

Top-down proteomics

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Abstract

Proteoforms, which arise from post-translational modifications, genetic polymorphisms and RNA splice variants, play a pivotal role as drivers in biology. Understanding proteoforms is essential to unravel the intricacies of biological systems and bridge the gap between genotypes and phenotypes. By analysing whole proteins without digestion, top-down proteomics (TDP) provides a holistic view of the proteome and can decipher protein function, uncover disease mechanisms and advance precision medicine. This Primer explores TDP, including the underlying principles, recent advances and an outlook on the future. The experimental section discusses instrumentation, sample preparation, intact protein separation, tandem mass spectrometry techniques and data collection. The results section looks at how to decipher raw data, visualize intact protein spectra and unravel data analysis. Additionally, proteoform identification, characterization and quantification are summarized, alongside approaches for statistical analysis. Various applications are described, including the human proteoform project and biomedical, biopharmaceutical and clinical sciences. These are complemented by discussions on measurement reproducibility, limitations and a forward-looking perspective that outlines areas where the field can advance, including potential future applications.

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Introduction

The central dogma of biology describes the flow of information from DNA to processed mRNA and finally proteins, which are the primary effectors of biological function^{1,2}. Numerous proteoforms lead to a vast range of chemically diverse protein families. Proteoforms occur due to post-translational modifications (PTMs), RNA splice variants and genetically defined amino acid sequences, including genetic polymorphisms² (Fig. 1a). As a result, a comprehensive knowledge of proteoforms is essential to understand biological systems and establish the link between genotypes and phenotypes³. However, the number of possible proteoforms greatly exceeds the number of genes, presenting an analytical challenge⁴.

Top-down proteomics (TDP) has emerged as the most powerful experimental strategy for comprehensive analysis of proteoforms^{5–8}. The base experiment is top-down mass spectrometry (TDMS)⁹, which analyses intact proteins without digestion to provide a holistic view of the proteoforms. Importantly, unlike intact mass spectrometry¹⁰, a TDMS experiment requires both an accurate intact molecular mass measurement (top) and controlled fragmentation of the gas-phase molecule (down). Top-down sequencing was challenging until electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) could be sufficiently used for tandem mass spectrometry (MS/MS or MS2) measurements. Although MALDI-MS can fragment intact protein ions, the multiply charged ions generated by ESI are more effectively dissociated in tandem mass spectrometry to produce sequence-informative product ions¹¹. A variation of TDMS, termed native TDMS (nTDMS)^{12,13}, performs both ionization and backbone cleavage in a way that maintains higher order structure. The ability of nTDMS to yield sequence information directly from protein complexes is enhanced by using electron-based fragmentation methods, such as electron capture dissociation (ECD)^{14,15}, and ultraviolet photodissociation (UVPD)^{16,17}. Native mass spectrometry¹⁸ and nTDMS are now a viable complement to traditional structural biology tools and are starting to be applied more broadly in biopharmaceutical research¹⁹.

The alternative to TDP, bottom-up proteomics (BUP), involves extensive proteolysis to yield peptides that are typically <3 kDa. BUP is currently used more widely than TDP as peptides are easier to separate, ionize and fragment than proteins. There is also a greater technological maturity and more established informatics tools for BUP²⁰. However, there is an intrinsic limitation of BUP owing to the peptide-to-protein inference problem, as only a limited number of peptides are detected per protein, with generally low protein sequence coverage. This leads to a loss in proteoform information and connectivity when mapping sequence variations and PTMs^{1,3,21,22}. Another limitation of BUP is an inability to infer different combinations of modifications on various proteoforms. Capturing this combinatorial information is important to understand proteoform function and regulation (Fig. 1b). Consequently, BUP is not optimal for profiling the complete repertoire of proteoforms²³.

By contrast, TDP forgoes protein digestion and analyses the intact protein directly to achieve unambiguous, proteoform-resolved molecular details. This enables accurate protein identification, PTM localization and quantification for different proteoforms. The top-down strategy (Fig. 2) starts by measuring the intact protein mass. As modifications change the molecular mass of the protein, TDP can inherently capture proteoform information. Subsequent fragmentation of intact proteins identifies the protein and all its modifications, as well as any correlations that exist between modifications²⁴. Classically, the three basic pillars of TDP²⁵ are front-end sample preparation; top-down

mass spectral data acquisition of the intact mass and corresponding fragmentation; and informatics for proteoform identification, characterization and quantification (Fig. 2). In a typical TDP experiment, proteins are separated through either offline fractionation coupled with direct infusion mass spectrometry²⁶ or online separation²⁷. For example, online separation could use liquid chromatography (LC) or capillary electrophoresis (CE) with MS/MS detection²⁷. This type of setup was used to map intact proteoforms with a 4D separation system and identified 1,043 gene products from human cells dispersed over 3,000 proteoforms²⁸.

A final requirement in the TDP workflow is software to compare experimental TDP data with possible protein sequences. Without databases of sequenced genomes, BUP as it is currently used would not exist. The same is true for TDP. Multiple tools have been developed for large-scale TDP projects involving direct fragmentation of intact protein ions^{24,29,30}. Current TDP platforms are largely the same as originally established. However, advances in sensitivity and efficiency for all TDP components – sample preparation, separation/fractionation, ionization, mass analysis, ion dissociation and bioinformatics – enable exceptional breadth and depth. An example of this was the identification of approximately 30,000 unique proteoforms expressed from human genes across 21 cell types and plasma from human blood and bone marrow³¹.

This Primer focuses on the methodology of TDP. Experimental approaches required for TDP are described, as well as key issues related to sample preparation, proteoform separation and identification and data acquisition and processing. Example applications of TDP are described to show current capabilities and highlight the challenges of extending the technology in the future.

Experimentation

Sample preparation and controls

Sample preparation is a critical step for TDP (Fig. 3a). Traditionally, protein extraction methods use Good's buffers, which have high salt concentrations (>100 mM), protease and phosphatase inhibitors and surfactants, such as sodium dodecyl sulfate (SDS) or Triton X-100 for total protein solubilization³². These conventional reagents are often incompatible with TDP because they can interfere with protein ion detection and suppress the mass spectrometry signal. As a result, they must be removed for high-quality data. Incompatible salts and small molecules can be removed by ultracentrifugation filters or replaced using size exclusion chromatography (SEC) spin columns. The broader term buffer exchange is sometimes used to refer to solvent replacement. However, this is an inaccurate term for TDP workflows, which often require complete removal of buffer salts or other solution stabilizing agents, rather than a simple exchange. A protocol describing typical biological buffers, standardized sample preparation and performance benchmarks was developed from a best practices and benchmark study by the Consortium for TDP (CTDP)³³. TDP performance can be evaluated using a standard intact protein mixture containing ubiquitin, myoglobin, trypsinogen and carbonic anhydrase, established by the [National Resource for Translational and Developmental Proteomics](#). Care should be taken to minimize the introduction of artefactual proteoform changes during sample preparation. For example, protease and phosphatase inhibitors are commonly included in the extraction buffers to minimize in vitro protein degradation and dephosphorylation, respectively³⁴. Temperature-sensitive protein modifications, such as oxidation, should always be considered during TDP experiments. Samples

