

REVIEW

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Mass spectrometry-based proteomics of FFPE tissues: progress, limitations, and clinical translation barriers

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Abstract

Background Formalin-fixed paraffin-embedded (FFPE) tissue proteomics has emerged as a promising approach for precision medicine, offering access to vast clinical archives. Despite technological advances enabling identification of thousands of proteins from FFPE samples, no proteomic diagnostic tests based on FFPE tissues have achieved regulatory approval for clinical diagnostics, raising fundamental questions about the translational viability of this approach.

Main body This review critically evaluates the realistic barriers preventing clinical translation of FFPE proteomics and identifies targeted applications with genuine promise for near-term implementation. We demonstrate that while comprehensive discovery-based proteomics faces insurmountable challenges including validation failure rates exceeding 90%, targeted proteomic strategies focused on specific clinical questions show substantially greater potential. Current implementation barriers extend beyond technical limitations to encompass economic constraints (5-10-fold higher costs than immunohistochemistry), regulatory uncertainties, and fundamental incompatibilities with clinical laboratory workflows. The persistent emphasis on increasingly complex analytical platforms may represent misallocated resources given unresolved standardization and validation challenges.

Conclusion Strategic redirection toward targeted proteomic applications addressing specific diagnostic needs, rather than comprehensive molecular profiling, offers the most viable pathway for clinical translation. Success will require prioritizing applications where FFPE proteomics provides unique, actionable information that justifies its complexity and cost relative to established methodologies. We propose specific criteria for identifying high-impact applications and outline a pragmatic roadmap for achieving clinical implementation within realistic timeframes.

Keywords FFPE tissues, Clinical proteomics, Targeted proteomics, Biomarker validation, Clinical translation, Precision medicine, Mass spectrometry

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Introduction

Clinical significance and scope of FFPE tissue archives

FFPE tissues constitute the most extensive repository of preserved human biological specimens worldwide, encompassing billions of archived samples housed within pathology departments and biobanks that contain decades of longitudinally linked clinical outcome data [1]. These archives represent an invaluable and irreplaceable resource for translational research, offering unique opportunities for retrospective biomarker validation across diverse patient populations, rare disease investigation where prospective sample collection is impractical, and longitudinal studies spanning disease progression from early lesions to metastatic disease.

The clinical value of FFPE archives extends beyond simple sample availability to encompass critical advantages for precision medicine implementation. These specimens are routinely collected during standard diagnostic procedures, eliminating the need for additional invasive sampling while providing direct correlation with histopathological diagnosis and clinical outcomes. The ability to perform molecular analysis on morphologically defined regions of interest enables investigation of tumor heterogeneity, microenvironmental interactions, and spatial organization of disease processes that cannot be captured through liquid biopsy or bulk tissue analysis approaches.

Current molecular diagnostic landscape for FFPE tissues

The contemporary molecular diagnostic landscape for FFPE tissues encompasses multiple established and emerging technologies, each with distinct capabilities, limitations, and clinical applications that define the competitive environment within which FFPE proteomics must demonstrate value. Immunohistochemistry (IHC) remains the gold standard for protein-level analysis in clinical pathology, providing targeted protein detection with subcellular spatial resolution at costs typically ranging from fifty to one hundred dollars per marker [2–4]. The technology's widespread adoption reflects multiple advantages including established clinical workflows integrated into routine pathology practice, extensive validation across thousands of clinically relevant targets, regulatory approval pathways with clear precedent, and direct visual correlation with tissue morphology enabling pathologist interpretation. Modern IHC platforms have evolved to include multiplexed immunofluorescence approaches capable of detecting 40–60 targets simultaneously, automated staining systems ensuring reproducibility, and digital pathology integration enabling quantitative analysis [2–4]. However, IHC faces inherent limitations that create opportunities for complementary technologies. The requirement for specific, validated antibodies limits analysis to known targets, preventing

discovery of novel biomarkers [5]. Antibody-based detection provides limited quantitative dynamic range, typically 2–3 orders of magnitude, particularly when using fluorescent labels (chromogen-based methods are even more restricted, around 1–2 logs), compared to mass spectrometry's 5–6 orders of magnitude [6, 7]. Furthermore, IHC cannot distinguish between protein isoforms, post-translational modification states, or degradation products that may have distinct biological and clinical significance [8, 9].

Genomic analysis of FFPE tissues has achieved routine clinical implementation through targeted sequencing panels, comprehensive genomic profiling platforms, and emerging whole exome/genome approaches, demonstrating that molecular analysis of chemically fixed tissues is feasible when appropriate technological and regulatory frameworks are established [10–12]. The success of FFPE genomic analysis provides important precedent and benchmarks for proteomic applications. DNA's inherent stability compared to proteins, combined with PCR amplification capabilities, enables robust analysis despite fixation-induced fragmentation and crosslinking. Established bioinformatics pipelines, standardized variant calling algorithms, and clear clinical actionability for identified mutations facilitate interpretation and clinical decision-making. The FDA approval of multiple FFPE-based genomic tests, including FoundationOne CDx and MSK-IMPACT, demonstrates successful navigation of regulatory pathways for complex molecular diagnostics using fixed tissues [13, 14]. These approvals established critical precedents for analytical validation, clinical validation, and quality control requirements that inform development of proteomic applications. However, genomic analysis cannot capture post-transcriptional regulation, protein abundance, post-translational modifications, or protein-protein interactions that often determine therapeutic response and resistance mechanisms.

Liquid biopsy approaches analyzing circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and extracellular vesicles offer non-invasive molecular profiling with capabilities for serial monitoring and early detection of resistance [15, 16]. These technologies have gained rapid clinical adoption for specific applications including resistance mutation detection such as EGFR T790M, minimal residual disease monitoring, and treatment selection when tissue biopsy is not feasible [17]. The non-invasive nature enables repeated sampling for dynamic monitoring of treatment response and emerging resistance mechanisms [18]. However, liquid biopsies inherently lack spatial context, cellular heterogeneity information, and tissue architecture preservation that characterize solid tissue specimens, limiting their applicability for diagnosis requiring morphological correlation, assessment of tumor microenvironment, and

spatial biomarker patterns [12, 13]. Furthermore, sensitivity limitations for early-stage disease and inability to detect non-secreted proteins restrict their utility as comprehensive tissue replacements [19, 20].

Theoretical advantages and unique capabilities of FFPE proteomics

Mass spectrometry-based proteomics theoretically offers unique analytical capabilities that address fundamental limitations of existing approaches while providing complementary information essential for comprehensive molecular characterization [21, 22]. Unlike targeted approaches requiring prior knowledge of analytes, discovery proteomics can theoretically identify and quantify thousands of proteins simultaneously without predetermined bias [23]. This capability enables discovery of unexpected biomarkers, characterization of novel disease mechanisms, and identification of previously unrecognized therapeutic targets. Modern data-independent acquisition (DIA) approaches can achieve comprehensive proteome coverage with improved reproducibility compared to traditional data-dependent methods, creating permanent digital proteome maps that can be retrospectively interrogated as new hypotheses emerge [24–27]. Proteins, not genes, are the primary functional molecules in cells, serving as enzymes, structural components, signaling molecules, and drug targets. The correlation between mRNA and protein abundance is only moderate, with Pearson correlation coefficients typically ranging from 0.4 to 0.6, indicating that transcriptomic analysis provides insufficient information for predicting protein-based phenotypes [28, 29]. This limited correlation is further complicated by proteogenomic analyses revealing that typically less than 20% of genomic variants are confirmed at the protein level, highlighting systematic sensitivity limitations that may miss clinically relevant therapeutic targets [30]. These observations underscore the necessity of direct protein measurement rather than relying on genomic or transcriptomic surrogates for clinical decision-making. Post-translational modifications (PTMs) including phosphorylation, acetylation, ubiquitination, and glycosylation fundamentally alter protein function, localization, and stability without changes in protein abundance. Mass spectrometry uniquely enables comprehensive PTM profiling, providing insights into signaling pathway activation, epigenetic regulation, and metabolic states not accessible through genomic or transcriptomic analysis.

Modern mass spectrometry platforms provide quantitative measurements across 5–6 orders of magnitude with coefficients of variation below 20% for optimized workflows [25, 31, 32]. This dynamic range substantially exceeds immunohistochemistry's 2–3 orders of magnitude while avoiding antibody-related artifacts including

non-specific binding, epitope masking, and batch-to-batch variability. Absolute quantification using stable isotope-labeled standards enables precise protein concentration measurements for therapeutic target assessment and diagnostic threshold establishment [33]. Mass spectrometry inherently provides multiplexed analysis without the spectral overlap limitations of fluorescence-based approaches or the sequential staining requirements of traditional IHC [34]. A single analysis can quantify hundreds to thousands of proteins, enabling comprehensive pathway analysis, network reconstruction, and systems-level understanding of disease mechanisms [35]. This capability is particularly valuable for identifying multi-protein signatures that capture disease heterogeneity more effectively than single biomarkers.

The translation challenge and current status

Despite theoretical advantages and over two decades of technological development, the translation of FFPE proteomics from research tool to clinical diagnostic has proven exceptionally challenging. No FDA-approved FFPE proteomic diagnostic tests currently exist in routine clinical practice [36–38], while genomic approaches using identical tissue types have achieved widespread clinical adoption [10–14]. This striking translation gap reflects multiple interconnected challenges that extend beyond simple technical limitations to encompass analytical, economic, regulatory, and operational barriers.

Currently, while RNA-based molecular profiling tests such as the Pathwork® Tissue of Origin Test achieved FDA clearance for FFPE tissues over a decade ago [39–41], mass spectrometry-based proteomic tests remain in various stages of development and validation. Some CLIA laboratory-developed tests have been implemented in specialized oncology applications, and several targeted MS assays are progressing through various stages of regulatory validation, though their timelines for clinical approval remain undetermined. This implementation lag reflects systematic challenges in analytical validation, clinical utility demonstration, and regulatory pathway navigation that distinguish proteomic applications from their genomic counterparts [42–44].

A fundamental distinction must be established between discovery-based (untargeted) and targeted proteomic approaches, as they represent distinct analytical strategies with profoundly different clinical translation potential. Discovery proteomics employs comprehensive profiling methodologies but faces substantial implementation challenges including extended analysis times of 8–12 h per sample, complex data interpretation requiring specialized bioinformatics expertise, and poor inter-laboratory reproducibility with coefficients of variation typically 15% to 36% for protein quantification [42, 45–47]. In contrast, targeted proteomics using selected reaction

monitoring (SRM/MRM) or parallel reaction monitoring (PRM) approaches achieve coefficients of variation below 15% for optimized assays, analysis times of 15–30 min per sample, and the precision, throughput, and validation feasibility essential for clinical implementation [48–52]. This distinction underlies our conclusion that targeted, rather than discovery-based proteomics, represents the most viable pathway for clinical translation within realistic timeframes.

The comprehensive analysis of these barriers and potential solutions, along with strategic recommendations for overcoming them, forms the core of this review.

Review objectives and scope

This review critically evaluates the translational potential of FFPE proteomics with a specific focus on identifying applications that are realistically achievable within the next decade. Rather than cataloging all methodological innovations, we concentrate on approaches most likely to overcome existing barriers and reach clinical implementation. Our objective is to distinguish between (i) surmountable technical challenges, (ii) inherent limitations that require strategic workarounds, and (iii) contextual factors such as regulatory and economic constraints that define real-world feasibility. By doing so, we aim to provide clinicians, researchers, and diagnostic developers with a pragmatic understanding of where FFPE proteomics can deliver true clinical impact.

Throughout this review, we distinguish between ‘proteomic diagnostic tests’ (clinical applications for patient care), ‘MS-based assays’ (analytical laboratory methods), and ‘proteomic analysis’ (research applications). This terminology reflects different regulatory and validation requirements across application domains.

Fundamental barriers to clinical translation

Molecular basis of Formaldehyde-induced protein modifications

The fundamental challenge of FFPE tissue proteomics originates from the complex chemical modifications induced during formaldehyde fixation, which create analytical barriers that distinguish FFPE analysis from all other proteomic applications. Formaldehyde, typically used as a 10% neutral buffered formalin solution containing 3.7–4.0.7.0% formaldehyde in phosphate buffer at pH 7.0–7.4.0.4, exists predominantly as methylene glycol ($\text{CH}_2(\text{OH})_2$) in aqueous solution, with only a small fraction present as the reactive aldehyde species [53–56]. Upon tissue penetration, formaldehyde undergoes nucleophilic addition reactions with primary amines, particularly the ϵ -amino groups of lysine residues and the N-terminal amino groups of proteins, forming hydroxymethyl adducts (methylol groups) that serve as intermediates in crosslink formation. These methylol

intermediates can undergo dehydration to form Schiff bases (imines), which, although present at low concentrations under physiological pH, are highly reactive electrophiles that drive subsequent crosslinking reactions. The predominant crosslinking pathway involves direct condensation between methylol adducts and another nucleophilic amino group, yielding stable methylene bridges ($-\text{CH}_2-$) that constitute approximately 85–95% of formaldehyde-induced crosslinks in tissue specimens [55, 57–60].

The crosslinking chemistry extends beyond simple methylene bridge formation to encompass diverse reaction products that create complex three-dimensional protein networks. Secondary crosslinking pathways include Mannich-type reactions between Schiff base intermediates and electron-rich aromatic amino acids such as tyrosine, tryptophan, and histidine, forming heterogeneous crosslinks that account for 5–15% of total modifications [57, 58]. Extended fixation periods, particularly those exceeding 24–48 h, lead to formation of more complex structures including dimethylene ether linkages ($-\text{CH}_2-\text{O}-\text{CH}_2-$), polymeric formaldehyde adducts, and higher-order crosslinked networks involving multiple proteins [61]. The reaction kinetics are influenced by multiple factors including temperature, with fixation at room temperature (20–25 °C) proceeding more slowly than at 37 °C, pH effects where slightly alkaline conditions favor Schiff base formation while neutral pH promotes methylol stability, and tissue composition where tissues rich in lysine and arginine show more extensive crosslinking [62, 63]. These chemical modifications have profound implications for protein extraction and analysis, as the extensive crosslinking creates a stable protein matrix that resists solubilization under conditions that would completely dissolve fresh tissue proteins (Fig. 1).

