proteins compared to label-free methods (Li et al. 2012). A disadvantage, on the other hand, is that the labels are only integrated after protein reduction, alkylation, and digestion, creating thus variability. In the context of FFPE proteomics, the labeling efficiency of crosslinked peptides is also major concern. Jain et al. though demonstrated that the iTRAQ technology was able to quantify 114 FFPE extracted proteins, which could lead to discrimination between HIV(+) and HIV(−) patients (Jain et al. 2008). Xiao et al. (2010) used iTRAQ quantification in combination with MudPIT technology to perform a proteome analysis on nasopharyngeal carcinoma. According to the author, a good iTRAQ labeling performance was achieved, despite the presence of crosslinked proteins. To elucidate the pathology of diabetic nephropathy, Nakatani et al. (2012) used iTRAQ and could find 55 proteins that were upregulated and 45 downregulated proteins in glomerular cross sections from diabetic patients with nephropathy. Most of them are linked to renal and urological disease. Again, no special adjustments were made to deal with the crosslinking problem.

## Mass spectrometry imaging

Mass spectrometry imaging (MSI) is a mass spectrometric technique that enables the visualization of proteins without losing spatial information. In proteomics applications, mostly matrix assisted laser desorption ionization (MALDI)-MSI is applied (Amstalden van Hove et al. 2010). In MALDI-MSI, matrix is first deposited on a thin tissue section, the sample surface is rastered according to a predefined rectangular *X*, *Y* grid, and full mass spectra are acquired in each pixel where analytes are deserted upon laser irradiation. The intensity values of different ions can be plotted upon the initial raster, generating a spatial image (Walch et al. 2008; Cornett et al. 2007). Furthermore, the technique allows to map hundreds of proteins/peptides simultaneously in a thin tissue section with a spatial resolution of 50 µm (Minerva et al. 2012). A major advantage using imaging techniques is that no extensive extraction procedures, purifications, and separations are conducted. Also, there is no need to develop specialized antibodies, which makes it an ideal approach for biomarker discovery. The nature of the proteins and their distribution in the tissues can be directly attributed to morphological or biochemical changes (Goodwin et al. 2008). Therefore, it is complementing classical immunohistological approaches, such as immunohistochemistry and in situ hybridization, that require prior knowledge of the target proteins (Angel and Caprioli 2013). However, the MALDI-imaging method works best for small hydrophilic and abundant proteins, but fails to detect a large number of proteins, even in fresh material. Also, absolute quantification of proteins using MSI is not possible yet (Angel and Caprioli 2013).

The use of FFPE tissue slices as an alternative for fresh frozen material for MSI is known for several years now. In 2007, Lemaire et al. could show that direct tissue MSI of FFPE tissues stored <1 year was feasible for peptides smaller than 5 kDa using 2,4-dinitrophenylhydrazine as matrix. To analyze the proteins in the FFPE sample, they implemented an enzymatic digestion protocol, as MSI imaging of FFPE tissues is mainly limited to peptide analysis, because chemical crosslinked proteins are difficult to assess with MS (Lemaire et al. 2007). More recent reports illustrate that FFPE imaging of proteins/peptides by MSI is feasible by combining the antigen retrieval technique and in situ enzymatic digestion before matrix application (Gustafsson et al. 2010; Djidja et al. 2009; Seeley and Caprioli 2011; McDonnell et al. 2012).

The lab of Caprioli could show that the spatial expression of some proteins, including S100-A9, differs between adenocarcinoma and squamous cell lung carcinoma by application of tissue microarrays (TMA) (Groseclose et al. 2008). In TMAs, several tissue cores are placed into a

single paraffin block, sectioned, and in this way analyzed simultaneously. TMAs have gained tremendous interest as these allow high throughput analysis from large clinical cohorts (Groseclose et al. 2008). In 2010, TMAs and MALDI-MSI were applied to detect cancer specific or histological type-specific proteins using gastric cancer FFPE samples (Morita et al. 2010). Morgan et al. (2013) also combined TMA and MALDI-MSI and found a peptide signature that could accurately distinguish malignant from normal renal tissue. MALDI-MSI of FFPE tissues was also used to distinguish spitz nevus from spitzoid malignant melanoma. Five peptides were found to be differentially expressed and could classify spitz nevus with 97 % sensitivity and 90 % specificity using a validation cohort of 59 samples (Lazova et al. 2012).