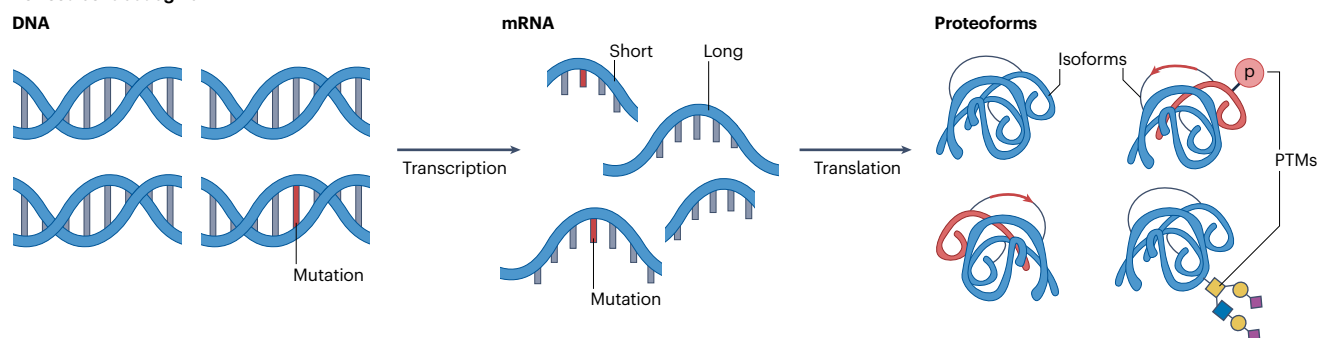
should be handled at low temperatures (-4°C) to slow the rate of any modification processes³⁵.

Surfactants are often used for general biological sample preparation and can facilitate cell permeabilization and solubilization of hydrophobic membrane proteins^{36–38}. However, surfactants are a particular challenge for downstream mass spectrometry analysis owing to signal suppression³⁹. Protein precipitation methods, which usually involve a chloroform/methanol mixture or acetone, can remove surfactants and other mass spectrometry-incompatible contaminants^{40–42}. However, protein precipitation methods can be time-consuming and may lead to protein loss, experimental variability or solubilization challenges^{41,43}. Cleavable surfactants have been developed – such as Rapigest⁴⁴, ProteaseMAX⁴⁵ and MaSDeS⁴⁶ – that are acid-labile and compatible with BUP after acid degradation. However, these acid-labile surfactants are not directly compatible with TDP. To address this, a photocleavable surfactant, 4-hexylphenylazosulfonate, was developed, referred to as Azo⁴⁷. Azo can effectively solubilize proteins, including membrane proteins, with performance comparable to SDS and rapidly degrades on exposure to ultraviolet radiation. Photodegradation of Azo requires ultraviolet B irradiation (maximal absorbance $\sim 305\text{ nm}$), rather than the conventional ultraviolet C (254 nm), plus additives – such as isopropanol, L-methionine and

tri(2-carboxyethyl) phosphine – to prevent protein precipitation and radical-induced oxidation⁴⁷. Surfactant-aided TDP workflows require careful sample handling steps and future optimization will enhance the depth of coverage, especially for the membrane proteome⁴⁸. For instance, a non-ionic, redox-cleavable surfactant, *n*-decyl-disulfide- β -D-maltoside, was developed as a mass spectrometry-compatible surfactant that mimics the properties of *n*-dodecyl- β -D-maltoside to facilitate protein solubilization, in particular for membrane proteins⁴⁹.

Front-end fractionation and enrichment strategies (Fig. 3b and Table 1) can selectively isolate subproteomes to capture and enrich low-abundance proteins from intricate biological samples before mass spectrometry analysis^{50,51}. Organelle fractionation is performed by differential centrifugation. This captures most subcellular components, including nuclear, cytosolic, mitochondrial and mixed microsomal – Golgi, endoplasmic reticulum, other vesicles and plasma membrane – fractions⁵². Proteins can be extracted from subcellular fractions for the downstream mass spectrometry-based proteomic analysis. For example, a TDP study of a mitochondrial fraction identified 347 mitochondrial proteins with comprehensive profiling of proteoforms specific to organelle targets⁵³. An alternative approach is to use affinity-based enrichment methods, traditionally with antibodies for protein capture and quantification^{51,54,55}. Antibody-based affinity purification has been

a Revised central dogma



b Top-down vs bottom-up

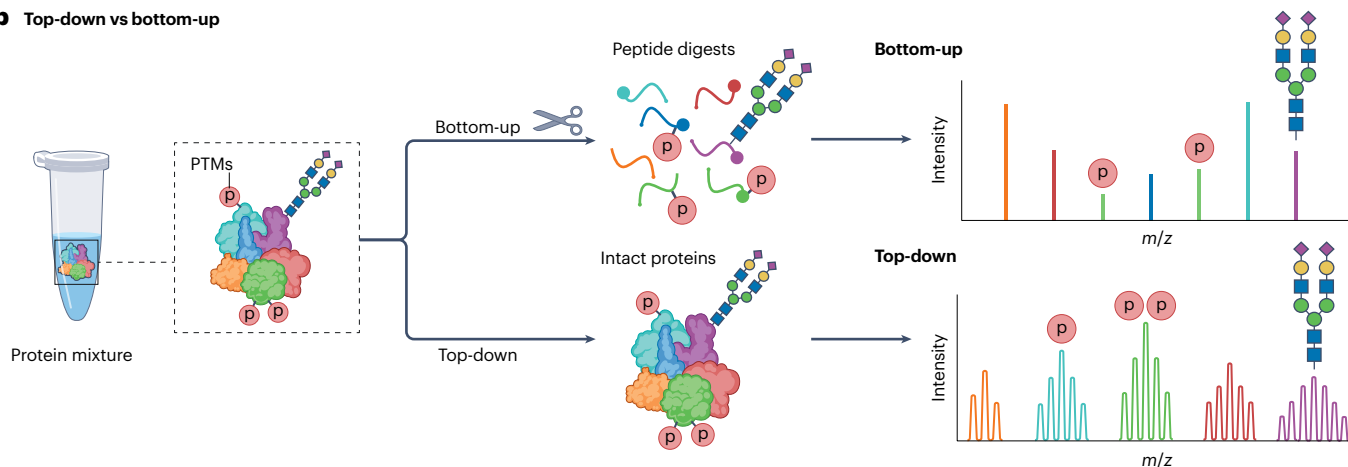
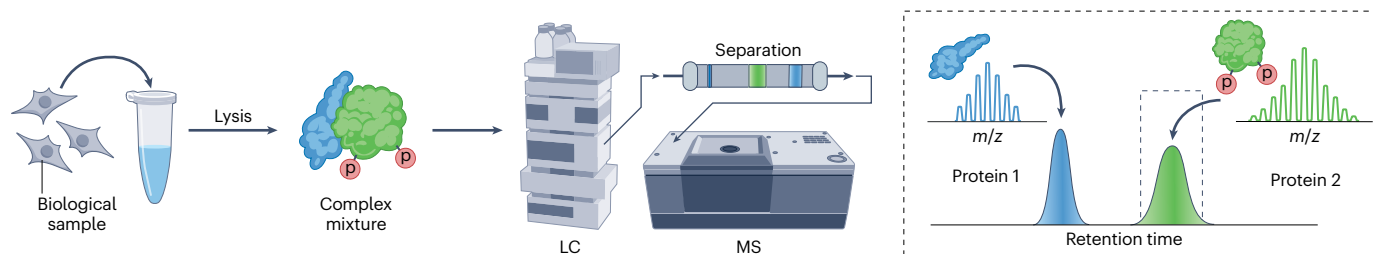