Pre-analytical variables and their cumulative impact

Cold ischemia time and biomolecular degradation

The period between tissue devascularization and fixation initiation, termed cold ischemia time, represents a critical pre-analytical variable that profoundly impacts protein integrity and post-translational modification states. During this interval, cessation of blood flow triggers immediate cellular responses including ATP depletion, pH decline from 7.4 to 6.5–6.8, activation of degradative enzymes, and initiation of autolytic processes [64]. Phosphoproteins are particularly vulnerable to cold ischemia-induced alterations, with quantitative phosphoproteomic studies demonstrating that a substantial proportion of phosphorylation sites show significant changes within 30 min of tissue excision, and some labile phosphorylation events are completely lost within 10 min [65, 66]. The activation of endogenous phosphatases during cold

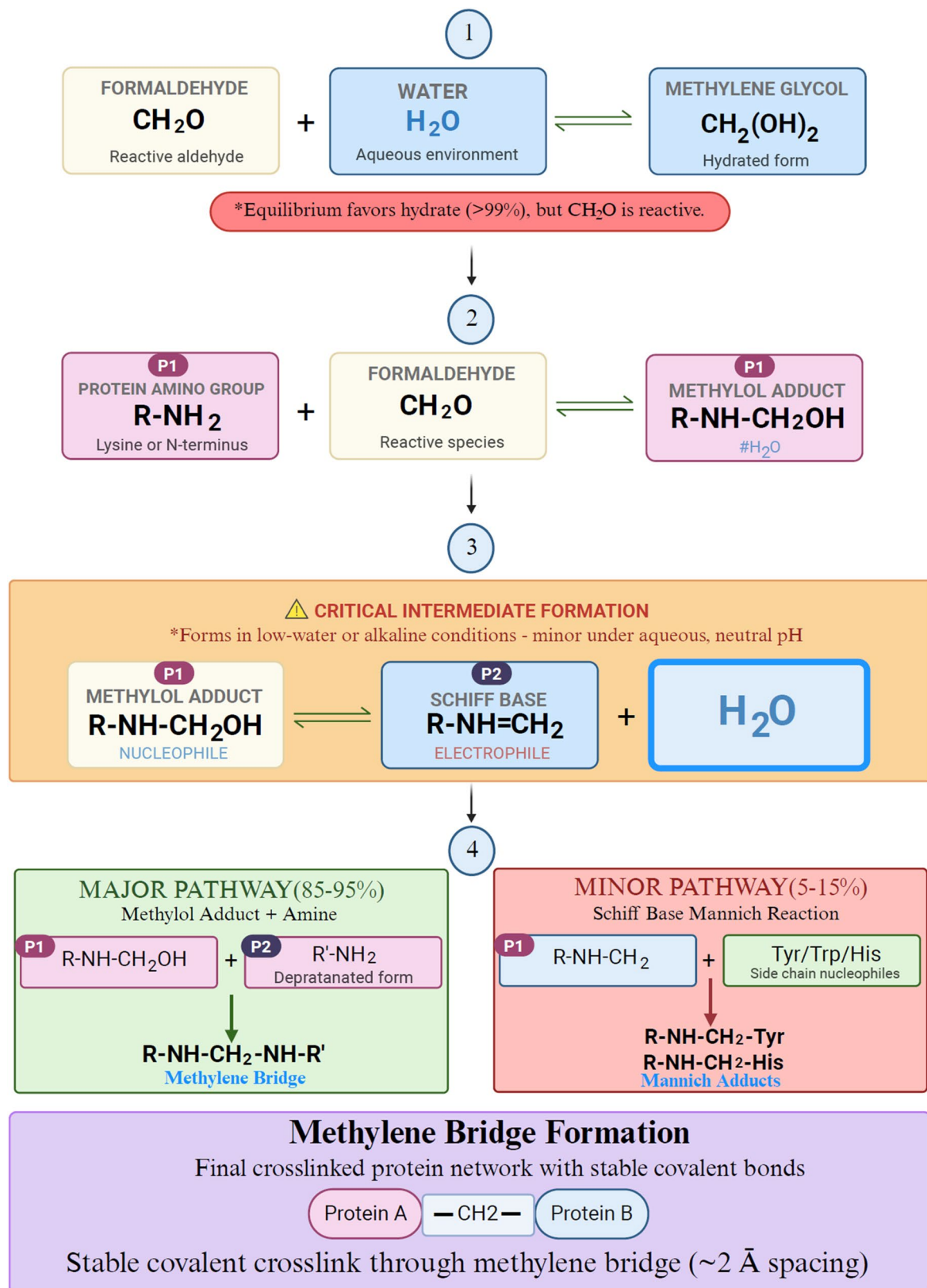


Fig. 1 (See legend on next page.)

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Fig. 1 Formaldehyde-induced protein crosslinking mechanisms in FFPE tissues. This diagram outlines the chemical pathways by which formaldehyde covalently crosslinks proteins during formalin fixation—a process that preserves tissue architecture but complicates proteomic analysis. In dilute aqueous solutions (e.g., buffered formalin), formaldehyde exists predominantly as methylene glycol ($\text{CH}_2(\text{OH})_2$) in rapid equilibrium with the reactive aldehyde (CH_2O), the latter driving crosslink formation. The carbonyl carbon of CH_2O undergoes nucleophilic attack by primary amines, most often lysine ϵ -amino groups, yielding hydroxymethyl (methylol) adducts ($\text{Protein-NH-CH}_2\text{OH}$). These adducts can reversibly dehydrate to form transient Schiff bases ($\text{Protein-N}=\text{CH}_2$), which, although low in abundance under aqueous neutral pH, are highly electrophilic. Crosslinking proceeds via two main routes: [1] Major pathway (≈ 85 – 95%)—direct reaction of methylol adducts with another primary amine, producing stable methylene bridges ($-\text{NH-CH}_2-\text{NH}-$); and [2] Minor pathway (≈ 5 – 15%)—Mannich-type reactions between Schiff bases and electron-rich aromatic residues such as tyrosine, tryptophan, or histidine, forming mixed amine–aromatic crosslinks. The resulting covalent network is dominated by methylene bridges between lysine residues, with a smaller contribution from aromatic Mannich adducts, providing long-term stabilization of tissue ultrastructure while limiting protein extraction and antigen accessibility in proteomics workflows

ischemia preferentially dephosphorylates specific signaling proteins, with documented losses exceeding 80% for certain pAKT and pERK sites that serve as critical biomarkers for pathway activation status and therapeutic response prediction [66].

Protein degradation during cold ischemia follows predictable patterns, with specific protein classes showing differential susceptibility. Cytoskeletal proteins and metabolic enzymes remain relatively stable for 1–2 h, while signaling proteins, transcription factors, and cell cycle regulators show significant degradation within 30 min [65].

The impact of cold ischemia extends beyond simple degradation to encompass complex biochemical changes that alter the tissue proteome in ways that confound biological interpretation. Hypoxia-induced activation of HIF-1 α signaling cascades triggers expression of hypoxia-responsive proteins within minutes of ischemia onset, creating artifactual protein signatures that may be misinterpreted as disease-related changes [67]. Cellular stress responses activate heat shock proteins, unfolded protein response pathways, and autophagy mechanisms that substantially alter the protein landscape [68, 69]. The release of proteases from damaged cells initiates proteolytic cascades that generate protein fragments and neo-epitopes not present *in vivo*.

These ischemia-induced changes show significant variation across tissue types, with metabolically active tissues such as liver and kidney showing more rapid degradation than structural tissues like skin or bone. Current guidelines recommend limiting cold ischemia to less than 30 min for phosphoproteomic applications and less than 1 h for general proteomic analysis, though achieving these targets consistently in clinical practice remains challenging due to surgical logistics and specimen transport delays [70].

Fixation parameters and their optimization

The fixation process itself introduces multiple variables that significantly impact downstream proteomic analysis, with fixation duration representing the most critical parameter affecting protein extractability and modification patterns. Systematic studies examining

fixation kinetics have demonstrated that formaldehyde penetration proceeds at approximately 1 mm per hour at room temperature [71], implying that thicker specimens require many hours for complete penetration [72, 73]. These findings support clinical experience that routine specimens often exhibit well-fixed outer rims but poorly-fixed inner cores after overnight processing.

The temperature during fixation significantly influences reaction kinetics: fixation at 4 °C slows crosslinking, potentially aiding antigen preservation, while fixation at 37 °C accelerates crosslinking but may also increase protein degradation and epitope masking [74, 75].

Buffer composition and pH during fixation create additional sources of variability that impact proteomic analysis. While 10% neutral-buffered formalin (pH 7.0–7.4.0.4) represents the standard fixative, variations in buffer capacity, phosphate concentration, and pH stability can significantly alter fixation chemistry. Acidic pH shifts below 6.0, which can occur in unbuffered formalin solutions, promote formaldehyde polymerization and formation of paraformaldehyde precipitates that create heterogeneous fixation patterns [76, 77].

The presence of methanol as a stabilizer in commercial formalin (typically ~ 1 – 2%) may cause protein precipitation independently of crosslinking, particularly affecting membrane proteins and hydrophobic domains [76, 77].

Tissue thickness represents another critical variable, as specimens exceeding 3–4 mm thickness show gradient fixation patterns with peripheral over-fixation and central under-fixation, complicating quantitative analysis. A minimum 10:1 fixative-to-tissue volume ratio is recommended to optimize fixation uniformity and maintain buffer capacity [78, 79].

Long-term storage effects on protein integrity

The stability of proteins in FFPE blocks during long-term storage represents a critical consideration for retrospective biomarker studies utilizing archival specimens. Contrary to the assumption that FFPE tissues represent stable, inert specimens, mounting evidence demonstrates progressive molecular changes during storage that impact proteomic analysis [80]. Proteins in FFPE blocks stored at room temperature undergo continued

slow crosslinking reactions, with formation of additional methylene bridges and more complex crosslinked structures that progressively reduce extractability [81].

Quantitative studies have documented a significant decline in extractable protein yield from blocks stored at room temperature, with accelerated degradation under suboptimal conditions including high temperature (>25 °C), high humidity (>60%), or exposure to light [82]. The oxidation of proteins during storage, particularly affecting methionine and cysteine residues, creates additional modifications that complicate mass spectrometric analysis and may alter antibody recognition epitopes [80].

The heterogeneous nature of storage-induced changes creates systematic biases in retrospective studies comparing specimens with different storage histories. Blocks stored for more than 10 years show not only reduced total protein yield but also altered protein profiles, with preferential loss of high molecular weight proteins, decreased detection of membrane proteins, and accumulation of protein fragments from slow hydrolytic degradation [83]. Post-translational modifications show differential stability during storage, with some phosphorylation sites remaining stable for years while others, particularly those on serine residues, show progressive loss through slow hydrolysis [81].

The physical integrity of FFPE blocks also deteriorates during storage, with dehydration causing tissue shrinkage and cracking that creates barriers to uniform protein extraction [80]. These storage effects show significant variation across tissue types and fixation conditions, with well-fixed tissues showing better long-term stability than marginally fixed specimens [82]. Recent studies suggest that storage at 4 °C–20 °C can substantially reduce storage-related degradation, though the vast majority of archival specimens have been stored at room temperature without climate control [81].

While optimization of formalin fixation and storage conditions can improve proteomic outcomes, alternative fixation technologies have been explored to fundamentally address crosslinking limitations [84].

Analytical variability and reproducibility challenges

Technical variability in FFPE proteomic workflows

The cumulative effect of pre-analytical and analytical variables in FFPE proteomics creates levels of technical variability that substantially exceed those observed in fresh tissue or plasma proteomics. Technical variability inherent in FFPE proteomic workflows exceeds that observed in fresh tissue analysis due to heterogeneous fixation conditions across different specimens, pathology laboratories, and temporal storage periods, with coefficients of variation substantially higher than fresh tissue approaches [43]. Studies have demonstrated CV values of 49–65% for FFPE tissues compared to approximately

41% for fresh frozen tissues, representing a roughly 1.2–1.6-fold increase in variability [80, 85]. This increased variability stems from multiple sources throughout the analytical workflow.

Protein extraction efficiency shows considerable run-to-run variation even when using identical protocols, attributed to heterogeneous tissue composition, variable crosslinking patterns, and incomplete resolubilization of protein aggregates [80]. The efficiency of crosslink reversal during extraction shows stochastic variation, with some proteins showing consistent extraction while others demonstrate substantial variability across technical replicates. Enzymatic digestion efficiency varies significantly across samples, influenced by residual crosslinks, protein aggregation state, and presence of fixation-induced modifications that alter protease accessibility, particularly affecting lysine residues due to formaldehyde-induced methylation [85].

Inter-laboratory reproducibility barriers

The translation of FFPE proteomic biomarkers to clinical practice requires demonstration of inter-laboratory reproducibility, yet significant challenges remain in achieving consistent results across different sites. Studies have shown that inter-laboratory coefficients of variation for FFPE proteomics typically range from 30 to 60%, exceeding the 20% threshold generally considered acceptable for clinical assays [43]. These reproducibility challenges arise from multiple, interconnected sources of variation that are difficult to standardize across laboratories.

At the pre-analytical level, differences in protein extraction protocols introduce systematic biases even when laboratories nominally follow the same published methods. These variations stem from heating equipment calibration differences, buffer preparation precision, and operator technique variations that compound throughout the workflow. Similarly, the use of different mass spectrometry platforms and configurations creates platform-specific biases in protein detection and quantification. The Clinical Proteomic Technology Assessment for Cancer (CPTAC) studies have demonstrated that data-dependent sampling of peptides constitutes a stochastic element contributing to variability in discovery proteomics [86]. These comprehensive interlaboratory studies, spanning 144 LC-MS/MS experiments across multiple Thermo LTQ and Orbitrap instruments, revealed that repeatability and reproducibility varied significantly between platforms and laboratories, with each analyzer type exhibiting distinct performance characteristics [87, 88].

Beyond instrumental factors, bioinformatics processing represents a major yet often underappreciated source of inter-laboratory variation. The computational

pipeline introduces variability at multiple levels: different laboratories employ varying database search algorithms, parameter settings, and false discovery rate control methods, which can lead to substantial differences in protein identifications from identical raw data [86, 89]. Furthermore, the choice of protein inference algorithms, normalization methods, and missing value imputation strategies adds additional layers of variation that can fundamentally alter biological conclusions [90, 91]. Post-processing decisions—including protein filtering criteria, batch effect correction approaches, and statistical analysis methods—create further divergence in final results [92, 93].

To address these computational challenges, the proteomics community has developed several mitigation strategies. Researchers now recommend using multiple search engines to improve identification confidence [89], performing adequate technical replicates to assess measurement precision [86], and employing probabilistic methods for more robust protein inference [94]. The development of standardized data formats, open-source analytical tools, and centralized data repositories has begun to address some of these reproducibility issues, though implementation remains inconsistent across laboratories.

Despite these inherent challenges, significant progress has been made in improving inter-laboratory reproducibility through systematic standardization efforts. Multi-center initiatives have focused on harmonizing protocols, implementing rigorous system suitability checks, and incorporating isotope-labeled standards as internal controls [95–97]. These coordinated efforts have demonstrated that with proper standardization and real-time monitoring of LC-MRM-MS performance through system suitability protocols, interlaboratory coefficients of variation can be reduced to < 20% for many peptides. Such improvements have achieved median intra- and interlaboratory reproducibility levels sufficient for most biological studies and candidate protein biomarker verification [98].

However, substantial challenges persist, particularly for complex samples like FFPE tissues where the inherent variability of the sample preparation process compounds analytical variability [99]. The combination of formaldehyde-induced protein modifications, variable crosslinking reversal efficiency, and the stochastic nature of data-dependent acquisition creates a particularly challenging matrix for achieving consistent quantitative results across laboratories.

Continued efforts to optimize and validate proteomic assays remain essential for clinical translation. These include the ongoing development of certified reference materials, implementation of harmonized protocols across institutions, and establishment of proficiency

testing programs. Only through such systematic approaches to quality assurance can the proteomics field meet clinical laboratory standards and enable the successful translation of FFPE proteomic biomarkers to routine clinical practice. The ultimate goal is to transform FFPE proteomics from a research tool into a reliable clinical diagnostic platform that can support precision medicine initiatives.

Technological strategies to overcome ffpe proteomic barriers

Chemical crosslink reversal strategies

Heat-Induced antigen retrieval approaches

Heat-induced antigen retrieval protocols, originally developed for immunohistochemistry, have been successfully adapted for proteomic applications through systematic optimization of temperature, pH, and buffer composition [1, 54, 83, 100–104]. The application of high temperature (95–120 °C) in combination with alkaline pH (8.0–9.0) and reducing agents promotes partial reversal of methylene crosslinks through hydrolysis reactions while maintaining protein solubility [104, 105]. Advanced protocols incorporating guanidine hydrochloride-based lysis buffers at 95 °C for 1 h followed by 80 °C for 2 h have demonstrated extraction of previously inaccessible protein populations, particularly nuclear and chromatin-associated proteins [105]. Recent developments from 2020 to 2023 have further refined these approaches, with S-Trap micro spin columns combined with TMTpro 16plex labeling achieving high-quality quantitative proteome analysis at 97 °C for 10 min [103], and systematic temperature optimization studies demonstrating that controlled variation between 60 and 100 °C can reduce extraction time while maintaining protein yield [104]. The integration of heat-induced retrieval with modern detergent systems has yielded protocols achieving high protein recovery from FFPE tissues [54, 102], though extraction efficiency may still vary compared to fresh tissues and may exclude some clinically relevant biomarkers.