### Gel-based proteomics

Although gel-based proteomics is still the only technique which can visualize thousands of proteins in one image, interest in the workhorse of proteomics has decreased. Also in FFPE-applied proteomics, the number of research articles is limited. Ahram et al. (2003) showed that the application of 2D gels to ethanol-fixed paraffin embedded tissues was more successful than using tissues fixed with formaldehyde. The ethanol fixed protein spots in the gel were a bit fuzzy, but a good comparison with the frozen material was possible. The 2D gel separation of FFPE extracted proteins, on the other hand, did not show any spot, only streaking and blurring. Ono et al. (2009) did use 2D DIGE to compare the FFPE proteome of uterine cervix squamous cell carcinoma and healthy tissues, but could only show a low-quality 2D map with smears. Donadio et al. (2011) applied 2D electrophoresis using FFPE material from patients with sporadic primary hyperparathyroidism and did only found a low quality pattern of 16 spots and smears. Until now, the research groups of Alessandro Tanca and Maria Filippa Addis are the only one managing to successfully separate proteins extracted from FFPE tissues using 2D gel electrophoresis (Addis et al. 2009; Tanca et al. 2011b, 2012c). Although their patterns are more comparable with fresh frozen tissues than other research groups ever achieved, the quality of FFPE 2D gels remains questionable.

### Targeted proteomics

Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), is a technology that also complements the shotgun proteomics strategy as it allows the quantification of a predefined set of proteins. In an SRM workflow, the mass spectrometer will first target a specific peptide of interest in a first stage followed by targeting one (SRM) or more (MRM) of its fragment ions in a second stage. The combination of the precursor ion and fragment ions allows to specifically target the protein of interest in different samples, as all other acquired signals can be ignored (Picotti and Aebersold 2012; Lange et al. 2008).

The performance characteristics of FFPE extracted protein quantitation by MRM is assessed by Sprung et al. Their results did show that, as expected, the formalin-induced chemical modifications decrease the sensitivity of the MRM measurements, meaning that fewer targets will be available for quantification compared to fresh tissues. However, in their proof-of-concept experiment in which they quantified the HER2 receptor expression profiles in breast tumors, the assay sensitivity of FFPE extracted proteins and fresh frozen samples was comparable, when using unmodified peptides (Sprung et al. 2012). Grüzel et al. also made use of MRM quantification to find pre-eclampsia related calcyclin peptides in placental FFPE tissue. Their MRM assay demonstrated that in pre-eclamptic patients, elevated levels of calcyclin is observed in placental trophoblast cells compared to normal trophoblast cells (Guzel et al. 2011). An MRM quantitative analysis for stage-related proteins upon non-metastatic lung adenocarcinoma was performed by Nishimura et al. This study suggested that napsin-A and anterior gradient protein 2 homolog (hAG-2) would be useful for determining stage IA or IIIA lung adenocarcinoma. Both proteins could also be related to metastasis (Nishimura et al. 2010). In the context of FFPE extracted proteins for MRM applications, the Absolute QUAntification (AQUA) technique is also introduced. In the AQUA workflow, a peptide containing a stable-isotope labeled amino acid is synthesized based on the sequence of the targeted peptide of interest. This synthesized 'heavy' peptide is then spiked into the complex proteome sample and used as internal standard for quantification (Ye et al. 2009). The research group of Han used the AQUA technique to quantify PSA in prostate FFPE tissues and did find a trend of increasing levels of PSA in more advancing tumors (Hwang et al. 2007). To quantify pyruvate kinase M2 in endometrial samples and to confirm overexpression in cancer, DeSouza et al. (2010) used Tags for Relative and Absolute quantification (mTRAQ). In mTRAQ, up to three non-isobaric labels can be used to label a known quantity of a synthetic peptide whose sequence is identical to the tryptic peptide of interest and, on the other hand, the sample containing tryptic peptides. After mixing and LC separation, the labeled peptides provide unique MRM transitions, both in MS mode (three non-isobaric parent ions) and in MS/MS mode (non-isobaric sequence ions) (DeSouza et al. 2008).

## Pitfalls and challenges

Although a vast archive of clinical samples is present, FFPE tissues have been considered as obstinate to proteomic analysis. The reason why molecular analysis of these tissues is often questioned is through the many challenges and pitfalls one is confronted with.

The presence of protein/DNA/RNA crosslinks is one of the most important issues which hamper proteomic analysis. Extraction of proteins from archival FFPE tissues is mostly accompanied by a combination of exposure to organic solvents, heat, and proteolysis. Although shown to be efficient, it even might be the question whether all proteins stay soluble in this combination. Also, the reversal of crosslinks renders several by-products, inaccessible for mass spectrometric analysis and possibly disturbing high quality LC separations. However, it is also known that only a small percentage of formaldehyde-reactive amino acids form irreversible chemical modifications. *In vitro* models do show that the crosslink event is more complicated than theoretically assumed and thus more unspecified modifications will turn up. Also, predicted peptide modifications, such as methylol derivatives, are often not detected in experimental data. In addition, modifications due to chemical crosslinking might hinder tryptic digestion in some extent.

This will lead to a second major challenge: the confident identification of proteins. Not only will unknown and unexpected modifications on (tryptic) peptides lead to a decreased protein and proteome coverage; also biases exist compared to fresh material in identified basic proteins and in the lysine/arginine ratio of identified proteins, as basic amino acids, and in particular, lysine are known to be involved in the crosslinking process (Tanca et al. 2012a). Therefore, new bioinformatic approaches are needed to unlock the full FFPE proteome. The development of new algorithmic data-analysis software for LC–MS/MS data could solve this problem to a major extent.