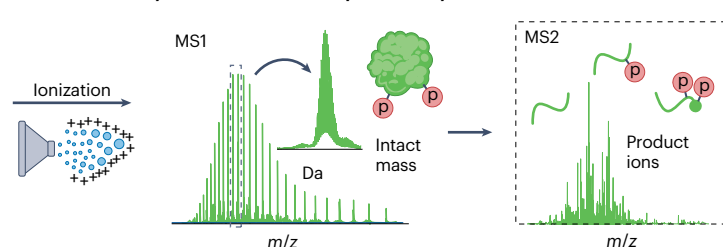
Fig. 1 | Proteoforms and the top-down approach. **a**, A revised central dogma of biology describing the flow of information from DNA to RNA, and, after processing, from RNA to mRNA and finally protein. Genetic variations, alternative splicing and post-translational modifications (PTMs) can form many proteoforms, all originating from the same gene. **b**, Illustration of

the conventional bottom-up proteomics approach that analyses peptides obtained from protein digests and the alternative top-down proteomics approach that analyses intact proteins. The red p represents protein phosphorylation.

a Front-end sample preparation and separation



b Intact mass analysis and tandem mass spectrometry



c Data analysis

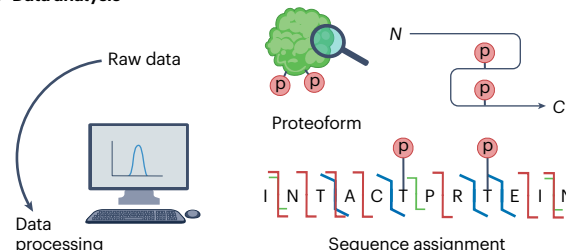


Fig. 2 | The pillars of top-down proteomics. **a**, Front-end sample preparation including sample fractionation; in this example, a protein mixture is separated by liquid chromatography (LC). The resulting separated proteins are analysed by high-resolution mass spectrometry (MS) for intact mass measurement (the top portion)

and then fragmented (the down portion) to obtain proteoform sequence-informative product ions. **b**, Data analysis and database searching are performed on the resulting tandem mass spectra for proteoform identification, characterization and quantification. The red p represents protein phosphorylation.

favoured for targeted analysis of intact proteins and protein complexes^{56,57}. However, it has major limitations, such as challenges in generating highly specific antibodies, limited availability of high-quality antibodies, batch-to-batch antibody variability, relatively low stability and high costs^{58–61}. To address these challenges, surface-functionalized multivalent superparamagnetic nanoparticles were designed as a versatile affinity platform for highly specific capture and enrichment of low-abundance proteoforms. This approach is based on nanoparticles being functionalized with an appropriate affinity reagent^{62–65}. For example, superparamagnetic nanoparticles functionalized with a multivalent ligand specific to phosphate groups have a high specificity for global capture of phosphoproteins^{62–64}. Another example is an integrated nanoprotoeomics method that combines peptide-functionalized nanoparticles with TDP to enrich and analyse cardiac troponin I – a gold-standard biomarker for cardiac injury – directly from serum to uncover proteoform–pathophysiology relationships^{65,66}. However, functionalized nanoparticles specific to TDP are not yet broadly commercially available. Engineered nanoparticles with tunable nanobiological interactions have been developed for deep plasma BUP; however, they have not yet been applied to TDP^{67,68}.

Equipment

The top-down approach requires three major steps (Fig. 2b): ionization to produce gas-phase ions from the protein of interest that can be transported in the mass spectrometer; intact mass analysis of the ionized protein by MS1 (the top portion) and intact gas-phase fragmentation to generate sequence-informative product ions (the down portion)⁸ by MS2; and data processing, including database searching, for proteoform identification, characterization and quantification. As TDP is performed on protein mixtures, the workflow typically requires analyte separation. Direct infusion, which involves introducing the analyte solution directly to the mass spectrometer, can be used for TDP⁶⁹.

Although methods for TDP by MALDI have been explored^{70,71}, TDP is conventionally performed with ESI⁹. Early TDP experiments relied on single-quadrupole and triple-quadrupole (Q and QqQ, respectively) mass spectrometers for intact protein analysis^{72,73}. These systems have poor mass resolving power, making charge state determination difficult, and limited mass-to-charge (m/z) range resulting in lower applicability to large proteins. High mass resolving power is particularly important for TDP, as fragment ions produced from intact proteins can generate convoluted mass spectra, in which various ions with different charge states can partially overlap. Many modern mass spectrometry instruments can reliably achieve high resolving power, including Fourier transform mass spectrometry systems, such as ion cyclotron resonance (FTICR)⁷⁴ and Orbitrap⁷⁵ mass spectrometers, as well as time-of-flight (TOF) and quadrupole TOF (QTOF) instruments⁷⁶.