Enzymatic digestion challenges and optimization

Trypsin cleavage barriers

The enzymatic digestion of FFPE-extracted proteins represents a critical bottleneck that fundamentally limits the sensitivity and comprehensiveness of proteomic analysis (reviewed and discussed in FFPE-methodology comparisons) [61, 106]. Trypsin, the predominant protease in bottom-up proteomics, cleaves proteins specifically at the C-terminus of lysine and arginine residues, generating peptides of optimal length (7–25 amino acids) for mass spectrometric analysis [107]. However, formaldehyde-induced modifications at lysine residues create multiple impediments to efficient tryptic digestion: formaldehyde

chemistry produces methylol adducts, methylations, and crosslinks that alter ϵ -amino groups and neighboring residues, and these modifications are frequent enough in FFPE material to measurably perturb downstream proteolysis and identification [57, 108].

Methylene-type crosslinks and dimeric modification products between side chains can physically block trypsin access to cleavage sites or change the local chemistry of the cleavage site, resulting in missed cleavages that produce abnormally long peptides suboptimal for LC-MS/MS [57, 61]. Even when crosslinks are partially reversed during extraction, residual methylol or mono-/di-methyl modifications at lysine residues substantially reduce trypsin recognition and catalytic efficiency compared with unmodified proteins [106, 108]. The three-dimensional protein aggregates formed through extensive crosslinking create steric barriers that prevent trypsin access to internal cleavage sites; in practice, this often forces FFPE workflows to employ longer digestions (commonly overnight or longer) or accelerated/harsh protocols, such as pressure cycling, acoustic, or heat-assisted digestion, to approach the digestion completeness typical of fresh samples [109, 110].

The consequences of compromised digestion efficiency cascade through the analytical workflow. Missed cleavages generate longer peptides, often beyond the ideal proteomic mass range, that ionize and fragment poorly in MS/MS, reducing identification efficiency and spectral quality [111, 112]. The heterogeneous nature of missed cleavages, where the same protein may yield different peptide patterns across samples depending on modification extent, complicates quantitative analysis because peptide-level measurements are not directly comparable [111, 113]. Database search strategies optimized for fully tryptic peptides lose sensitivity when accommodating semi-tryptic or non-tryptic matches, as the expanded search space reduces identification rates and complicates false discovery rate control [113, 114].

Optimization with enzyme-to-substrate ratios

To overcome steric hindrance and chemical modification, FFPE workflows often use higher enzyme-to-substrate ratios than fresh-tissue protocols. SP3-based methods for low-input and FFPE samples may employ sequencing-grade trypsin at a 1:20 (w/w) enzyme-to-protein ratio to maximize cleavage efficiency in the presence of persistent crosslinks and aggregates [115]. Other optimized workflows, including FASP and direct-digestion approaches, frequently apply trypsin or sequential Lys-C/trypsin digestion at 1:50 (w/w) ratios with overnight incubation, with pressure-assisted setups further validating this ratio for robust processing under denaturing conditions [109, 116, 117]. While bead-based digestion methods across proteomics generally span 1:100 to 1:20 ratios,

FFPE-specific confirmation of values as high as 1:50 remains limited [118]. Higher enzyme loads can improve digestion completeness but increase reagent costs and the risk of non-specific proteolysis or trypsin autolysis, emphasizing the need to balance efficiency with reproducibility.

Multi-enzyme approaches and alternative proteases

FFPE tissues are valuable for proteomic studies but present challenges due to formaldehyde-induced protein modifications. Lysine methylation is the most frequent modification in FFPE samples [108], affecting 2–6% of peptide-spectrum matches. These modifications significantly interfere with isobaric labeling strategies like TMT and iTRAQ, which rely on lysine reactivity for quantitative multiplexing [103, 108]. The formaldehyde-induced lysine modifications, including methylation and crosslinking, reduce labeling efficiency and potentially compromise quantitative accuracy. Precursor mixing in isobaric labeling can further distort quantification, necessitating careful optimization of labeling conditions and potentially requiring alternative quantification strategies that do not depend on lysine reactivity [119, 120].

Despite these challenges, FFPE tissues can yield comparable results to frozen samples when using optimized protocols [103, 121]. Strategies to improve FFPE proteomics include using MS3 scans to eliminate ratio distortion [120, 122] and employing de novo interpretation to elucidate unspecified modifications [121]. These advancements are crucial for leveraging FFPE tissue archives in biomarker discovery and clinical research [123].

Enzymatic digestion efficiency represents a fundamental challenge in FFPE proteomic workflows, often resulting in missed cleavages and generation of atypically large peptides that exceed optimal mass ranges for LC-MS/MS analysis [124, 125]. This compromised digestibility necessitates extended digestion periods (often >18 h) or elevated enzyme-to-substrate ratios (>1:20), which can introduce non-specific proteolysis and reduce spectral quality and quantification accuracy.

To address these digestion and modification challenges in FFPE tissues, various multienzyme approaches have been explored. Tandem LysC/trypsin digestion improves cleavage efficiency over trypsin alone [126], while alternative proteases offer complementary cleavage specificities. LysargiNase, for example, enhances digestion of methylated peptides and improves proteome identification by 13.4% compared to trypsin alone [125]. RapiGest-based extraction protocols have also shown improved protein recovery and enhanced overlap between FFPE and frozen tissue proteomes [54, 106].

Multienzyme digestion protocols such as sequential LysC followed by trypsin generate complementary

peptide populations that enhance proteome coverage and improve reproducibility and quantification accuracy [126, 127]. Proteases like chymotrypsin (targeting aromatic residues), elastase, and proteinase K (broad specificity) have been explored to access regions of crosslinked protein networks that are inaccessible to trypsin alone [128]. These strategies have demonstrated substantial increases in protein identifications under optimized conditions [129, 130]. In specific contexts, sequential digestion with trypsin followed by proteinase K has achieved up to a 731% increase in protein identifications, though such dramatic gains are not typical in routine workflows [131].

However, multienzyme approaches introduce operational complexity that limits widespread clinical implementation. Extended processing times, increased reagent costs, and the need for precise optimization of enzyme combinations and incubation conditions challenge standardization and regulatory approval. Analytical variability introduced by sequential enzymatic steps requires careful evaluation, especially when processing specimens with diverse fixation histories and tissue characteristics.

While these strategies may not be suitable for routine diagnostics requiring rapid turnaround, they hold promise for specialized clinical applications in biomarker discovery and validation, where maximal proteome coverage is essential. Ongoing research into simplification and automation may eventually enable broader clinical use. Nonetheless, certain enzyme combinations—particularly those involving broad-specificity proteases like proteinase K—can complicate database searching and reduce identification confidence, further emphasizing the need for careful protocol design.

Alternative fixation technologies

Recent fixation technologies have emerged to address traditional limitations while maintaining compatibility with proteomic applications. However, clinical adoption faces regulatory and practical barriers that limit implementation. Advanced fixation technologies including UMFIX, Glyo-fixx, NEO-FIX, and FineFIX demonstrate superior protein preservation characteristics compared to conventional formalin fixation [132–134], but lack regulatory approval for routine diagnostic use and require specialized procurement and handling protocols that increase operational complexity.

Ethanol fixation demonstrates promise for proteomic applications, yielding protein profiles comparable to frozen tissues through preservation via dehydration and coagulation without extensive covalent crosslinking [135]. However, ethanol fixation produces inferior morphological preservation compared to formalin, creating conflicts with standard pathological evaluation requirements.

Glyoxal fixation offers novel approaches with unique advantages in tissue processing. Compared to formaldehyde (molecular weight 30 Da), glyoxal (molecular weight 58 Da) is a larger dialdehyde molecule that creates different crosslinking patterns. Despite its larger size, glyoxal demonstrates efficient tissue penetration and distinct reactivity profiles, forming potentially reversible crosslinks that facilitate subsequent molecular analysis [136]. Cleavable crosslinkers, including disuccinimidyl tartrate (DST) and dithiobis(succinimidylpropionate) (DSP), represent specialized approaches designed to facilitate subsequent molecular analysis through selective crosslink reversal under mild reducing conditions [137] (Table 1).

The choice of fixation method has profound implications for multi-omic applications. While formaldehyde fixation remains the clinical standard, it creates differential effects across biomolecule classes, with proteins showing more extensive modifications than nucleic acids. Modern extraction protocols such as AllPrep have been developed to enable simultaneous isolation of DNA, RNA, and proteins from FFPE specimens [138], though differential extraction efficiencies across biomolecule classes create systematic biases that complicate integrative analyses [139, 140]. These technical limitations, combined with the computational complexity of multi-omic data integration, suggest that single-analyte or focused proteomic approaches may be more viable for clinical translation than comprehensive multi-omic strategies.

Advanced protein extraction workflows

Pressure-assisted and temperature-controlled extraction methods

Recent technological innovations in protein extraction have substantially improved protein recovery from FFPE tissues, though fundamental limitations persist that constrain clinical applicability. Pressure Cycling Technology (PCT) represents the most significant advancement, employing alternating cycles of high pressure (up to 45,000 psi) and ambient pressure to physically disrupt crosslinked protein networks while enhancing solvent penetration into tissue matrices [157, 158]. Systematic optimization studies have demonstrated that PCT-assisted extraction yields 5,000–8,500 protein identifications from FFPE specimens, with investigations documenting 5,192 proteins from liver specimens and up to 8,541 protein groups from various tissue types [157]. The reported 97% concordance with fresh-frozen tissues in comparative studies represents optimal performance under research conditions using abundant tissue samples and may not be reproducible with small clinical specimens typical of diagnostic practice [159]. The workflow depends on proprietary consumables such as PCT MicroTubes, MicroCaps, and MicroPestles ($\approx$ \$9–\$10 per sample) together with downstream items including

Table 1 Comparative analysis of chemical fixatives for MS-Based Proteomics

Fixative	Advantages	Limitations	Application notes	MS compatibility	Primary MS types	Key citations (DOIs)
Formaldehyde (FA)	• Preserves architecture • Histology-compatible	• Creates extensive crosslinks that require harsh reversal conditions • Blocks tryptic digestion • Carcinogenic	Requires heat/pressure for partial crosslink reversal; introduces artifactual modifications	Yes (major limitations)	LC-MS, MALDI-MS	[55, 57–60]
Glutaraldehyde (GA)	• Superior ultrastructure • Minimal denaturation	• Irreversible lysine crosslinks • Prevents digestion • Tissue hardening	EM exclusive; incompatible with bottom-up proteomics	No	–	[141–144]
Ethanol	• Zero crosslinking • PTM-friendly	• Poor morphology • Shrinkage	Gold standard for frozen sections & phosphoproteomics	Yes	LC-MS, MALDI-IMS	[135, 145–147]
Methanol	• Optimal precipitation • MALDI-IMS compatible	• Degrades morphology • Partial denaturation	Occasionally used in spatial proteomics, but ethanol fixation is preferred	Yes	MALDI-IMS, LC-MS	[148–150]
Glyoxal	• Reversible crosslinks • Faster than FA	• Limited validation • Antibody incompatibility	Emerging FA alternative; limited RNA-protein crosslinks compared to formaldehyde	Yes (emerging)	LC-MS	[151, 152]
DTBP	• Reversible amine crosslinks	• Not histological • Requires reduction	Specialized XL-MS for protein interactions	Yes (XL-MS only)	LC-MS (XL-MS)	[153, 154]
DST	• Cleavable crosslinks • Amine-specific	• Hydrolysis-sensitive • Complex cleavage	Cleavable by periodate oxidation; XL-MS workflows	Yes (XL-MS only)	LC-MS (XL-MS)	[137, 155]
NHS-Ester-Based	• Stable amide bonds • Lysine-specific	• Rapid hydrolysis • Limited peer-reviewed data	Low independent validation; commercial systems only	Limited	LC-MS	[154, 156]

This table evaluates chemical fixatives and crosslinking agents for mass spectrometry-based proteomics applications. Traditional histological fixatives (formaldehyde, glutaraldehyde, ethanol, methanol) vary significantly in MS compatibility. Emerging alternatives (glyoxal) and NHS-ester approaches require further validation. Specialized crosslinking reagents (DTBP, DST) are designed for crosslinking mass spectrometry (XL-MS) to probe protein interactions rather than tissue preservation. Glyoxal May induce RNA-protein crosslinks [136]

S-Trap columns, MS-grade enzymes, and optional isobaric tags, which can raise per-sample consumable costs to approximately \$50–\$100. Typical processing times for complete tissue-to-peptide preparation range from 3 to 6 h, which may be incompatible with some clinical workflows [109, 160].

Advanced detergent systems and surfactant chemistry

Advanced proteomic analyses of fixed tissues employ carefully optimized detergent formulations that substantially exceed traditional SDS-based approaches [161]. These specialized formulations incorporate multiple surfactant classes with complementary physicochemical properties to address multifaceted challenges inherent to protein extraction from crosslinked specimens by targeting different aspects of crosslinking networks and protein solubilization requirements. The strategic combination of ionic, zwitterionic, non-ionic, and acid-labile detergents has enhanced protein recovery while maintaining compatibility with downstream analytical workflows, addressing both aggressive solubilization needs and mass spectrometry-compatible sample preparation requirements.

SDS and sodium deoxycholate (SDC) constitute essential components in advanced extraction buffers due to

their exceptional solubilizing capabilities, with systematic concentration optimization studies establishing that intermediate concentrations of 2–4% w/v provide optimal protein extraction efficiency while minimizing inhibition of enzymatic activity during subsequent proteolytic digestion. SDC has received particular attention due to its demonstrated compatibility with protease functionality at elevated concentrations, simultaneously enhancing protein solubilization and digestion efficiency in single reagent systems [161]. Optimized protocols integrating SDS and SDC have yielded significantly increased identification rates for membrane proteins compared to conventional methodologies, with quantitative analyses demonstrating 25–40% higher identification rates for transmembrane proteins relative to formulations containing exclusively ionic detergents [162, 163]. Efficient removal of SDC from peptide solutions can be accomplished through acid precipitation or phase transfer techniques, with acid precipitation demonstrating superior efficiency and cost-effectiveness for most applications [164].

The incorporation of zwitterionic detergents, specifically CHAPS, CHAPSO, and MEGA 10, has produced substantial improvements in membrane protein extraction from fixed specimens for two-dimensional gel

electrophoresis applications [163]. Amidosulfobetaines, particularly those with 14–16 carbon alkyl chains, have proven effective in extracting previously undetectable membrane proteins, while combinations of detergents including CHAPS, MEGA 10, and LPC have demonstrated additive improvements in protein spot number, density, and resolution [165]. These advancements have been successfully applied to various biological samples, including erythrocyte membranes, liver, and brain tissues, and have enhanced protein analysis in MALDI imaging mass spectrometry (MALDI-IMS) of fixed tissue specimens [163, 166].