A third challenge is related to working with human tissues. It is already known that the variability between human samples is partly due to genetic diversity of humans, different environments, but also differences such as age, gender, and race that need to be controlled whenever one wants to perform proteomic profiling studies. Also, some ethical issues need to be considered, such as permissions that must be obtained from the patient before using the clinical sample for scientific purposes. This might not be a problem for samples gathered these days, but must also be present for FFPE samples that are already stored for over 10 years.

Finally, several other, more technical factors determine the variability of FFPE proteome data. One of the major parameters that should be controlled is the time between the resection of the tissue and the fixation in formalin. Ideally, samples should be fixed immediately and completely from the living state. In animal models, this can be achieved using in vivo perfusion. In humans, however, surgically removement is the only option, and anoxic periods due to anesthesia and surgical clamps can influence the expression patterns of proteins. These factors cannot be strictly controlled because they impact patient care, but could, on the other hand, be recorded in detail (Hewitt et al. 2008). Also the way of conservation of the tissue between resection and fixation (either dry or bading in a solution) can have implications and should be standardized.

Another source of variability (and probably the easiest to control) is the fixation procedure. Three aspects are important during this protocol: the thickness of the tissue, the volume of the fixative, and the fixation time. Unsuccessful optimization of these three elements results in under- or overfixation of the clinical samples, resulting in higher variation between different specimens. Through the large diversity of size of resected specimens, fixation procedures might be difficult to standardize. Therefore, we suggest that larger specimens should be sectioned as soon as possible after their resection, and the volume of the fixative is adapted to the size of the specimen, starting from a minimum formalin:tissue ratio 10:1. Concerning different fixation times, Tanca et al. made an evaluation of the efficiency of protein extraction and the quality of the obtained quantitative data. Their results show that successful identification of peptides decreases, as the fixation time increases (Tanca et al. 2012b). Unfortunately, it is common practice to fix specimens overnight or even over the weekend. Failure to standardize these steps will thus pose problems to the researchers working with the material.

The archival time is also an important parameter that can introduce variability in datasets, as it influence the retrieval of proteins over time (Balgley et al. 2009). On top, differences in storage conditions, e.g., temperature, humidity,… will also have an effect on the variation of the FFPE proteome. For data interpretation, the way of isolation of the tissue, e.g., cutting cylindrical section, working with tissue slices, or laser microdissected cells, will also influence the data. Overcoming all these technical issues and accomplish the goal of standardization is thus a major challenge.

## Future perspectives

Recent advances in the field of FFPE proteomics demonstrate that unlocking the FFPE proteome might have major potential for retrospective and translational biomarker research. Unfortunately, current practices lack the required standardization. Therefore, the use of fresh tissue remains the gold standard. To establish the use of FFPE material as primary

tissue source for proteome studies, more investigations are needed to reduce the numerous variables and to achieve the many challenges. In the first place, protocol refinement is needed to manage the confounding FFPE data analysis which arises from different fixation times, storage periods, incomplete crosslink reversal, etc. Therefore, more standardization in procedures of fixation and storage are needed to reduce biases and to maximize the dependability of the obtained proteomic results. These standard operation procedures should be systematically used in every hospital with an FFPE archive, which should create the possibility to perform multicentric proteomic studies. We also recommend obtaining as much information as possible regarding the surgery parameters, fixation times, etc., which can only improve the quality and reproducibility of the scientific research. Also, existence of measures to assess the sample quality of archival tissues, both at molecular and morphological level, is critical and should reduce the variability in degradation effects between samples. Finally, the unraveling of all the peptide modifications induced by formalin crosslinking by means of bioinformatics and understanding the full process of crosslinking might lead the FFPE proteome research toward complete acceptance. This translational (biomarker) research strategy depends thus on the interdisciplinary contributions of clinicians, scientists, and biostatisticians.

When evaluating different analytical platforms used in proteomics today, LC–MS/MS will probably have the most potential to unlock the FFPE proteome because of its capability to identify thousands of proteins in just a couple of hours. However, obtaining such results is only possible when new developments in the interpretation of FFPE raw data files succeed. Also the combination of LC–MS/MS and MALDI-MSI might have opportunities for future clinical research, but with limitations concerning the mass range of proteins. This setup is a powerful tool because detecting differences in cells with specific spatial coordinates combined with the identification of these specific candidates by LC–MS/MS make it possible to merge biomarker discovery and histopathology. Regarding targeted analysis, only non-modified peptides are applicable for high quality absolute quantification, making the development of MRM methods even more difficult than it is these days using fresh material.

In conclusion, while considerable progress has been achieved, analysis of FFPE tissue proteome is still a major challenge. Using FFPE tissue in future, proteome biomarker studies has potential, if more standardized methods will be applied and appropriate data-analysis software is available.

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