Intact protein separations

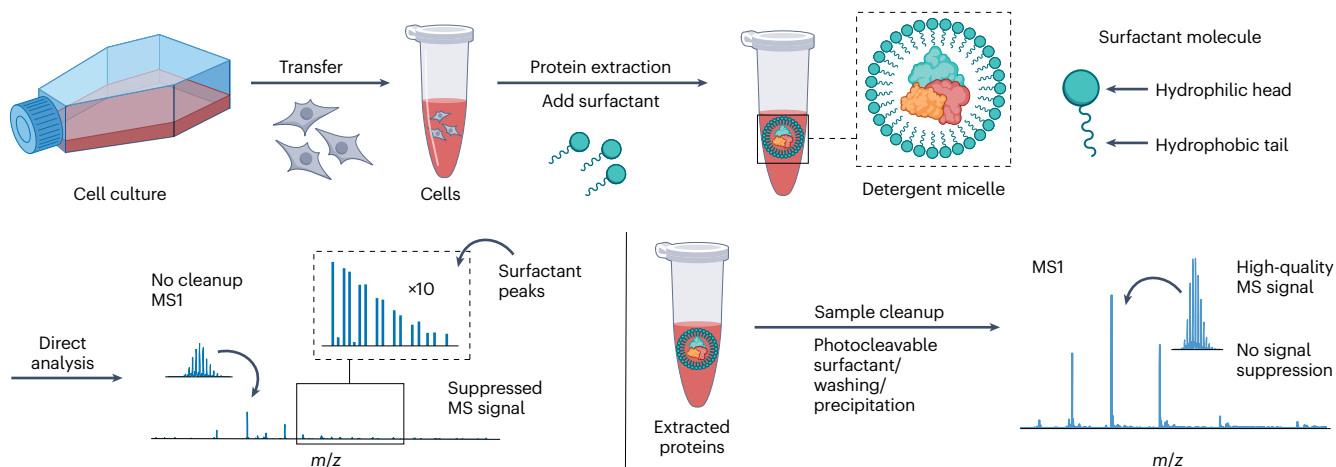
The proteome complexity presents a substantial challenge for TDP, requiring separation of intact proteins before mass spectrometry analysis⁵. This challenge is particularly pronounced when dealing with larger proteins (≥ 30 kDa) because, as protein size increases, ion signals in ESI mass spectra rapidly decrease⁷⁷. To address this issue, deep proteome profiling with TDP first separates intact proteins⁷⁸. Early demonstrations used gel-electrophoresis-based fractionation techniques, such as gel-eluted liquid fraction entrapment electrophoresis⁷⁹ or 2D gel electrophoresis⁸⁰. One example, termed the integrative approach, involves front-end 2D gel electrophoresis separation of complex protein mixtures, followed by in-gel extraction and LC–MS/MS analysis⁸¹. Another example is the virtual 2D gel mass spectrometry platform, which combines high-resolution isoelectric focusing with immobilized pH gradient polyacrylamide gels to separate complex protein mixtures. These mixtures are then incubated with a MALDI matrix and analysed by MALDI MS directly from the matrix-embedded dry gels,

referred to as xerogels⁸². A recent method – passively eluting proteins from polyacrylamide gels as intact species for mass spectrometry (PEPPI-MS) – was developed as a TDP-compatible front-end separation approach for size-based proteome fractionation⁸³. Although PEPPI-MS is promising for enhancing proteoform coverage, further optimization is needed to improve protein recovery rates for large-scale proteomics

analysis. Serial SEC was developed as an online or offline technique to separate smaller proteoforms from larger ones. Using serial SEC followed by reversed-phase LC (RPLC) enables detection of proteoforms up to 223 kDa on a QTOF mass spectrometer^{84,85}.

Advances in chromatographic stationary phases, liquid chromatographs and new column chemistry have improved the resolution

a Surfactant-aided sample preparation for TDP



b Front-end fractionation and enrichment for TDP

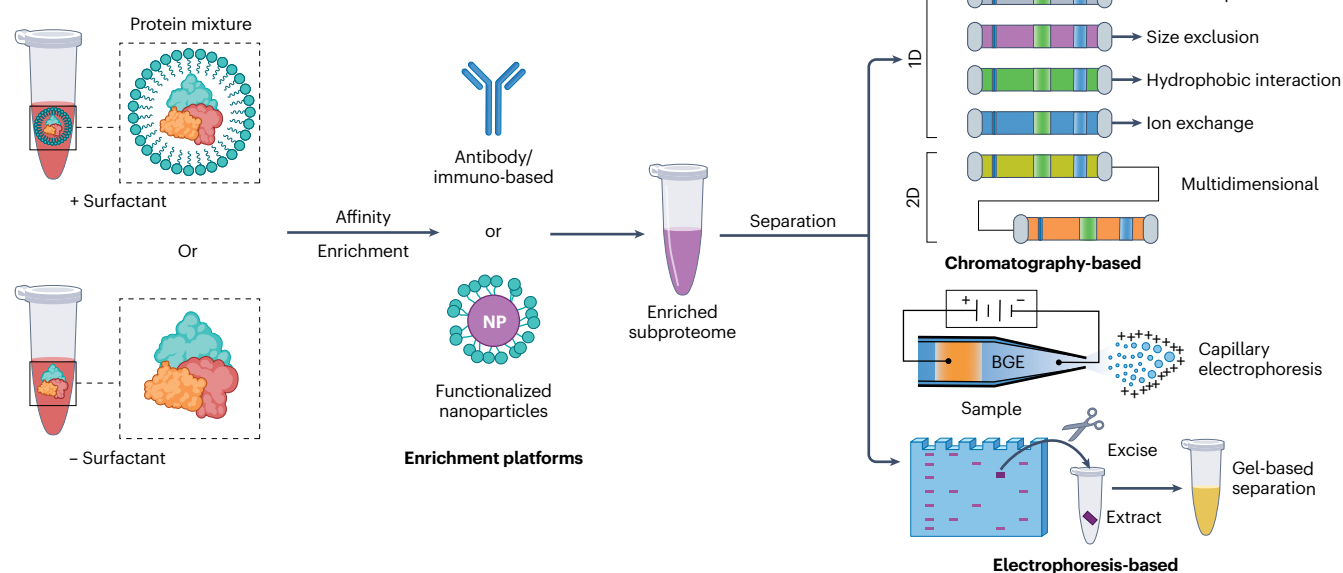


Fig. 3 | Top-down proteomics sample preparation. **a**, General surfactant-aided sample preparation methods for top-down proteomics (TDP). Surfactant-aided preparation typically proceeds by extracting proteins from a biological sample using a chaotropic buffer with a surfactant to efficiently solubilize proteins and yield a complex protein mixture. Without additional cleanup, top-down mass spectrometry (MS) signals suffer from immense signal suppression, leading to low-quality data. With proper sample cleanup using either wash methods, MS-compatible surfactants or protein precipitation methods, high-quality top-down MS data can be acquired. **b**, Illustration of front-end fractionation and enrichment strategies for TDP. Protein-containing samples are first extracted using a chaotropic buffer with or without

(indicated by +/- in the illustration) surfactant. Affinity-based enrichment with antibodies or functionalized nanoparticles (NPs) is often used to enrich specific protein targets or protein families from a complex lysate to give an enriched subproteome. Front-end fractionation of the starting lysate and the enriched subproteome are performed using chromatographic methods – such as reversed-phase liquid chromatography, size exclusion chromatography, hydrophobic interaction chromatography, ion-exchange chromatography or multidimensional liquid chromatography – or electrophoresis-based methods, for instance, capillary electrophoresis or gel-based separation. BGE refers to the background electrolyte used in capillary electrophoresis.