RapiGest, ProteaseMAX, and PPS Silent Surfactant represent significant methodological advancements for fixed tissue proteomics through their unique properties addressing multiple analytical challenges simultaneously. These specialized detergents provide robust solubilizing functionality during extraction and digestion phases but undergo selective degradation under mild acidic conditions prior to liquid chromatography-tandem mass spectrometry analysis, eliminating potential interference with peptide ionization efficiency and chromatographic separation. This distinctive property has facilitated development of streamlined sample preparation protocols with reduced purification requirements, minimizing sample loss while enhancing analytical reproducibility when working with precious fixed tissue specimens. RapiGest has demonstrated superior performance metrics in comparative studies, enabling identification of significantly more peptides and proteins compared to SDS and PPS methodologies without detectable bias regarding peptide physicochemical properties, making it particularly valuable for comprehensive proteome characterization [167].

Specialized detergent formulations, while offering superior protein recovery compared to traditional approaches, create significant standardization and quality control challenges that impact clinical implementation feasibility. The complexity of multi-component detergent systems introduces batch-to-batch variability and requires careful optimization for different tissue types and fixation conditions, making protocol standardization difficult for routine clinical applications. The incorporation of multiple surfactant classes with different physicochemical properties can create unpredictable interactions affecting extraction efficiency and downstream analytical performance in ways that are difficult to predict or control across diverse clinical specimens. Advanced surfactants such as RapiGest and ProteaseMAX represent significantly higher reagent costs compared to conventional detergents, while their specialized nature creates supply chain dependencies that may compromise laboratory operations. The requirement for careful handling and storage conditions for acid-labile surfactants adds operational complexity that increases personnel training

requirements and quality control overhead, creating economic and logistical considerations that must be weighed against improved analytical performance when evaluating cost-effectiveness for routine clinical applications.

Quantitative impact on protein recovery and detection

The practical consequences of formaldehyde-induced modifications manifest as systematic reductions in protein recovery that vary significantly across protein classes and cellular compartments. For example, Wolff et al. demonstrated that longer fixation times (up to 144 h) result in reduced protein yield, achieving only around 66% recovery compared to 6-hour fixation, while older archival blocks yield even less with conventional buffers—though a newer buffer system improved this significantly [168].

Membrane proteins are particularly affected due to dense lysine content and lipid environment. While not always reported explicitly with percentages, receptor tyrosine kinases and other surface antigens are known to be under-recovered in comparison to cytoplasmic proteins—a fact acknowledged in reviews highlighting the need for aggressive buffers (e.g., SDS-containing, high pH) and heat treatment [169].

These quantitative distortions introduce systematic bias, complicating biomarker studies—what appears as differential abundance may reflect extraction artifacts, not true biological variation. Additionally, extraction efficiency can vary stochastically even for identical protein targets, due to variable crosslinking in different cellular locales or conformational states [170].

Bottom-up proteomics methodological advances

Methodological evolution and current capabilities

Bottom-up proteomics remains the predominant analytical strategy for FFPE tissue analysis, with recent advances substantially improving its performance despite inherent limitations. The established workflow encompasses tissue deparaffinization, protein extraction, enzymatic digestion, peptide purification, and LC-MS/MS analysis [171] (Fig. 2). Modern implementations have achieved significant improvements through systematic optimization of each step. Hydrogel-mediated approaches have introduced novel extraction strategies, with enzyme solutions containing trypsin (125 ng/μL), TEAB (100 μM), and deoxycholate (1%) activated through microwave digestion achieving rapid protein extraction in 2 min at 320 W [172]. Recent methodological developments over the past decade have systematically addressed these challenges through diverse approaches across multiple tissue types and analytical platforms (Table 2).

The evolution of sample preparation has addressed many historical limitations through integrated workflows. Filter-aided sample preparation (FASP) protocols

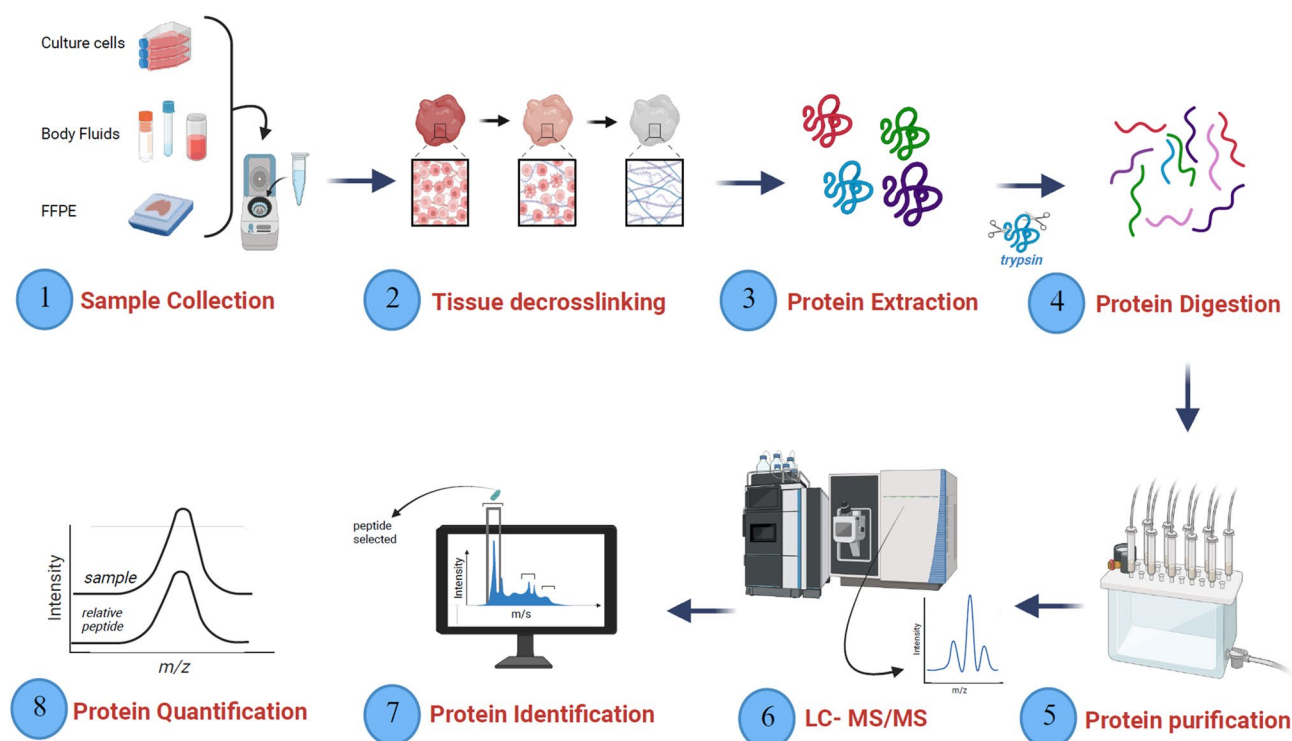


Fig. 2 Sequential Workflow for Bottom-Up Proteomics Analysis. Comprehensive workflow for Bottom-Up Proteomics implementation. Initiating with sample acquisition from diverse biological matrices, encompassing cultured cell systems, biological fluids, and FFPE tissues (Step 1), tissue undergo decrosslinking (Step 2) followed by proteins extraction (Step 3) followed by enzymatic cleavage into constituent peptides, predominantly utilizing tryptic digestion (Step 4). The resultant peptide mixtures undergo purification and enrichment procedures to reduce sample complexity and enhance detection sensitivity (Step 5). High-resolution LC-MS/MS analysis generates peptide fragmentation spectra (Steps 6–7), facilitating peptide identification and subsequent protein composition inference. Quantitative analyses proceed through peptide intensity measurements, enabling proteome-wide profiling of biological systems (Step 7). This methodology underpins proteome characterization at the peptide level, distinguishing it from top-down approaches that analyze intact protein species directly

optimized for FFPE tissues now incorporate multiple wash steps to remove crosslink reversal reagents, on-filter alkylation and reduction for complete cysteine modification, and extended digestion times of 18–24 h to accommodate reduced enzyme accessibility [83]. Single-pot solid-phase-enhanced sample preparation (SP3) using carboxylate-modified paramagnetic beads has demonstrated particular promise, enabling protein cleanup and digestion in a single tube with minimal sample transfers, reducing losses by 30–50% compared to conventional multi-step protocols [115, 173–175]. Suspension trapping (S-Trap) technology combines the benefits of FASP and precipitation-based methods, achieving 90% of protein identifications from matched fresh-frozen tissues while reducing processing time to 4–6 h [103].

Direct tissue analysis and microproteomics approaches

Laser capture microdissection (LCM) has transformed proteomic analysis of fixed tissues by enabling precise isolation of specific cellular populations from heterogeneous specimens, addressing fundamental limitations of bulk tissue analysis where signals from disparate cell types become averaged together [178–180]. This

technology represents critical advancement for investigating disease mechanisms at the cellular level, particularly in complex tissues where bulk analysis would obscure cell type-specific molecular alterations crucial for understanding disease pathogenesis (Fig. 3). LCM has been successfully combined with various molecular analysis methods, including proteomics, genomics, and transcriptomics [181, 182].

LCM systems employ two predominant mechanisms for tissue procurement: infrared capture systems utilizing thermoplastic films to bond with selected tissue regions, and ultraviolet cutting approaches employing focused laser energy to excise targeted areas with high precision. Comparative studies applied to FFPE tissues demonstrate that infrared-based systems typically yield superior protein recovery and better preserve protein integrity due to reduced thermal damage during capture, while ultraviolet-based approaches offer superior spatial precision for isolating morphologically intricate structures [184, 185]. Both methodologies show comparable results for phosphoprotein preservation, though ultraviolet LCM enables

Table 2 Advances in Bottom-Up proteomics of FFPE tissues in the last decade

Publication year and Author(s)	Tissue type(s)	Tissue processing method	Extraction buffer composition	Temperature and duration of extraction	Protein analysis application(s)	Notes or Observations
2015/ Negm et al. [177]	Breast cancer tissue	FFPE	Q-proteome FFPE Tissue Kit (Qiagen); Laemmli buffer	Q-proteome: 100 °C for 20 min, 80 °C for 2 h; Laemmli: 105 °C for 20 min or 2 h	RPPA	Comparison of protein extraction methods for RPPA analysis of FFPE breast cancer tissues
2016/ Azimi et al. [100]	Cutaneous squamous cell carcinoma (cSCC)	FFPE	0.1% w/v RapiGest SF Surfactant in 50 mM triethylammonium bicarbonate (TEAB)	95 °C for 30 min	LC-MS/MS	Label-free MS-based proteomics for analyzing FFPE cSCC tissues
2016/ Broeckx et al. [105]	Murine liver/ murine colon/ Human colon	FFPE	– 20 mM Tris HCl, 2% SDS, 200 mM DTT, 20% glycerol, 1% protease pH 8.8 – RIPA lysis buffer, 2% SDS, pH 8 – 20 mM Tris HCl, 0.5% SDS, 1.5% CHAPS, 200 mM DTT, 10% glycerol, pH 8.8	98 °C for 20 min, 80 °C for 2 h	GeLC-MS/MS	Comparison of multiple protein extraction buffers for proteomic analysis of FFPE tissues
2018/ Longuespée et al. [101]	Lung and colon adenocarcinomas; healthy tissue from skeletal muscle, liver, kidney, testis, myometrium, breast, prostate, and skin	FFPE	MQ water	95 °C for 20 min	LC-MS/MS	Laser microdissection-based microproteomics for small FFPE tissue samples
2018/Föll et al. [83]	Human tonsil mouse kidney	FFPE	0.1% RapiGest SF, 0.1 M HEPES, 1 mM DTT	95 °C for 4 h	LC-MS/MS	Comparison of direct trypsinization (DTR) and FASP protocols for proteomics of FFPE tissues
2019/ Mantsiou et al. [178]	Prostate cancer	FFPE	SDS-based buffer following beads homogenization and boiling	Boiling	LC-MS/MS	Characterization of tissue protein changes associated with castration-resistant prostate cancer
2019/Taverna et al. [173]	Cardiac myxoma tissue	FFPE and frozen	Hydrogels activated by enzyme solution containing 125 ng μ L ⁻¹ of trypsin, 100 μ M TEAB, 2 mM DTT, and DOC 1% w/v	Microwave digestion: 2 min at 320 W	LC-MS/MS	Hydrogel-mediated on-tissue proteomic characterization and TMT labeling for quantitative analysis
2020/ Coscia et al. [102]	Ovarian cancer, glioma, colorectal adenoma, urachal carcinoma	FFPE	- TFE-based extraction: - RapiGest-based extraction: RapiGest SF - FASP: Filter-Aided Sample Preparation	- TFE: Long heating (~90 min, precise temperature not specified) - RapiGest: 80 °C for 1 h - FASP: Room temperature	LC-MS/MS	Developed a robust, high-throughput proteomic workflow for FFPE tissues, demonstrating scalability and broad applicability to various tumor types.
2020/ Kuras et al. [103]	Lung adenocarcinoma tissues	FFPE	S-Trap micro spin columns; TMTpro 16plex labeling for quantitative proteomics	97 °C for 10 min followed by cooling on ice	LC-MS/MS	High-quality quantitative proteome and post-translational modification analysis of FFPE tissues
2021/ Davalieva et al. [54]	Prostate cancer tissue	FFPE	RapiGest SF surfactant; FASP	RapiGest extraction at 80 °C for 1 h; FASP at room temperature	LC-MS/MS	Comparative evaluation of two methods for LC-MS/MS proteomic analysis of FFPE tissues

Table 2 (continued)

Publication year and Author(s)	Tissue type(s)	Tissue processing method	Extraction buffer composition	Temperature and duration of extraction	Protein analysis application(s)	Notes or Observations
2022/Dressler et al. [104]	Urothelial carcinoma tissue	FFPE	Deparaffinization buffers: xylene and ethanol; Protein extraction buffers: Tris-HCl, pH 8.0, with SDS; Antigen retrieval buffers: citrate buffer, pH 6.0, and Tris-EDTA buffer, pH 9.0	Systematic variation of temperature (60 °C to 100 °C) and exposure duration (10 to 60 min)	Western blot, LC-MS	Optimization of preanalytical protein extraction to improve unbiased protein extraction and reduce time and costs
2023/Obi et al. [1]	Liver, kidney, and lung tissues	FFPE	Urea-based buffer (8 M urea, 2 M thiourea); SDS-based buffer (4% SDS in Tris-HCl, pH 7.5)	Urea buffer extraction at room temperature for 1 h; SDS buffer extraction at 95 °C for 10 min followed by cooling on ice	LC-MS/MS, protein arrays	Biomarker analysis of FFPE clinical tissues using proteomics

Comprehensive overview of bottom-up proteomics investigations conducted over the preceding decade, emphasizing FFPE tissue analysis. Documentation encompasses diverse tissue types, extraction protocols, buffer compositions, and conditions employed for protein analysis. Featured applications, including LC-MS/MS and RPPA, demonstrate progression in quantitative proteomics, methodological optimization, and biomarker discovery, providing insights into enhanced reproducibility and scalability