Table 1 | Summary of various top-down proteomics-compatible front-end enrichment strategies

Technique	Description	Useful for
Chromatography-based separation		
Reversed-phase liquid chromatography (RPLC)	A separation method using a nonpolar stationary phase and polar mobile phase for biomolecule separation based on hydrophobic interactions	Separation of denatured intact proteins for offline sample fractionation or online separation before mass spectrometry. A high-resolution separation applicable to most top-down proteomics (TDP) samples
Size exclusion chromatography (SEC)	Chromatographic separation of proteins based on their apparent hydrodynamic size. Conventionally, protein molecular mass is used as an estimate or analogue for size	Separation of native and/or denatured proteins with different molecular masses. Also used to fractionate a complex protein mixture into specific bins based on a range of sizes. Low resolution compared with other methods
Hydrophobic interaction chromatography (HIC)	Based on reversible interactions between hydrophobic protein surface regions and weakly hydrophobic ligands in the stationary phase. A high salt buffer is used to separate proteins based on hydrophobicity. A decreasing salt concentration gradient is used to elute bound proteins from low to high hydrophobicity	Under suitable conditions, HIC can preserve native protein structures and separate aggregated protein species from lower oligomeric states. HIC is also commonly used for antibody purifications
Ion-exchange chromatography (IEX)	Uses a charged stationary phase for separation based on the protein net charge. Depending on the buffer and protein isoelectric point (pI), positively charged proteins are separated with a negatively charged stationary phase (cation exchange) at pH < pI, whereas negatively charged proteins are separated with a positively charged stationary phase (anion exchange) at pH > pI	Separation of native and/or denatured proteins and protein purification. Used for downstream processing of antibodies and separation of highly charged proteins or protein mixtures with abundant charged species. IEX is commonly used to purify histidine-tagged proteins
Multidimensional liquid chromatography	Interface of two or more columns to incorporate multiple separation modalities based on different retention mechanisms to increase separation dimensionality and enhance analyte separation	Separation of complex mixtures with distinct chemical retentivities or separation selectivities. Examples include RPLC×RPLC, IEX×RPLC, HIC×RPLC, hydrophilic interaction chromatography (HILIC)×RPLC and SEC×RPLC
Affinity-based enrichment		
Antibody	A protein produced by the immune system capable of binding to specific antigens with high affinity	A reliable, low toxicity approach for enriching protein targets when high-quality antibodies are available and validated. Also used for native protein purification. Antibodies and their epitopes can be engineered to enhance target specificity but can have batch-to-batch reproducibility issues
Nanoparticles	Inorganic, organic or hybrid synthetic nanomaterials that can be functionalized for various biological applications	Used for highly specific and efficient enrichment when functionalized with specific affinity ligands. Can be modified with pan-selective ligands for broader enrichment specificity. Useful for native protein purification. Cost-effective, efficient and reproducible from batch-to-batch
Electrophoresis-based separation		
Capillary electrophoresis	Involves separation of charged molecules in a narrow capillary tube under the influence of an electric field	High-sensitivity separation of protein mixtures from low starting sample amounts. Can suffer from low sample loading capacity compared with RPLC
Gel separation/extraction	Separation of proteins by polyacrylamide gel-based fractionation, typically based on protein molecular mass. Involves one or more modifications to the conventional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) approach	Simple and reproducible method for partitioning protein mixtures into discrete mass ranges by SDS–PAGE. Proteins separated on gel can be resolved for TDP by gel-eluted liquid fraction entrapment electrophoresis or passively eluting proteins from polyacrylamide gels as intact species for mass spectrometry

and efficiency of intact protein separations^{5,86,87}. Compatibility of the mobile phase with ESI is crucial when developing new separation methods⁸⁸. To stabilize the protein tertiary structure and optimize separation selectivity, techniques such as hydrophobic interaction chromatography (HIC)⁸⁹ and ion-exchange chromatography (IEX)⁹⁰ require high concentrations of buffer salt in the mobile phase. Conventional non-volatile buffers – such as sulfate, phosphate or citrate salts – are typically used in HIC and IEX^{89,91–93}. Direct online coupling of HIC and IEX with TDMS was demonstrated, using the volatile buffer ammonium acetate for TDP analysis^{90,94}.

Despite the rapid growth of new intact protein separation modalities, no single modality can fully resolve all species in a proteome of interest. Multidimensional liquid chromatography (MDLC) presents opportunities to increase resolution by combining multiple

separation modalities for TDP^{95,96}. Two-dimensional LC, coupling HIC and RPLC, can greatly enhance the range of separable proteins in an *Escherichia coli* cell lysate⁹². A 3D LC approach, coupling HIC–IEX–RPC – offline first-dimension HIC and second-dimension IEX separation, before third-dimension online RPLC–MS – showed a 14-fold improvement in protein identifications compared with 2D IEX–RPLC–MS⁹³. However, offline MDLC methods are time-consuming and labour-intensive. It is expected that MDLC coupled with automation will lead to exciting new approaches, such as active solvent modulation and stationary-phase-assisted modulation^{97,98}.

Recent developments in CE–MS enable it to be used as both a denaturing and non-denaturing separation technique for TDP^{99–103}. The orthogonality of separation selectivity to conventional LC–MS methods, low sample volume requirements and commercial systems

make CE-MS an attractive technique for TDP^{104–106}. Alongside the increasing array of liquid-phase separation methods, gas-phase ion mobilities can also be used to separate intact proteins^{107–109}. Ion mobility spectroscopy (IMS) is based on the gas-phase transport properties of a molecule in the presence of an electric field and its rotationally averaged collision cross-sections (CCSs). The CCS is a unique physical property that captures information related to individual conformers in the population of gas-phase structures. CCS can be related to molecular conformation and structural dynamics¹¹⁰. IMS has expanded to include new techniques and devices. Drift tube ion mobility spectrometry involves ion separation under a uniform electric field that propagates through a buffer gas drift region. Trapped ion mobility spectrometry uses radially confining radiofrequency voltages and an axial electric field to counteract the drag force from a gas flow to trap and release ions according to their mobility. Field asymmetric ion mobility spectrometry (FAIMS) separates ions in a carrier gas by their behaviour in strong and weak electric fields under atmospheric pressure. Differential mobility spectrometry performs ion separation under atmospheric pressure with a similar operating principle to FAIMS, but using a different electrode geometry. Travelling wave ion mobility spectrometry uses an oscillating electric field to produce a set of voltage waves that pushes ions through a drift gas towards the mass analyser¹¹⁰. High-resolution IMS is promising for fast separation of proteoforms, with a high level of sequence homology. For example, travelling wave ion mobility spectrometry with pervasive charge solvation was integrated with TDMS to analyse chemically derivatized native-like protein ions with greatly improved TDMS sequencing¹¹¹. Trapped ion mobility spectrometry was shown to be effective for characterizing complex glycoproteins by TDMS^{112,113}, and FAIMS was shown to enhance TDP coverage in complex protein mixtures^{114–116}.

Tandem mass spectrometry techniques

Tandem mass spectrometry (MS/MS) is a powerful analytical technique used to identify and characterize molecules. It usually involves two consecutive stages of mass spectrometry to elucidate the identity and structure of a molecule. In TDP, MS/MS typically involves analysing

intact proteins by selecting a precursor protein ion, dissociating it into smaller fragment ions and analysing the fragment ions to derive the primary structure and modifications of a protein. Mass spectrometers used for TDP tend to be hybrid instruments, in which precursor ion selection (MS1) is followed by measurement of product ions generated by fragmentation of the precursor (MS2) (Fig. 4a). Such instruments could be tandem in space designs – such as hybrid QTOF and quadrupole Orbitrap platforms with two separate mass analysers – or tandem in time designs, such as ion traps that perform MS1, MS2 and higher MS n in the same mass analyser.

Various activation/dissociation methods are available to generate product ions (Fig. 4b). Most instruments can perform collision-induced dissociation (CID), also known as collisionally activated dissociation, to generate backbone *b*–*y* ions (Fig. 4b) through collisional activation from interactions with neutral gas molecules, such as N₂ or argon. Infrared multiphoton dissociation involves the absorption of low-energy infrared photons to produce *b*–*y* ions and potentially generate secondary and higher order fragment ions upon the absorption of multiple photons to yield more extensive protein sequence information^{117,118}. Historically, TDMS used CID to fragment protein ions⁹, either through a formal MS2 process from a precursor ion or through in-source fragmentation of all ions at the atmosphere–vacuum interface⁷³. CID processes usually generate enough product ions for identification, but the depth of sequence coverage may not be sufficient for unequivocal proteoform identification of, for example, PTMs. Electron-based dissociation methods (ExD)¹¹⁹, such as ECD¹⁵ and electron-transfer dissociation (ETD)¹²⁰, are often better than CID at generating high sequence coverage. ExD leads to *c*–*z*[•] products that can be used for confident proteoform characterization and PTM localization. More complex tandem mass spectra are generated by UVPD using 193 nm or 213 nm lasers¹²¹, with sequence coverage comparable to or higher than ExD methods. Tribrid platforms, combining a quadrupole mass filter, linear ion trap and Orbitrap, can perform proton transfer charge reduction (PTCR) to simplify product ion spectra¹²². PTCR reduces the product ion charge states, pushing product ions to higher *m/z*, owing to a lower *z*, and reducing overlap with other product ions at a similar *m/z* but different *z* values.

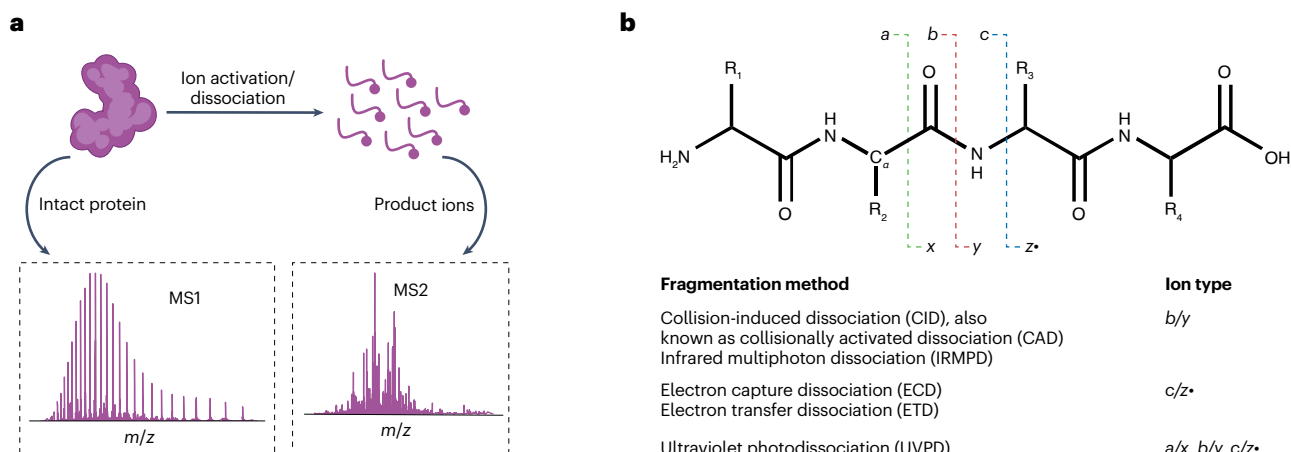


Fig. 4 | Tandem mass spectrometry techniques for top-down proteomics.

a, Illustration of the process of an intact protein undergoing ionization/dissociation events in a mass spectrometer to yield various fragment ions. The corresponding intact protein precursor ion spectrum (MS1) and product ion spectrum (MS2) are shown for the beginning and end stages of the process. **b**, Peptide backbone fragmentation scheme showing selected tandem mass

spectrometric techniques. Fragment ion nomenclature is depicted with *a*, *x*, *b*, *y*, *c*, *z*[•] notation depending on the specific cleavage along the amino acid backbone. Various fragment ion types are shown for the common tandem mass spectrometry (MS/MS or MS2) methods used in top-down proteomics.

Data collection

Generally, TDP analyses multiple proteins that could coelute at similar chromatographic times, convoluting the mass spectrometry analysis. The number of MS2 spectra that can be collected depends on the peak width of the separation technique and the spectrometer duty cycle; the amount of time the mass spectrometer is actively acquiring data in a given instrument setting. Key considerations for data acquisition involve selecting appropriate high-resolution instrumentation and methods to provide suitable peak resolution, analytical separation, sensitivity and depth of coverage for tandem mass spectra. Such evaluation steps are essential to improve the downstream calculation of accurate intact masses and resolve proteoforms with unusual and combinatorial PTMs, or single amino acid substitutions not easily separated by chromatography. The goal is to obtain unit mass resolution across the entire observed mass range¹²³ and isotopically resolve each protein molecular ion. The most common TDP data acquisition method is data-dependent acquisition¹²⁴. In data-dependent acquisition, a full mass spectrometry scan is collected and several precursor ions, usually the most abundant, are selected for fragmentation⁵³. Data-independent acquisition methods¹²⁵, which involve fragmentation of a mass spectrometry scan without precursor ion isolation, are being rapidly developed and adopted in BUP workflows¹²⁶ and offer exciting opportunities for TDP.

Results

Raw data interpretation and visualization

TDP data sets are rich in information but have a high level of complexity. As a result, analysis and interpretation can be a challenge for newcomers¹²⁷. Accounting for the effects of isotopes and charge states on instrument signal to noise (S/N), in addition to the high dynamic range (10^8 – 10^{12}) and broad mass range of the human proteome^{77,128}, makes intact protein spectra complicated to analyse and detection of low-abundance proteins difficult (Fig. 5a). Unlike mass spectra of smaller biomolecules or peptides, in which the most abundant isotopologue typically corresponds to the monoisotopic mass – the sum of the atom masses based on the most abundant isotope for each element – proteins have complex isotopic envelopes, often without an observable monoisotopic peak (Fig. 5b). Spectral deconvolution is a critical step to simplify TDP data by converting a complex isotope and charge state distribution to a single monoisotopic mass^{129–135}. For isotopically resolved spectra, collected with sufficient resolution for various possible isotopic peaks of a molecule to be observed, most tools rely on the Averagine model¹²⁹ to deisotope and predict theoretical isotopic distributions. Predictions are then fit to experimental isotopic envelopes to extrapolate a monoisotopic mass. Mass spectra are acquired continuously across an LC gradient and precursor ions are often represented by multiple charge states. As a result, additional information from extracted ion current chromatograms and multiple charge state peaks can aid spectral deconvolution^{131,133,135}. When spectra are not isotopically resolved, spectral deconvolution can use multiple charge state ions to derive the average neutral mass of a proteoform¹³⁶.