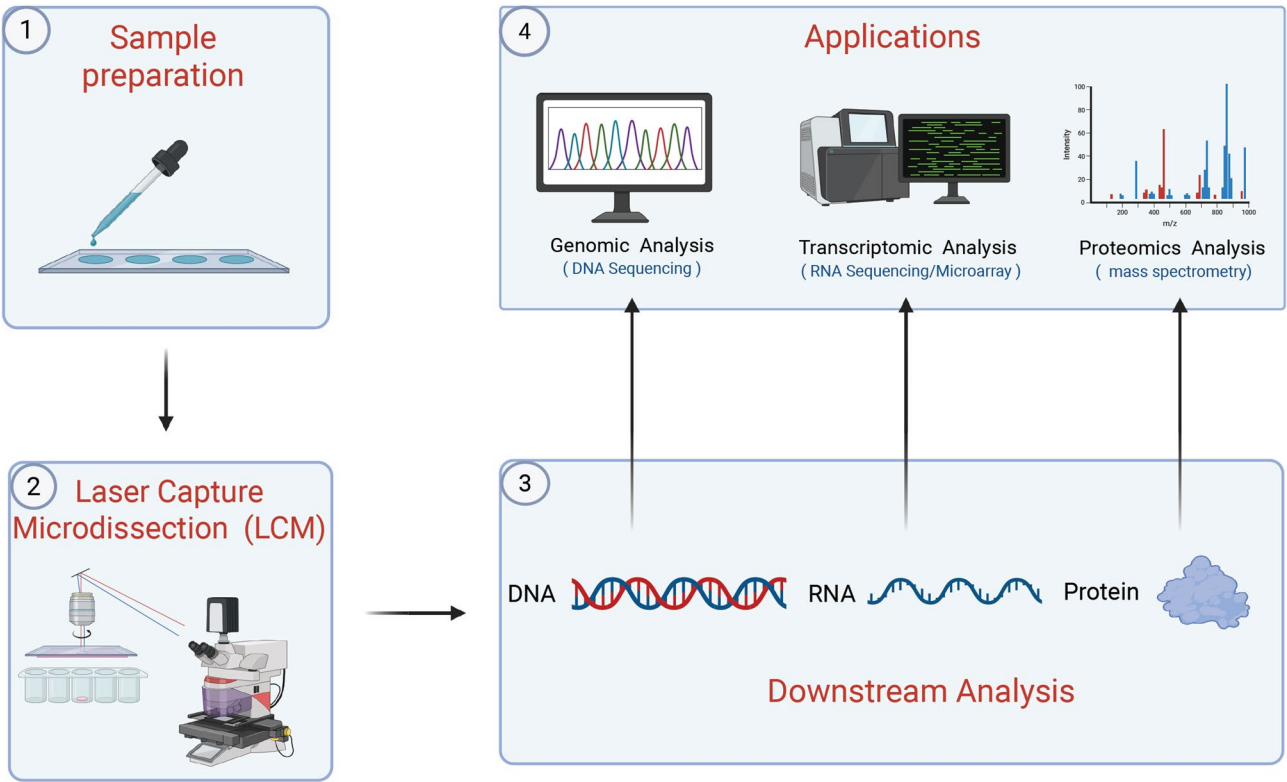


Fig. 3 Overview of LCM and downstream analysis. This schematic illustrates the workflow of LCM and its subsequent analytical applications, encompassing proteomics analysis. The process consists of four main components: [1] Sample Preparation, wherein tissue specimens are prepared on specialized slides; [2] LCM, which employs a precise laser beam to isolate specific cells or regions of interest from the tissue and collect them in tubes; [3] Downstream Analysis, depicting the types of biomolecules that can be analyzed (DNA, RNA, and proteins); and [4] Applications, highlighting three major analytical approaches. The proteomics analysis is performed using MS, which generates characteristic intensity versus mass-to-charge (m/z) ratio plots for protein identification and quantification. This approach complements other analytical methodologies shown, including transcriptomic analysis through RNA sequencing or microarray, and genomic analysis through DNA sequencing, allowing for comprehensive molecular characterization of the microdissected samples

faster and more precise sample collection when processing large specimen numbers.

Scale limitations of LCM-based proteomics have been progressively addressed through systematic refinements in sample processing workflows optimizing every aspect of the analytical pipeline. Early applications typically required 50,000–100,000 captured cells to achieve meaningful proteome coverage, severely limiting analysis to abundant cell populations and making rare cell type analysis practically impossible. Contemporary optimized protocols have reduced input requirements by more than an order of magnitude through innovations across entire workflows, with studies reporting identification of 1,500–3,000 proteins from as few as 3,000–5,000 LCM-captured cells from FFPE tissues [102, 185, 186]. Identification rates vary substantially by methodology; for example, MALDI-IMS typically yields 200–400 proteins per section versus 2,000 + via bulk LC-MS/MS [187]. These advancements result from improvements in extraction buffer efficiency, reduction of surface adsorption losses through carrier proteins and optimized consumables, and enhanced mass spectrometry instrumentation sensitivity.

The reduction in input requirements to 3,000–5,000 cells, while representing substantial technical progress, still exceeds cellular content available in many clinically relevant scenarios including micrometastases, rare cell populations, or small needle biopsy specimens [178, 186]. Protein identification rates of 1,500–3,000 proteins from optimized LCM protocols represent only 15–30% of proteome coverage achievable through bulk tissue analysis, creating substantial analytical gaps that may miss clinically relevant biomarkers. The manual nature of cellular selection creates processing bottlenecks limiting daily specimen throughput, while required operator training and expertise in morphological identification creates personnel dependencies unsustainable in routine clinical settings. Time-consuming nature of the technique create workflow conflicts with clinical diagnostic timelines requiring rapid turnaround. Economic barriers extend beyond instrument acquisition to include specialized consumables creating per-sample costs substantially exceeding conventional tissue processing expenses, while personnel time requirements may exceed economic thresholds acceptable for routine diagnostic applications compared to established immunohistochemical approaches providing targeted protein information with proven clinical workflows. Despite these challenges, LCM continues to evolve with the integration of artificial intelligence and automation, offering potential for enhanced multi-omic profiling in both research and clinical settings [188].

Mass spectrometry instrumentation advances

Data-Independent acquisition (DIA) strategies

DIA has transformed fixed tissue proteomic analysis through systematic fragmentation of all precursor ions within predefined mass-to-charge windows, providing comprehensive and unbiased proteome sampling that proves particularly valuable for degraded or chemically modified proteins characteristic of preserved specimen matrices [24]. This analytical approach demonstrates enhanced quantitative accuracy, precision, and reproducibility compared to traditional DDA methodologies when applied to chemically fixed tissues, effectively addressing several critical limitations inherent in conventional analytical approaches while providing superior analytical coverage [25]. The systematic acquisition strategy eliminates the stochastic sampling bias that historically compromised the reproducibility of proteomic analyses in complex biological matrices, particularly those containing extensive chemical modifications resulting from fixation procedures.

DIA generates permanent digital proteome maps that enable comprehensive retrospective analysis of cellular and tissue specimens without requiring additional sample preparation procedures, proving particularly valuable for precious fixed tissue specimens where sample availability may be substantially limited due to clinical constraints or specimen scarcity [26, 27]. The systematic fragmentation strategy employed ensures that chemically modified peptides, which might be systematically excluded in DDA approaches due to altered chromatographic retention times or modified ionization properties resulting from fixation-induced chemical alterations, are consistently fragmented and can be reliably identified through targeted data extraction methodologies. This comprehensive analytical coverage provides significant advantages for characterizing the complete molecular landscape of chemically preserved specimens where conventional approaches may demonstrate systematic biases against modified protein species.

Methodological innovations including overlapping isolation windows have substantially improved precursor ion selectivity and analytical sensitivity when processing complex peptide mixtures generated from chemically fixed tissues, wherein extensive chemical heterogeneity and modification diversity create particularly challenging analytical conditions that compromise conventional analytical approaches [25]. The unbiased analytical nature of DIA provides distinct advantages for fixed tissue analysis applications, wherein the complete extent and diversity of chemical modifications may not be predictable in advance of analysis, thereby enabling comprehensive characterization of the modified proteome without requiring prior knowledge of specific modification types or target proteins. This analytical capability represents

a fundamental advance over targeted approaches that require prior specification of analytes of interest.

The computational complexity inherent to DIA analytical workflows requires specialized bioinformatics expertise that substantially exceeds the technical capabilities of most clinical laboratories currently performing routine molecular diagnostic procedures. Data processing workflows demand significant computational resources and substantial time investments that create fundamental conflicts with the rapid analytical turnaround expectations characteristic of clinical diagnostic environments where timely results directly impact patient care decisions. The comprehensive nature of DIA data generation, while providing substantial advantages for research-oriented applications, creates complex regulatory validation challenges as the extensive datasets require sophisticated quality control metrics and interpretation frameworks not currently established or validated in routine clinical practice.

The implementation of DIA approaches for routine clinical diagnostic use requires substantial infrastructure investments that extend significantly beyond standard proteomic analytical capabilities currently available in most healthcare institutions. High-resolution mass spectrometry instrumentation capable of optimal DIA analytical performance represents a substantial capital investment that may exceed the budgetary constraints of many clinical laboratories, while the sophisticated computational infrastructure necessary for comprehensive data processing and long-term storage creates ongoing operational expenses that substantially impact cost-effectiveness analyses. The mandatory requirement for specialized software licenses and dedicated bioinformatics support personnel adds significant operational complexity that may exceed the cost-benefit thresholds acceptable for routine diagnostic applications, particularly when compared to established immunohistochemical approaches that provide targeted protein information at substantially lower operational complexity and reduced infrastructure requirements.

Super-SILAC is an advanced quantitative proteomics technique that uses a mixture of SILAC-labeled cell lines as an internal standard for analyzing unlabeled samples, including clinical tissues [189, 190]. This method improves quantification accuracy and enables the study of complex proteomes, post-translational modifications, and biomarkers in various biological systems [191, 192]. Super-SILAC has been successfully applied to analyze FFPE tissues, overcoming limitations of traditional proteomics approaches and allowing for accurate quantification of proteins in tumor samples to study disease progression and molecular genetic abnormalities [5–7].

However, important limitations must be acknowledged when applying Super-SILAC to clinical FFPE

tissue analysis. The proteomes of cell lines used to generate Super-SILAC standards may not fully represent the molecular complexity and heterogeneity of clinical FFPE tissues, particularly regarding cell-type-specific proteins, tissue microenvironment factors, and disease-specific protein modifications [8, 193]. This limitation can result in incomplete coverage of clinically relevant biomarkers and may introduce systematic biases when extrapolating quantitative measurements from cell line-based standards to patient specimens. The analysis of FFPE tissues presents additional challenges due to protein modifications and cross-links, limiting confident identification of low-level proteins and biologically relevant modifications [194]. Furthermore, the metabolic labeling requirements for Super-SILAC generation cannot accommodate the post-mortem tissue processing inherent to clinical FFPE workflows, limiting its applicability primarily to research contexts rather than routine clinical diagnostics. Despite these constraints, Super-SILAC's versatility and robustness make it a valuable tool for biomarker discovery in clinical research and studying various biological systems [8].

Enhanced sensitivity through ion mobility and improved instrumentation

The integration of ion mobility spectrometry (IMS) with mass spectrometry has provided an additional dimension of separation that substantially improves analysis of complex FFPE-derived peptide mixtures. Ion mobility separates ions based on their collision cross-section in a drift gas, providing separation of isobaric peptides, resolution of co-eluting peptides, and reduction of chemical noise from contaminants [195]. For FFPE tissues, where incomplete digestion and chemical modifications create complex peptide mixtures with many near-isobaric species, IMS improves analytical performance compared to LC-MS alone [195]. High-field asymmetric waveform ion mobility spectrometry (FAIMS) combined with parallel reaction monitoring has demonstrated improved signal-to-noise ratios and increased assay sensitivity in FFPE tissue analysis for clinically relevant proteins including HER2, EGFR, cMET, and KRAS [196].

Trapped ion mobility spectrometry (TIMS) coupled with time-of-flight mass analyzers has demonstrated particular utility, with the parallel accumulation-serial fragmentation (PASEF) technology enabling over 100 Hz sequencing speeds while maintaining high sensitivity [197]. TIMS achieves high resolving power ($R \sim 300$), duty cycle (100%), and efficiency ($\sim 80\%$) [198]. At a resolving power exceeding 250, TIMS reveals features otherwise hidden by lower resolution IMS analyzers, offering up to 3–8 times greater resolution than modern drift tube or traveling wave IMS techniques [199].

Contemporary high-resolution mass spectrometers have achieved performance specifications that address many historical limitations in FFPE proteomics. The speed and accuracy of TIMS and PASEF enable precise measurements of collisional cross-section values at the scale of more than a million data points and the development of neural networks capable of predicting them based only on peptide sequences [197]. Recent advances include the timsTOF Ultra 2, which demonstrates exceptional capabilities for analyzing FFPE tissue proteins with limited sample quantities, enabling precise mapping to specific tissue regions [200].

Spatial and targeted proteomics: clinically relevant applications

Spatial proteomics technologies with clinical potential

Digital Spatial profiling for multiplexed protein analysis

Digital Spatial Profiling (DSP) represents one of the most advanced spatial proteomic research technologies, enabling simultaneous quantification of 40–100 proteins (or up to 20,000 RNA targets) within morphologically defined regions of FFPE tissue Sects [201, 202]. The technology employs oligonucleotide-conjugated antibodies that bind to specific protein targets, followed by UV-mediated photocleavage of oligonucleotide tags from user-selected regions of interest. Spatial resolution depends on region-of-interest (ROI) selection, ranging from 10 μm for single-cell analysis to 650 μm for larger tissue areas, with most clinical studies utilizing 10–20 μm ROIs for cellular-level analysis [203]. The cleaved oligonucleotides are collected and quantified using nCounter technology or next-generation sequencing, providing digital counts proportional to protein abundance within each selected region.

DSP's key analytical advantages include validated quantitative performance with strong correlation to traditional IHC ($r > 0.9$ for validated antibodies), superior multiplexing capability measuring 40–100 proteins simultaneously, and exceptional analytical precision with coefficients of variation below 15% for proteins expressed above background levels [201, 204]. The technology achieves protein detection limits of approximately 10–50 molecules per cell for abundant targets, enabling analysis of regions containing as few as 10–20 cells for investigation of rare cell populations and microscopic disease foci [205].

Research applications of DSP have demonstrated substantial promise in biomarker discovery and immunotherapy prediction. The Yale University and 12 de Octubre collaboration discovered and independently verified CD44 as a candidate biomarker for PD-1 axis inhibition treatment in NSCLC. Using GeoMx DSP, 71 proteins were measured in spatial context, and CD44 expression in the tumor compartment was identified as a predictor of prolonged progression-free survival

(confounder-adjusted multivariate HR = 0.68, $p = 0.043$) [206]. Additional NSCLC studies identified spatially informed biomarkers, with high CD56 and CD4 levels in the CD45 compartment predicting clinical outcomes (HR: 0.24–0.31, $P < 0.011$).

DSP's compatibility with standard FFPE workflows and ability to analyze archival specimens make it particularly suited for retrospective research studies using existing clinical trial cohorts. While standardized research panels for immuno-oncology, neurodegeneration, and cell signaling are commercially available, these remain investigational and are not yet clinically validated for diagnostic use [203, 207]. A key limitation is that antibody panels are predetermined and fixed, preventing novel biomarker discovery beyond pre-selected targets.

However, clinical translation faces challenges including specialized instrumentation requirements, CLIA validation rather than FDA approval for protein applications, and need for specialized bioinformatics expertise to manage batch effects that can dominate data variation [203].

MALDI imaging mass spectrometry for Label-Free Spatial analysis

Matrix-Assisted Laser Desorption/Ionization Imaging Mass Spectrometry (MALDI-IMS) enables label-free, discovery-based detection of hundreds of proteins while preserving tissue architecture and spatial context essential for understanding disease processes in fixed specimens [208–210]. Unlike targeted approaches requiring predetermined protein panels, MALDI-IMS provides unbiased protein discovery with direct visualization of protein distributions across tissue sections without prior target knowledge, addressing fundamental limitations of extraction-based proteomics that lose spatial information during homogenization [106, 211].

MALDI-IMS excels in spatial resolution and discovery capabilities, typically achieving 10–200 micrometer resolution with specialized instruments reaching 5–10 micrometers under optimized conditions. The technology reveals spatial protein gradients, metabolic zonation, and drug distribution patterns that cannot be captured through targeted multiplexed approaches. Some clinical laboratories already utilize MALDI-IMS for specific applications such as glioblastoma subtyping under CLIA validation (e.g., specialized neuro-oncology centers and academic medical centers with research-focused pathology departments), though broader clinical adoption remains limited [212, 213].

However, MALDI-IMS faces significant analytical limitations that constrain widespread clinical translation, particularly for FFPE tissues where formaldehyde crosslinking reduces peptide recovery efficiency. MALDI-IMS faces challenges in protein identification due to gas-phase fragmentation inefficiency [214] and

ionization suppression effects, which limit detection of low-abundance species [215]. Protein identification rates from FFPE tissues using MALDI-IMS remain characteristically limited compared to extraction-based LC-MS/MS approaches, with studies reporting identification of hundreds rather than thousands of proteins per tissue Sect [216]. To address these issues, researchers have developed strategies combining MALDI-IMS with separation techniques [215] and improved sample preparation methods [217].

Emerging technical improvements include automated matrix application systems and advanced data processing algorithms that enhance reproducibility, though these advances have not yet overcome the fundamental limitations for routine clinical implementation. No regulatory approval pathways currently exist for MALDI-IMS diagnostic applications, requiring extensive validation studies and standardization before clinical implementation becomes feasible.

Integration with digital pathology and clinical Decision-Making

The integration of spatial proteomic data with histopathological analysis has begun to reshape diagnostic workflows, though significant implementation barriers persist. Deep learning approaches have demonstrated remarkable ability to predict molecular characteristics from morphological features in whole slide images across multiple cancer types, potentially identifying cases requiring proteomic analysis [218, 219]. Digital pathology platforms incorporating proteomics-derived annotations have been developed to highlight regions with specific molecular characteristics within routine histological specimens, enabling pathologists to visualize molecular information alongside traditional morphology [220, 221]. Molecular tumor boards increasingly utilize these integrated approaches to guide personalized therapy decisions, particularly for complex cases where standard morphological assessment proves insufficient [222]. However, the clinical utility of these sophisticated platforms remains limited by fundamental constraints. Despite technological capabilities, spatial proteomic applications have not achieved regulatory approval for clinical use. A striking discrepancy exists between the effort directed toward biomarker discovery and the number of markers that make it into clinical practice, with quite often the scientists working on biomarker discovery having limited knowledge of the analytical, diagnostic, and regulatory requirements for a clinical assay [223]. The number of new cancer biomarkers cleared or approved by the US FDA is rather limited, with most studies relegating protein signature analyses to exploratory rather than primary endpoints in clinical trials. Although technological advances are important, clearly defining intended use,

good study design and appropriate patient specimens are critical for the success of FDA approval [224].

The absence of established reimbursement mechanisms for multiparameter spatial analyses creates an economic barrier that technological advancement alone cannot overcome. A clinically successful device that makes it through the regulatory process can still fail to be integrated into medical practice if there is no or poor reimbursement, therefore it is often important to develop a reimbursement strategy early during product development and clinical planning [225]. Furthermore, the computational expertise required to interpret integrated histopathological and proteomic data exceeds the capabilities of most clinical laboratories. Quantitative mass-spectrometry-based spatial proteomics involves elaborate, expensive, and time-consuming experimental procedures, and data analysis is as critical as data production for reliable and insightful biological interpretation [224].

Fine needle aspirate analysis and clinical microproteomics

While fine-needle aspirates (FNA) are not always formalin-fixed, their inclusion in this discussion reflects emerging efforts to adapt FFPE-compatible analytical workflows to minimally invasive sampling contexts. Many clinical protocols now employ formalin fixation for FNA specimens to enable comprehensive molecular analysis, and the analytical challenges parallel those encountered in traditional FFPE tissue processing.

FNA specimens are increasingly utilized for proteomic analysis of cancer tissues due to their minimally invasive nature and diagnostic value [226, 227]. Proteomic profiling of FNA samples has demonstrated potential in distinguishing benign from malignant lesions [228], identifying cancer biomarkers [229], and evaluating treatment efficacy [230]. Various techniques have been applied to FNA proteomics, encompassing two-dimensional electrophoresis, MS [229], and novel methodologies like antibody barcoding [230]. Traditional proteomic workflows, designed for larger sample amounts, prove ineffective for microscale volumes due to substantial protein losses. To address this challenge, researchers have developed micro-scale techniques compatible with decreased sample sizes (100 µg or lower) [175]. These encompass single-tube preparation protocols using trifluoroethanol [231], on-microsolid-phase extraction tip (OmSET) methodologies [232], and integrated microscale analysis systems [233]. These approaches minimize sample handling steps, reduce processing volumes, and improve protein recovery [234]. Such innovations have enabled sensitive proteomic profiling of samples containing as few as 200 – 10,000 cells [235], paving the way for nanoproteomics applications [236].

Single-pot methodologies performing multiple processing steps in one vessel have demonstrated particular value for low-input samples by eliminating transfer-associated losses [237]. The SP3 approach has been successfully adapted for FNA specimens, enabling protein extraction, reduction, alkylation, and digestion within a single reaction vessel [115]. Automated sample preparation techniques like autoSP3 have been developed to improve reproducibility and enable high-throughput processing [238]. Novel approaches like nanoPOTS have significantly reduced sample losses through utilizing nanoliter-scale reactions, allowing deep proteome profiling of as few as 10–100 cells [239]. Alternative single-pot approaches including iST and FASP adaptations have similarly demonstrated utility for fixed FNA proteomics when optimized for microscale applications [240].

The integration of proteomic analysis with standard cytological evaluation represents a critical requirement for FNA specimens, where every cell may be precious for diagnosis. Sequential workflows that enable cytological examination followed by proteomic analysis of the same specimen have been developed to maximize diagnostic value of limited FNA material while ensuring cytological assessment is not compromised. These approaches typically involve careful specimen division, with portions processed for immediate cytological assessment while remainder is preserved for subsequent molecular analysis. Rapid on-site evaluation plays a crucial role in ensuring proper sample handling and allocation for both morphological and molecular studies [241]. Direct on-slide proteomic analysis represents an emerging approach eliminating the need to divide precious samples between different analytical approaches [242].

Mass spectrometry-based proteomic analysis of thyroid FNA specimens with indeterminate cytology has shown promise in improving diagnostic accuracy for indeterminate nodules, potentially reducing unnecessary surgeries [242, 243]. The application of proteomic approaches to pulmonary FNA specimens has demonstrated capability to distinguish primary lung adenocarcinoma from metastatic tumors with similar cytological appearance [244, 245]. The integration of FNA proteomics with therapeutic selection has demonstrated promise for individualizing cancer treatment based on protein-level drug target assessment and resistance marker quantification [228]. Proteomic analysis of breast cancer FNA specimens has demonstrated capability to quantify therapeutic targets including estrogen receptor, progesterone receptor, and HER2, with strong correlation to standard immunohistochemical assessment [246, 247].

Fine needle aspirate proteomics faces fundamental limitations that significantly constrain clinical utility despite demonstrated technical capabilities. The extremely limited cellularity typical of FNA specimens creates

analytical challenges that exceed capabilities of current proteomic technologies for comprehensive biomarker assessment. The reported capability to analyze samples containing as few as 200–10,000 cells provides limited proteome coverage that may miss clinically relevant biomarkers required for therapeutic decision-making. The heterogeneous cellular composition of FNA specimens complicates quantitative interpretation and may confound biomarker measurements essential for diagnostic accuracy. The reliance on single-pot methodologies eliminates opportunities for replicate measurements and quality control assessments essential for clinical laboratory validation. The specialized instrumentation, reagents, and expertise required for microscale proteomic analysis create per-sample costs that substantially exceed conventional cytological approaches. Extended processing times required for proteomic analysis conflict with rapid diagnostic timelines expected for FNA specimens, while complexity of data interpretation exceeds capabilities of most clinical laboratories currently performing FNA diagnostics.

Targeted proteomics for clinical translation

Selected reaction monitoring for biomarker validation

SRM, also termed multiple reaction monitoring (MRM), represents the most clinically advanced mass spectrometry approach for protein quantification in FFPE tissues, offering analytical performance approaching that of clinical immunoassays while providing superior multiplexing capability and standardization potential. SRM employs triple quadrupole mass spectrometers to specifically monitor predetermined precursor-to-product ion transitions corresponding to proteotypic peptides from target proteins, achieving exceptional specificity through the combination of retention time, precursor mass, and fragment ion selection. For FFPE tissues, SRM can achieve high precision (median CV 11–20%) and linearity across 4–5 orders of magnitude for multiplex quantification of 50–200 proteins in FFPE samples [96, 248, 249]. The use of stable isotope-labeled peptide standards enables absolute quantification with accuracy comparable to immunoassays while avoiding antibody-related artifacts [250].

Clinical validation studies have demonstrated the feasibility of SRM-based protein quantification in FFPE tissues for applications including HER2, estrogen receptor, and Ki-67 quantification, correlating well with standard clinical assays [251–253]. SRM has shown excellent correlation with gene amplification measurements for biomarkers like HER2 and EGFR in FFPE tissues [251, 254, 255]. Limits of detection in the attomolar range have been reported [252]. SRM offers advantages such as multiplexing capability, good reproducibility, and potential for standardization across laboratories [256]. Standardization efforts and protocol development continue to

improve reproducibility across laboratories. SRM's targeted nature and established mass spectrometry infrastructure provide advantages for clinical implementation. However, challenges remain including assay development requirements, limitation to predetermined targets, and need for high-quality peptide standards.

Parallel reaction monitoring and emerging targeted approaches

Parallel reaction monitoring (PRM) on high-resolution mass spectrometers represents an evolution in targeted proteomics, combining the specificity of selected reaction monitoring (SRM) with high-resolution detection [257, 258]. PRM acquires full MS/MS spectra for targeted precursors, enabling post-acquisition selection of optimal transitions and retrospective data analysis [259, 260]. This approach offers improved selectivity, sensitivity, and quantification reliability, particularly for complex samples like FFPE tissues [261, 262]. For FFPE tissues where chemical modifications and incomplete digestion create complex backgrounds, PRM's high mass accuracy and ability to extract multiple fragment ions per peptide enhance its performance in resolving interferences and quantifying low-abundance components [259].

Emerging targeted approaches are addressing limitations of traditional SRM/PRM while maintaining clinical feasibility. These hybrid approaches offer improved proteome coverage compared to pure targeted methods while maintaining superior quantification performance compared to discovery approaches. For FFPE clinical applications, these methods enable comprehensive therapeutic target assessment. While PRM shows promise for clinical applications and large-scale targeted experiments, its implementation currently requires specialized instrumentation and sophisticated data analysis, limiting its use to specialized laboratories [259, 262].

Clinical validation and implementation challenges

Systematic failures in biomarker validation

Proteomic biomarker discovery studies often suffer from inadequate sample sizes, leading to low statistical power and false positive findings [263, 264]. Studies with 40–50 samples per group can achieve 95% discovery power while minimizing false leads [265], though power analysis suggests that six samples per group may provide sufficient statistical power for detecting two-fold protein changes [266].

While MS-based assays have demonstrated analytical capabilities, translation to validated proteomic diagnostic tests remains challenging due to systematic validation failures. The common practice of optimizing multi-protein signatures on small discovery cohorts without independent validation often leads to severe overfitting, where models capture technical noise rather

than biological signal [140, 267–269]. This overfitting is evidenced by the systematic observation that biomarker panels with more proteins show worse validation performance than single markers [270, 271]. Studies frequently employ cross-validation practices that overestimate classifier performance, with median sensitivity and specificity dropping from 94% to 98% in cross-validation to 88% and 81% in independent validation [272].

The development of clinically useful biomarkers is hindered by methodological issues and poor reporting practices in research studies. Pre-analytical variables like fixation time and sample storage can significantly impact results [273]. Many studies fail to account for critical factors such as age, sex, and comorbidities, leading to spurious associations [274]. Publication bias and inconsistent validation criteria further distort the literature [275]. To address these issues, guidelines like REMARK have been developed to improve reporting standards [276]. However, adherence remains low, with fewer than 30% of studies meeting basic quality criteria [277].

Reverse Phase Protein Arrays (RPPA) represent a complementary validation approach for FFPE-derived biomarkers, offering high-throughput quantitative protein analysis with established clinical workflows [278, 279]. RPPA technology involves printing cellular lysates onto nitrocellulose slides, followed by probing with validated antibodies to quantify specific proteins and post-translational modifications across hundreds of samples simultaneously. This approach demonstrates particular compatibility with archival tissue analysis because it requires minimal sample input (< 5 µg of protein), offers sensitive detection of low-abundance proteins, and can accommodate the protein degradation and modification patterns characteristic of FFPE specimens [279–281]. RPPA has proven valuable for validating proteomic discoveries in clinical cohorts, providing quantitative measurements that bridge the gap between discovery proteomics and clinical implementation [282, 283]. The technology demonstrates high reproducibility and robustness, making it suitable for biomarker discovery and molecular classification of diseases like breast cancer [176]. Its workflows compatible with regulated clinical environments and cost-effectiveness compared to mass spectrometry-based approaches make it an attractive validation platform for FFPE-derived biomarkers before advancing to more complex mass spectrometry-based clinical assays [279, 282, 284].

Technical reproducibility across platforms and laboratories

The translation of FFPE proteomic biomarkers requires demonstration of analytical reproducibility across laboratories and platforms, yet current evidence indicates persistent challenges in achieving consistent quantification. Multi-center studies confirm that inter-laboratory

coefficients of variation for FFPE proteomics typically range from 30 to 60%, exceeding clinical acceptability thresholds [95, 285]. These challenges stem from platform-specific biases and protocol variations that resist standardization.

Mass spectrometry platforms exhibit systematic differences in protein detection, with 30–50% variability in measured abundance for identical proteins post-normalization. Even nominally identical sample preparation protocols introduce site-specific biases due to equipment calibration, reagent quality, and operator technique [286].

The complexity of data analysis pipelines creates additional reproducibility barriers that may exceed wet-lab variation. Different laboratories employ varying database search algorithms (Mascot, Sequest, MaxQuant, MSFragger), each with unique scoring systems and parameter optimizations that can result in 20–40% differences in protein identifications from identical raw data [89]. Protein inference algorithms that determine which proteins are confidently identified from observed peptides show poor concordance across platforms, with peptide overlap between technical replicates ranging from 35 to 60% [86]. Quantification strategies including spectral counting, extracted ion chromatograms, and TMT/iTRAQ reporter ions each have distinct biases and error profiles that affect reproducibility. Post-processing decisions regarding normalization methods, missing value imputation, and batch effect correction introduce additional variation that can alter biological conclusions [287]. The lack of raw data sharing in most publications prevents independent validation of analytical claims, while proprietary software and custom scripts create black boxes that cannot be reproduced externally. These technical reproducibility challenges are particularly problematic for regulatory approval, where analytical validation requires demonstration of consistent performance across multiple sites and operators.

Regulatory pathways and standardization requirements

Current regulatory landscape for proteomic diagnostics

The FDA has not yet approved any protein-based multiplex quantitative assays or MS-based protein/peptide diagnostic devices, highlighting the significant challenges in translating proteomic biomarkers to clinical practice [36–38].

The regulatory classification of FFPE proteomic tests remains ambiguous, potentially falling under multiple categories including Class II (moderate risk) in vitro diagnostics requiring 510(k) clearance, Class III (high risk) devices requiring premarket approval (PMA), laboratory-developed tests (LDTs) subject to evolving regulatory oversight, or companion diagnostics requiring co-development with therapeutic agents. Each pathway has distinct requirements for analytical validation,

clinical validation, and quality systems that substantially impact development timelines and costs [37, 288].