The greater complexity of TDP spectra requires specialized interpretation and processing software to extract molecular information. Continuous efforts aim to develop standardized file formats for storing mass spectrometry data^{137–139}. The most universal file format is mzML (latest version 1.1.1)¹³⁸, an XML format supported by the Human Proteome Organization Proteomics Standards Initiative (HUPO-PSI). Several open-source software libraries can convert, read and write mass spectrometry file formats, including ProteoWizard¹⁴⁰, JmzML¹⁴¹,

mzJava¹⁴² and pymzML¹⁴³. Many open-source visualization tools developed for BUP can be used for TDP, such as BatMass¹⁴⁴ and OpenMS¹⁴⁵, but there are also open-source tools developed explicitly for TDP data visualization, including MASH Explorer/MASH Native^{146,147} and TopMSV¹⁴⁸. In addition, instrument manufacturers and third-party companies offer commercial tools to directly process vendor file formats or convert files into mzML or another open-source format¹⁴⁹.

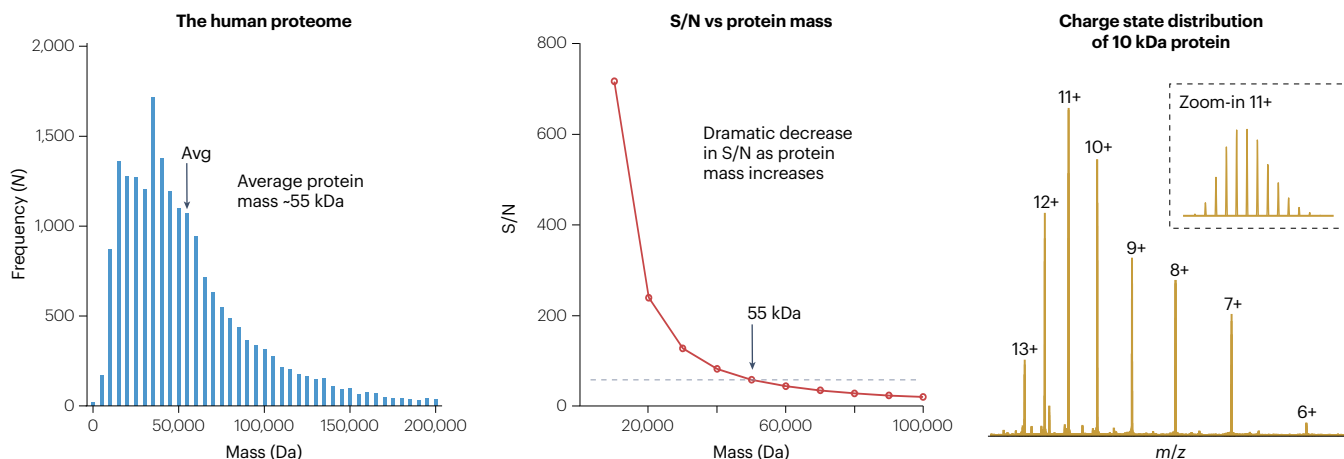
Data analysis

A TDP data analysis pipeline begins with top-down mass spectral pre-processing and deconvolution, which generates deconvolved mass spectra for proteoform spectrum matches (PrSMs). The next step involves searching the deconvolved mass spectra against a protein or proteoform sequence database to identify proteoforms with a false discovery rate (FDR) control and characterize PTMs. Finally, proteoform abundances are quantified and differentially abundant proteoforms between samples are identified. TDP workflows are often separated into two experiment types: targeted workflows, where an individual or set of proteins with a priori knowledge is used to inform measurement and analysis; or discovery workflows, where little-to-no information is known about the possible proteoforms and modification states.

There are several approaches for proteoform sequence database construction. As proteoforms from a biological sample often contain various alterations – such as gene mutations, alternative splicing events and PTMs² – building a database that accurately reflects proteoforms in the sample is essential for high-sensitivity proteoform identification¹⁵⁰. The most common approach is to directly use protein sequence databases from UniProt¹⁵¹, RefSeq¹⁵², GENCODE¹⁵³ or related resources. However, these sources only contain reference sequences and do not include proteoforms with various alterations. PTM annotations in protein knowledgebases and variable PTMs have been used to build proteoform sequence databases¹⁵⁴. Combining many PTMs or alteration sites leads to a combinatorial explosion of the search space, making it impractical to add all combinations to a database. To address this challenge, the number of PTM/alteration combinations can be constrained or all possible combinations of PTMs can be represented using graphs¹⁵⁵. Alternatively, DNA or RNA-seq data can be used to build proteoform sequence databases with sample-specific gene mutations and alternative splicing events¹⁵⁰.

Matching mass spectra and candidate proteoforms typically starts with a fast filtering method to reduce the number of candidates from thousands to tens¹⁵⁶. After this, a slower matching method is used to determine a match score between the mass spectrum and candidate proteoform from the first step¹⁵⁴. Many filtering methods have been developed for TDP spectral identification¹⁵⁶. When matching reference sequences, the precursor mass from tandem mass spectrometry is matched to the molecular masses of proteoforms, or proteoform fragments, in the database. When variable PTMs are included, a multi-notch search¹⁵⁷ is used, which allows multiple precursor mass differences. When unexpected mass shifts are allowed, the most common approaches include sequence tags¹⁵⁸, open search strategy^{159,160} and an unmodified protein fragment approach¹⁶¹. Proteoform candidates reported by filtering methods are aligned with the spectrum to identify proteoforms with variable PTMs or unexpected mass shifts¹⁶². Alignment algorithms for top-down mass spectra originated from BUP¹⁶³ and many variations exist^{131,155,162,164}. For example, the number of atoms replaces residue masses in MSPathFinder¹³¹, and the alignment between a mass spectrum and candidate proteoforms with variable PTMs is allowed in TopMC¹⁵⁵.

a The relationship of protein size and mass spectrometry signal to noise (S/N)



b Isotopologue distribution of small vs large proteins

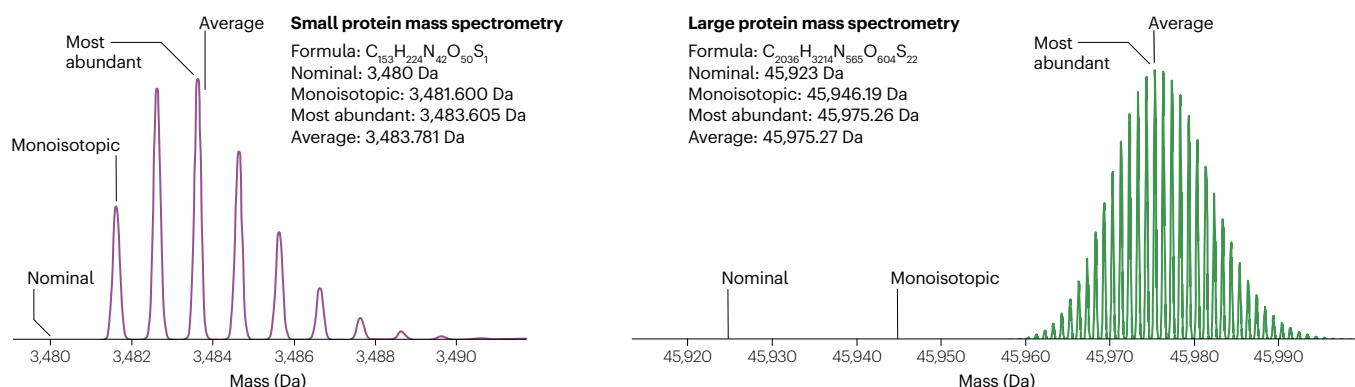


Fig. 5 | Fundamental concepts in protein analysis by top-down proteomics.