The FDA requires extensive clinical studies to demonstrate safety and effectiveness for new diagnostic tests, particularly those without predicate devices. This process can be time-consuming and costly, creating barriers to investment and commercial development [37]. Regulatory hurdles can negatively impact patient care and hospital finances [289]. To address these challenges, efforts have been made to develop mock 510(k) documents and improve dialogue between researchers and regulators [290–292]. Collaboration between the FDA and the clinical MS community could improve validation and regulatory review processes [38, 288]. Despite the difficulties, the potential impact of proteomic diagnostics on patient care makes continued efforts worthwhile [293].

Standardization initiatives and quality control frameworks

Multiple initiatives have emerged to address standardization challenges in FFPE proteomics, though implementation remains fragmented and incomplete. The Human Proteome Organization's Proteomics Standards Initiative (HUPO-PSI) has developed data standards including mzML for raw mass spectrometry data, mzIdentML for peptide and protein identifications, and mzQuantML for quantitative measurements, enabling data exchange between laboratories and software platforms [294, 295]. However, adoption remains incomplete, with many laboratories using proprietary formats that prevent data sharing and independent validation.

The Clinical Proteomic Tumor Analysis Consortium (CPTAC) has established standardized protocols for FFPE tissue processing, protein extraction, and mass spectrometry analysis, demonstrating feasibility of protocol standardization (Table 3). CPTAC developed a common data analysis pipeline to reduce variability in proteomics data processing [296] and created public repositories for proteomics data, assays, and antibodies [297–299]. To facilitate widespread adoption of targeted mass spectrometry assays, CPTAC launched an Assay Portal with detailed protocols and performance data [300].

Complementary advances in sample processing, such as the AllPrep methodology enabling simultaneous extraction of DNA, RNA, and proteins from single FFPE specimens [138], and recent developments like the HYPERsol method have enabled high-quality protein extraction and analysis from archival samples [102, 301]. However, the computational complexity of these integrated approaches and their infrastructure requirements continue to exceed most clinical laboratory capabilities, limiting their translation to specialized reference centers.

These developments demonstrate the feasibility of standardized protocols for large-scale clinical proteomics

Table 3 Standardization initiatives in FFPE tissue proteomics.

Framework	Lead organization	Technical scope	Primary protocols/SOPs	Performance standards (Evidence-Based)	Adoption status	Analytical benefits	Verified sources
NCI Biospecimen	NCI Biorepository Research Network	Pre-analytical variables (cold ischemia, fixation)	NCI Best Practices v2.0 (2016)	Storage: $\leq -70^{\circ}\text{C}$ (§ 4.1.1); IHC: CAP ANP22,900—“comparable staining” required	Widely implemented in CAP-accredited labs	↓28–35% pre-analytical variability (DNA/RNA focus)	- NCI Best Practices v2.0 (2016) https://biospecimens.cancer.gov/bestpractices/2016-NCIBestPractices.pdf - BRD SOP Portal (General Access) https://brd.nci.nih.gov/brd/ - CAP Guidelines
CPTAC Proteomics	NCI CPTAC Consortium	FFPE protein extraction and LC-MS/MS	PCT-SWATH SOP v3.1	Median CV: 8–12% (CPTAC 2020 data); Peptide yield: ↑22% (median) vs. standard	53 sites (incl. 9 international partners)	Optimized FFPE recovery; tissue-specific yield gains	- CPTAC Assay Portal https://assays.cancer.gov/available_assays - CPTAC Program Overview https://proteomics.cancer.gov/programs/cptac
NIST SRM 1950	NIST Metrology Group	Plasma biomarker quantification	SRM 1950 Certificate v3.1 (2023)	CV: 8.2–11.7% for 37 certified metabolites only	Widely adopted (850+ citations)	SI-traceable values for 107 analytes	- Certificate of Analysis (2023) https://tsapps.nist.gov/srmext/certificates/1950.pdf - NIST SRM 1950 Database https://srm1950.nist.gov/
ISO 15189:2022	ISO/TC 212	Clinical MS validation	ISO 15189:2022	Labs define criteria (§ 5.5.1.4); CLIA: HbA1c $\leq 15\%$, Troponin $\leq 25\%$ (example tests)	Mandated in 47 ILAC-MRA countries	Harmonized LDT validation requirements	ISO Standard https://www.iso.org/standard/76677.html

Table 3 (continued)

Framework	Lead organization	Technical scope	Primary protocols/SOPs	Performance standards (Evidence-Based)	Adoption status	Analytical benefits	Verified sources
HUPO-PSI MIAPE	HUPO Proteomics Standards Initiative	Experimental metadata standards	MIAPE v1.3	68.7% mandatory field completion (ProteomeXchange, 2023)	Required by journals (e.g., Nature Biotech)	FAIR data compliance; meta-data for reanalysis	- HUPO-PSI Main Site https://www.psdev.info/ - MIAPE Guidelines https://www.psdev.info/miape
GeoMx Spatial	NanoString Technologies	FFPE spatial protein mapping	GeoMx® DSP v3.0	Resolution: 10–20 µm (1–10 cells); ROI CV < 7% for high-abundance targets	12+ spatial omics core facilities (e.g., MD Anderson)	Single-cell-type ROI in archival FFPE	- GeoMx Protein Assays https://nanostring.com/products/geomx-digital-spatial-profiler/ - Digital Spatial Profiling Technology https://nanostring.com/blog/digital-spatial-profiling/

This table summarizes key initiatives and regulatory frameworks that have advanced reproducibility, quality control, and clinical translation in mass spectrometry-based proteomics of FFPE tissues. The NCI best practices for biospecimen resources provide sops for pre-analytical variables including cold ischemia and fixation protocols, with quality metrics for storage temperature ($\leq -70^{\circ}\text{C}$) and IHC validation requirements. The CPTAC proteomics sops offer FFPE-optimized extraction and LC-MS/MS workflows validated across 53 consortium sites, reporting median CVs of 14.7% and 22% improvements in peptide yield. The NIST reference materials program supports inter-laboratory comparability using plasma biomarker quantification standards such as SRM 1950, providing SI-traceable values for 107 analytes with CVs of 8.2–11.7% for certified metabolites. ISO 15189:2022 defines quality management requirements for clinical laboratories, with widely accepted performance criteria including $\leq 15\%$ total error for routine clinical assays such as HbA1c. The HUPO-PSI MIAPE guidelines standardize proteomics experimental metadata reporting, achieving 68.7% mandatory field completion in proteomexchange submissions and enabling FAIR data compliance. Finally, emerging Spatial FFPE proteomics initiatives using technologies such as GeoMx digital Spatial profiler are Establishing workflows for high-resolution protein mapping at 10–20 µm Spatial resolution with ROI CVs < 7%, enabling single-cell-type analysis from archival FFPE tissues

Abbreviations: CAP, college of American Pathologists; CPTAC, clinical proteomic tumor analysis Consortium; CV, coefficient of variation; DSP, digital Spatial profiler; FAIR, Findable, Accessible, Interoperable, and Reusable; FFPE, formalin-fixed, paraffin-embedded; HbA1c, hemoglobin A1c; HUPO-PSI, human proteome organization-Proteomics standards Initiative; IHC, immunohistochemistry; ILAC-MRA, international laboratory accreditation Cooperation mutual recognition Arrangement; ISO, international organization for standardization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MIAPE, minimum information about a proteomics Experiment; NCI, National cancer Institute; NIST, National Institute of standards and Technology; ROI, region of interest; SI, international system of Units; SOP, standard operating procedure; SRM, standard reference material

studies using both fresh and FFPE tissue samples, though implementation requires extensive training and quality control infrastructure.

The development of reference materials for FFPE proteomics remains a critical gap limiting standardization efforts. While NIST has developed SRM 1950 (human plasma) as a reference standard with certified values for 107 analytes, no equivalent exists for FFPE tissues (Table 3). Efforts to develop FFPE reference materials face challenges including heterogeneity of tissue composition preventing true homogenization, lot-to-lot variation in commercial cell line-derived FFPE blocks, inability to certify protein concentrations due to extraction variability, and lack of commutability between reference materials and clinical specimens [43, 104]. Quality control

frameworks adapted from clinical chemistry, including Westgard rules for analytical run acceptance, Levey-Jennings charts for trend monitoring, and external quality assessment schemes [303, 304], have been proposed for proteomic applications but require modification to address unique challenges including the high-dimensional nature of proteomic data, absence of true negative controls, and difficulty establishing clinically relevant acceptance criteria [304, 305]. The implementation of these quality systems requires resources and expertise that many laboratories lack, creating barriers to widespread adoption of standardized protocols.

Economic considerations and healthcare integration

Cost-Effectiveness analysis of FFPE proteomics

Direct and indirect economic barriers

The economic challenges facing FFPE proteomics extend beyond simple cost comparisons to encompass fundamental questions about value proposition and return on investment in resource-constrained healthcare systems. Historical cost analyses have reported immunohistochemistry costs of \$150 per antibody in academic hospital settings [306], though contemporary proteomic workflows require substantially higher investments covering complex multi-step processes from sample preparation through extensive bioinformatics analysis. Capital investment requirements create substantial barriers to implementation, with clinical-grade mass spectrometry systems ranging from mid-range instruments (\$400,000–1,000,000) to high-end systems exceeding \$1.5 million, plus ongoing operational expenses including annual service contracts and necessary ancillary equipment.

A streamlined mass spectrometry-based proteomics workflow for large-scale FFPE tissue analysis has demonstrated improved throughput and analytical consistency [102], while high-throughput workflows using adaptive focused acoustics technology have shown feasibility for scaling FFPE analysis [307]. Recent advances in comprehensive micro-scaled proteome and phosphoproteome characterization have enabled deep coverage from small FFPE biopsy specimens [116].

The indirect costs of FFPE proteomics implementation often exceed direct expenses and include personnel requirements that reflect the specialized nature of clinical laboratory operations. Recent American Society for Clinical Pathology wage surveys document the current compensation levels for clinical laboratory professionals [308], while longitudinal analyses of clinical laboratory economics demonstrate that labor costs represent a substantial and growing component of total operational expenses [309]. Economic analyses of clinical laboratory operations indicate that cost containment efforts must balance individual patient needs with limited societal resources [310]. The specialized expertise required for FFPE proteomics necessitates additional personnel categories including MS-qualified operators, bioinformaticians, and medical directors with proteomics expertise, each commanding premium compensation due to their specialized skills. Training and competency assessment requires substantial time investment for technical staff, and ongoing proficiency testing participation represents recurring operational expenses. Laboratory information system integration, often overlooked in cost projections, requires custom development to enable sample tracking, result reporting, and clinical decision support. Validation costs for each new biomarker panel represent substantial

investments, with the biomarker development pipeline facing significant economic barriers that contribute to the high failure rate in clinical translation [140]. These economic barriers are particularly challenging for smaller healthcare systems, as cost-effectiveness considerations increasingly influence reimbursement decisions and clinical adoption [37].

Reimbursement challenges and market dynamics

The absence of established reimbursement mechanisms for FFPE proteomic tests represents a fundamental barrier to clinical adoption that technology alone cannot overcome [311]. Current Procedural Terminology (CPT) codes for mass spectrometry focus on drug testing and therapeutic drug monitoring, with no specific codes for tissue-based proteomic analysis beyond basic protein identification [311]. Attempts to use miscellaneous molecular pathology codes result in inconsistent reimbursement ranging from \$100–500, often below the cost of test performance [312]. The complex multi-analyte algorithmic assays (MAAA) pathway potentially applicable to proteomic panels requires extensive clinical validation and utility evidence that few tests have achieved [313]. Private payer coverage determinations remain highly variable, with most considering FFPE proteomics investigational and denying coverage absent compelling clinical utility data [314].

The demonstration of clinical utility sufficient for reimbursement requires evidence that proteomic testing improves patient outcomes or changes clinical management in ways that justify additional costs. Randomized controlled trials comparing proteomic-guided treatment to standard care, while ideal, are prohibitively expensive (\$10–50 million) and require 5–10 years to complete [315, 316]. Observational studies and decision modeling provide weaker evidence often considered insufficient by payers [317]. The rapid evolution of proteomic technology creates moving targets for validation studies, as improved methods may obsolete clinical trials before completion.

Healthcare system integration challenges

Workflow integration and operational considerations

The integration of FFPE proteomics into existing clinical laboratory workflows presents operational challenges that extend beyond technical performance to encompass fundamental incompatibilities with established diagnostic processes. Current anatomic pathology workflows are optimized for rapid turnaround of histological diagnosis, with most routine cases requiring 24–48 h from specimen receipt to final report [318], while comprehensive proteomic analysis requires 3–5 days minimum for sample preparation, MS analysis, and data interpretation. This timeline discordance creates workflow bottlenecks

where proteomic results arrive after critical treatment decisions have been made, limiting clinical utility for time-sensitive diagnoses. The requirement for specialized sample handling including immediate freezing for phosphoproteomics, precise control of fixation time, and careful documentation of pre-analytical variables conflicts with routine pathology practices where specimens may experience variable processing depending on arrival time and workload.

The physical infrastructure requirements for proteomics create additional integration challenges. Despite MS advantages in specificity and sensitivity, MS-based protein analysis faces challenges in widespread clinical adoption due to specialized instrumentation, environmental controls, and space requirements [319, 320]. Automation and integration into existing laboratory workflows are crucial for overcoming these barriers [319]. Integration with laboratory information systems designed for discrete test results struggles with the complex, multidimensional data generated by proteomics, requiring custom interfaces and reporting mechanisms that IT departments may lack resources to develop and maintain. The disconnect between pathology and clinical chemistry organizational structures in most hospitals creates administrative barriers, as proteomics bridges traditionally separate departments with different accreditation requirements, quality systems, and management structures.

Clinical decision support and result interpretation

The translation of complex proteomic data into actionable clinical information represents a fundamental challenge that current healthcare information systems are not equipped to address [321, 322]. This challenge is compounded when considering multi-omic integration, where metabolomic and proteomic analyses reveal that post-translational modifications can dynamically regulate cellular metabolism without altering protein abundance [323, 324], adding layers of complexity beyond simple protein quantification. While specialized extraction methodologies now enable simultaneous recovery of proteins and metabolites from FFPE tissues, the integration and interpretation of these multi-dimensional datasets require computational infrastructure and expertise that exceed the capabilities of clinical laboratories focused on routine diagnostic testing [325]. Unlike genetic variants with established databases (ClinVar, COSMIC) or IHC results with defined scoring systems, protein expression data lacks standardized interpretation frameworks that enable consistent clinical decision-making. The continuous nature of protein expression measurements requires establishment of clinically relevant thresholds that may vary across populations, disease stages, and treatment

contexts, yet no consensus exists for determining these cutoffs.

The education and training requirements for appropriate utilization of proteomic information exceed current pathology and oncology curricula. Clinicians require understanding of mass spectrometry principles, protein biochemistry, statistical methods for multi-analyte interpretation, and integration of proteomic with genomic and clinical data. The absence of clinical decision support tools that synthesize proteomic findings with other diagnostic information means that physicians must manually integrate complex datasets, a cognitive burden that may lead to underutilization or misinterpretation. The lack of outcomes data demonstrating that proteomic-guided treatment improves patient survival or quality of life makes it difficult for clinicians to justify the additional complexity and cost of proteomic testing. Current electronic health records are not designed to capture, display, or trend proteomic data over time, preventing longitudinal analysis that might reveal treatment response or resistance patterns [326]. These interpretation and integration challenges create a chicken-and-egg problem where limited clinical use prevents accumulation of outcome data needed to develop interpretation guidelines and decision support tools.

Strategic pathways for successful clinical translation

Identifying High-Impact clinical applications

Criteria for selecting viable translation targets

The successful clinical translation of FFPE proteomics requires strategic focus on applications where unique analytical capabilities provide definitive clinical value that cannot be achieved through existing methods. Priority applications should meet multiple criteria including addressing unmet clinical needs where current diagnostics show poor performance, providing actionable information that changes patient management, demonstrating superior analytical performance compared to existing methods, showing feasibility within current laboratory infrastructure, and offering clear paths to regulatory approval and reimbursement. Based on these criteria, several applications emerge as promising candidates for near-term implementation. The quantification of therapeutic targets in oncology, particularly receptor tyrosine kinases and immune checkpoints, represents an area where mass spectrometry's superior quantification and multiplexing capabilities could improve therapeutic selection compared to semi-quantitative IHC [255, 327, 328]. Minimum residual disease detection in hematologic malignancies, where proteomic markers might complement flow cytometry and molecular methods, offers potential for improved sensitivity and standardization [329, 330]. The identification of resistance mechanisms in

targeted therapy, where post-translational modifications and pathway rewiring cannot be detected genomically, provides unique value for proteomics [331, 332].

Priority applications should meet multiple criteria including addressing unmet clinical needs where current diagnostics show poor performance, providing actionable information that changes patient management, demonstrating superior analytical performance compared to existing methods, showing feasibility within current laboratory infrastructure, and offering clear paths to regulatory approval and reimbursement. Importantly, applications requiring complex multi-omic integration should be avoided for initial implementation, as studies demonstrate that even with advanced tools for proteogenomic analysis, the correlation between genomic variants and protein expression remains limited, with less than 20% of variants typically confirmed at the protein level [333]. This reinforces the importance of focusing on direct proteomic measurements rather than complex integrative approaches for near-term clinical applications [334].

The common mistake of pursuing technologically impressive but clinically marginal applications has contributed to the field's translation failures. For example, attempting to replace established IHC markers with MS-based quantification offers limited value unless substantially superior performance can be demonstrated. Similarly, pursuing complex multi-omic integration before establishing utility of proteomics alone creates unnecessary complexity that delays translation. The focus should remain on applications where proteomics provides unique, actionable information that justifies additional complexity and cost.

Targeted panels versus comprehensive profiling

Targeted panels focusing on 20–100 carefully selected proteins are more suitable for clinical implementation due to reduced instrumentation requirements and faster turnaround times [335]. While progress has been made in developing reliable quantitative assays for plasma protein biomarkers, challenges remain in clinical validation and widespread adoption [322, 336]. Successful examples from related fields, such as the Oncotype DX gene expression panel, demonstrate that focused biomarker panels can achieve clinical adoption and impact patient care despite measuring fewer analytes than comprehensive approaches [116]. For FFPE proteomics, targeted panels for specific clinical indications such as breast cancer subtyping, lung cancer therapeutic selection, or colorectal cancer prognosis assessment offer the most viable path to near-term implementation.

Comprehensive profiling, while scientifically appealing and valuable for discovery research, faces insurmountable barriers for routine clinical use in the foreseeable future. The analytical complexity of measuring thousands

of proteins creates validation requirements that exceed practical feasibility, with each protein requiring individual validation across relevant sample types and clinical conditions. The data interpretation challenge scales exponentially with protein number, as the potential for spurious correlations and overfitting increases while clinical actionability often decreases. The economic argument for comprehensive profiling remains weak, as the marginal value of measuring additional proteins beyond validated markers rarely justifies proportionally increased costs. The regulatory pathway for tests measuring thousands of analytes remains undefined, with no clear precedent for validation standards or approval requirements. Healthcare systems and clinicians express preference for focused, interpretable tests over comprehensive profiles that require specialized expertise to understand. The evolution should be toward gradually expanding targeted panels as individual markers are validated, rather than attempting comprehensive profiling from the outset.

Implementation strategies and success factors

Phased implementation approach

Successful clinical translation of FFPE proteomics requires a phased implementation strategy that builds capabilities, evidence, and acceptance incrementally rather than attempting comprehensive deployment immediately [84, 102]. Phase 1 should focus on establishing technical capability through implementation of validated protocols for FFPE sample processing, acquisition of appropriate instrumentation and expertise, participation in proficiency testing and standardization initiatives, and development of quality management systems meeting clinical laboratory standards [296]. This foundation phase, typically requiring 12–18 months, enables laboratories to demonstrate technical competence before attempting clinical applications. Phase 2 involves pilot clinical studies on specific, well-defined applications where proteomic analysis provides clear value, such as quantification of established biomarkers in cases where IHC is equivocal, assessment of therapeutic targets in rare tumors lacking validated antibodies, or investigation of treatment resistance in patients failing standard therapy. These pilot studies, conducted under research protocols with appropriate consent, generate preliminary evidence of clinical utility while identifying operational challenges.

Phase 3 encompasses clinical validation through larger prospective studies demonstrating analytical validity, clinical validity, and ideally clinical utility for specific intended uses [337]. This phase requires collaboration between laboratories, clinicians, and potentially industry partners to generate evidence meeting regulatory standards. The validation phase typically requires 2–3 years and substantial investment but is essential for translation

beyond research applications. Phase 4 involves regulatory submission and approval, whether through FDA clearance/approval for commercial tests or CLIA validation for laboratory-developed tests. This phase requires comprehensive documentation of analytical performance, clinical evidence, and quality systems, often necessitating regulatory expertise many academic laboratories lack. Phase 5 represents clinical implementation and adoption, requiring education of clinicians, integration with clinical workflows, establishment of reimbursement mechanisms, and continuous quality improvement based on clinical experience [338]. Success factors throughout these phases include strong institutional support with protected resources, multidisciplinary collaboration between pathologists, oncologists, and laboratory scientists, focus on specific clinical needs rather than technology capabilities, rigorous adherence to quality standards and regulatory requirements, and realistic timelines acknowledging the complexity of clinical translation.

Conclusions and future perspectives

Current state and realistic assessment

The comprehensive analysis presented in this review reveals a complex landscape where significant technological advances in FFPE tissue proteomics coexist with persistent translational barriers that have prevented meaningful clinical implementation. Despite the ability to now identify 5,000–8,500 proteins from FFPE specimens using optimized protocols and advanced instrumentation, approaching the performance achieved with fresh tissues, the absence of FDA-approved FFPE proteomic diagnostic tests after decades of development indicates that technical capability alone is insufficient for clinical translation [157, 338]. This reality is exemplified by spatial proteomics, where despite remarkable technological sophistication enabling subcellular resolution and 50-target multiplexing, no applications have achieved regulatory approval for clinical use, with trials predominantly relegating protein signatures to exploratory endpoints rather than primary clinical decision points.

The economic analysis reveals that comprehensive FFPE proteomic analysis costs 5–10 fold more than established immunohistochemistry without demonstrated superior clinical utility in most applications, creating an unsustainable value proposition in resource-constrained healthcare systems. The validation crisis, with over 90% of discovered biomarkers failing to maintain performance in independent cohorts, reflects fundamental flaws in study design, statistical analysis, and biological understanding that cannot be resolved through technological advancement alone [140, 267–269]. The regulatory landscape remains poorly defined, with no clear pathway for approval of complex proteomic tests and no precedent for successful navigation of FDA requirements for FFPE

tissue-based mass spectrometry diagnostics. These challenges are compounded by operational barriers including incompatibility with clinical laboratory workflows requiring 24–48 h turnaround, lack of standardized protocols and reference materials enabling inter-laboratory reproducibility, absence of clinical decision support tools for complex proteomic data interpretation, and insufficient evidence of clinical utility to justify adoption and reimbursement.

Strategic recommendations for the field

Based on our comprehensive analysis, we propose that successful clinical translation of FFPE proteomics requires fundamental strategic reorientation away from comprehensive discovery approaches toward targeted, clinically focused applications. The field must acknowledge that targeted proteomics using SRM/PRM approaches represents the only viable pathway for near-term clinical implementation, offering analytical performance ($CV < 15\%$) approaching clinical requirements, simplified validation and regulatory pathways similar to existing multi-analyte tests, economic feasibility with costs approaching those of multiplex immunoassays, and compatibility with clinical laboratory workflows and expertise [48–52]. Priority should be given to applications where FFPE proteomics provides unique, actionable information not available through existing methods, such as quantification of therapeutic targets lacking validated antibodies, assessment of post-translational modifications determining drug response, detection of protein isoforms with distinct clinical significance, and multiplexed pathway analysis guiding combination therapy selection.

The development strategy should follow a disciplined phase-gate approach with clear go/no-go criteria at each stage. Initial proof-of-concept studies should demonstrate superior analytical performance compared to existing methods using archived specimens with known outcomes. Clinical validation should focus on specific, narrow indications where proteomic information would definitively change patient management, avoiding broad fishing expeditions that dilute clinical impact. Regulatory strategy should leverage existing pathways for laboratory-developed tests initially, building evidence for eventual FDA submission only after clinical utility is established. Economic modeling should be integrated from the beginning, ensuring that development focuses on applications where reimbursement is achievable based on demonstrated value. Collaboration structures should prioritize partnerships between academic laboratories providing scientific expertise, clinical centers offering patient access and outcome data, diagnostic companies contributing regulatory and commercialization capabilities, and payers engaged early to define evidence requirements.

Future technological directions

While acknowledging current limitations, several emerging technologies show promise for addressing specific translational barriers, though realistic timelines must acknowledge the lengthy development and validation cycles required for clinical implementation. Automated sample preparation platforms incorporating artificial intelligence for quality control could reduce technical variability from 20 to 50% to below 15%, though standardization across platforms remains challenging [238, 339, 340]. Miniaturized and microfluidic approaches may enable analysis of smaller specimens and reduce reagent costs, though scaling to clinical throughput requirements remains unproven [341–346]. Advanced data analysis algorithms incorporating machine learning could improve biomarker discovery and validation, though interpretability and regulatory acceptance of “black box” algorithms remain problematic [347–350]. Integration with digital pathology and artificial intelligence image analysis could enable selection of optimal tissue regions for proteomic analysis, though the added complexity may outweigh benefits for routine applications [351–356].

Novel chemical approaches to address formaldehyde crosslinking, including development of cleavable crosslinkers for prospective studies and improved reversal strategies for archived tissues, may fundamentally improve protein recovery and analysis, though widespread adoption would require substantial changes to pathology practice [137, 357]. The evolution of mass spectrometry instrumentation toward increased sensitivity, speed, and robustness will continue, though current instruments already exceed the analytical requirements for most clinical applications, suggesting that instrumentation is no longer the limiting factor. DIA methods will likely become standard for discovery applications, offering improved reproducibility and quantification, though the computational complexity remains challenging for clinical laboratories [24–27]. The integration of proteomics with other molecular data types will continue in research settings, though the added complexity and cost make multi-omic approaches unlikely to achieve routine clinical implementation within the next decade.

Realistic timeline and expectations

Based on current evidence and historical precedent from related fields, we project that meaningful clinical implementation of FFPE proteomics will require a minimum of 5–10 years for even the most promising applications. Within 2–3 years, we expect to see initial CLIA-validated laboratory-developed tests for targeted proteomic panels in specialized reference laboratories, focusing on specific applications such as therapeutic target quantification in rare tumors or assessment of resistance mechanisms in failed therapy. Within 3–5 years, the first FDA-cleared/

approved FFPE proteomic tests may emerge, likely as companion diagnostics developed in partnership with pharmaceutical companies where the economic incentive justifies investment. These initial tests will likely measure fewer than 20 proteins using targeted MS approaches on established clinical platforms. Within 5–10 years, broader adoption may occur if initial implementations demonstrate clear clinical utility and economic value, though this will likely remain limited to major academic medical centers and reference laboratories rather than community hospitals.

Beyond 10 years, the trajectory becomes less certain and will depend on multiple factors including technological breakthroughs addressing current limitations, accumulation of clinical evidence demonstrating improved patient outcomes, evolution of healthcare payment models favoring precision medicine, development of simplified platforms accessible to routine laboratories, and cultural shifts in pathology practice embracing molecular methods. However, it is equally possible that alternative technologies such as spatial transcriptomics, multiplexed immunofluorescence, or next-generation liquid biopsies may fulfill the clinical needs currently envisioned for FFPE proteomics, potentially limiting its role to specialized research applications. The field must therefore maintain realistic expectations while pursuing focused applications where success is achievable, avoiding the hype cycles that have previously damaged credibility and delayed meaningful progress.

Conclusion and roadmap for clinical translation

Despite substantial technological progress, mass spectrometry-based proteomics of FFPE tissues has yet to achieve routine clinical implementation. The central obstacles—chemical crosslinking, pre-analytical variability, reproducibility issues, economic burdens, and regulatory uncertainty—have collectively hindered progress. Yet, the successful adoption of genomic assays from identical archival materials demonstrates that translation is possible when the right combination of technological innovation, regulatory clarity, and clinical need converge.

The pathway forward requires a strategic shift in focus. Discovery-based proteomics, while powerful for hypothesis generation, remains fundamentally incompatible with clinical workflows due to its variability, long turnaround times, and extensive computational requirements. In contrast, targeted proteomic approaches such as SRM, MRM, and PRM offer the reproducibility, throughput, and validation feasibility needed for diagnostics. Concentrating efforts on targeted applications provides a pragmatic foundation for clinical translation.

Clinical adoption will also depend on defining applications where FFPE proteomics provides unique, actionable insights unavailable from other technologies.

This includes the detection of protein isoforms, post-translational modifications, and multi-protein signatures that directly inform therapeutic response or disease classification. Success in these areas will justify the additional complexity and cost of proteomic methods compared with more established techniques such as immunohistochemistry.

Equally critical is the establishment of rigorous standardization frameworks. Harmonization of pre-analytical and analytical protocols across institutions, development of certified reference materials, and inter-laboratory proficiency testing are essential to reduce variability and build the trust needed for clinical adoption. Without such quality control measures, even the most promising assays will struggle to achieve regulatory approval.

Economic feasibility must also be integrated into translation strategies. Workflows that approach the cost of multiplex immunohistochemistry are far more likely to be adopted than those requiring several hundred dollars per specimen. In parallel, regulatory alignment will be essential. Lessons from FDA-approved genomic assays provide a roadmap for proteomic assay development, but prospective, adequately powered validation studies will be indispensable for demonstrating clinical utility.

Taken together, these considerations suggest that FFPE proteomics should pursue a phased implementation strategy. Early adoption is most likely to occur in niche, high-value applications, such as rare cancers, resistant tumor subtypes, or retrospective studies using archival material, where alternative technologies cannot provide equivalent information. Broader clinical adoption will follow as workflows become more standardized, cost-effective, and supported by prospective clinical validation.

In outlook, if research efforts continue to prioritize targeted assays, practical standardization, and applications with direct clinical actionability, FFPE proteomics can realistically progress from a research tool to a diagnostic reality within the next five to ten years. By strategically narrowing its scope to areas of greatest clinical value, this field has the potential to evolve into a powerful complement to genomics and immunohistochemistry, ultimately advancing the goals of precision medicine.

Acknowledgements

Not applicable.

Author contributions

H.S. and M.A. conceptualized the review and developed the outline. H.S. conducted the primary literature analysis and wrote the initial draft of the manuscript. M.A. and L.B. critically revised the text and contributed to refining the clinical and translational sections. A.T. prepared the figures and supported the formatting of references. All authors reviewed and approved the final manuscript and agree to be accountable for all aspects of the work.

Funding

This review received no specific grant funding from any funding agency in the public, commercial, or not-for-profit sectors. The work was conducted as part of the authors' institutional responsibilities.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 11 July 2025 / Accepted: 3 October 2025

Published online: 28 November 2025

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