a, The effects of protein size on mass spectrometry signal to noise (S/N) and charge state distribution under electrospray ionization. A histogram of protein molecular masses for all known proteins in the human proteome is shown. The plot was created using 20,423 entries for *Homo sapiens* using the UniProt Knowledgebase released on 21 April 2023, and the bin size is 500 Da. Illustration of the decay in S/N as a function of increasing mass resulting from the increasing number of charge states observed for electrosprayed protein ions with the average protein mass (55 kDa) annotated. A typical top-down mass spectrum obtained for a 10 kDa protein under electrospray ionization with all charge states annotated. The most abundant charge state is given by $z = 11+$. **b**, Example of the

differences in isotopologue distribution between a small (3.4 kDa) and large (45.9 kDa) protein. For sufficiently large protein ions, the monoisotopic mass is no longer observed and the difference between the most abundant and average mass decreases. The monoisotopic mass is the sum of the masses of the atoms in a molecule using the principal (most abundant) isotope for each element, also known as the exact mass. The nominal mass is the sum of masses of the closest integer value of the most abundant mass of an atom. The average mass is the sum of the masses of the atoms from their respective weighted averages. The average mass of a compound is sometimes referred to as the relative molecular mass, denoted by M_r . The most abundant mass is the mass of the highest abundance peak in the entire isotopic cluster.

Proteoform identification and characterization

Understanding the functional role of proteoforms requires identification and characterization³. Unlike BUP, which uses a limited number of peptides as a proxy for proteins based on partial sequence information, TDP analyses whole proteins. Consequently, TDP offers a comprehensive insight into the proteoform landscape, enabling proteoform identification, novel proteoform discovery and in-depth sequence characterization^{5,32,34,124}. TDP has unique strengths, as it can characterize combinatorial PTMs alongside the isoforms encoded by different genes in a multigene family, which often have high sequence homology^{165,166}. For example, sarcomere proteins have diverse isoforms and PTMs, such as N-terminal di-methylation, acetylation, phosphorylation and

methylation. Proteoform variations from individual muscle cells can be investigated by TDP, enabling proteomics to be integrated with functional properties¹⁶⁵. In a practical example, TDP was used to investigate the expression of ventricular isoform myosin light chain 2 (MLC2v), a critical cardiac regulatory protein¹⁶⁷. MLC2v is considered the standard isoform marker of ventricular specification and is commonly used to assess human stem-cell-derived cardiomyocyte cultures. However, unlike previous genomic annotations for heart chamber specificity of MLC2, TDP revealed that MLC1v, but not MLC2v, exhibits ventricular-restricted expression. When multiple PTMs are present on a single protein molecule, TDP is the only technique that can resolve the complex proteoforms and combinatorial PTMs¹⁶⁸. For example, histones

are highly modified structural proteins associated with DNA. Histones have many PTMs – acetylation, methylation, phosphorylation and ubiquitylation – and are present as multiple isoforms¹⁶⁹. TDP is a crucial tool to decipher histone proteoform complexity and quantitatively describe molecular stoichiometries, such as connecting combinatorial histone H4 – an essential regulator of all eukaryotic DNA-templated processes – PTMs with potential biological functions^{170,171}. A recent example applied Nuc-MS as a top-down technique to characterize whole nucleosomes and unravel the histone code¹⁷². This approach can quantify histone variants and their PTMs with results highly concordant with chromatin immunoprecipitation sequencing.

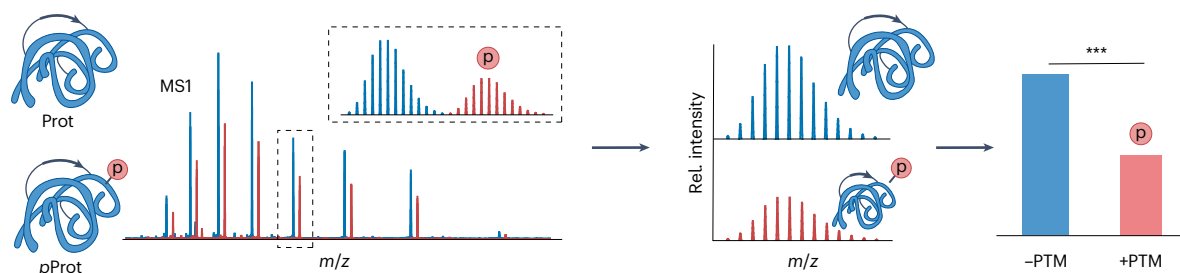
Proteoform quantification

TDP can quantitatively analyse proteoform changes in response to changes in the environment, disease state and differential cellular development in biological pathways¹⁷³. Similar to BUP, three distinct quantitative approaches have been developed for TDP (Fig. 6): label-free, in which proteoforms are quantified using proteoform intensity^{174,175};

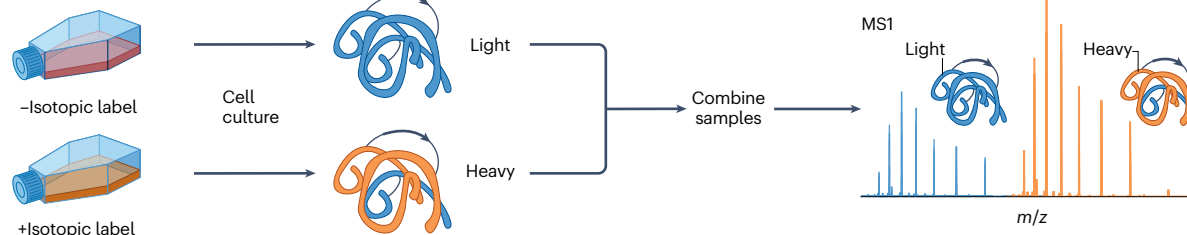
isotope labelling, in which proteoforms are quantified by differential isotope labelling^{176–178}; and chemical labelling, in which proteoforms are quantified with a chemical reporter, typically at the MS2 level¹⁷³. The advantages of label-free quantification are simplicity, high throughput and adaptability to most experiments and sample types^{179,180}. Label-free quantification can be applied to any protein sample and facilitates analysis of highly complex samples. Additionally, label-free quantification can be used with direct infusion or online separation techniques such as LC or CE^{181–183}. Many studies have demonstrated the accuracy and reproducibility of the label-free approach^{175,180,184–187}. For example, a label-free top-down LC–MS quantification method was developed to simultaneously quantify protein expression based on extracted ion chromatograms and PTMs derived from relative quantification in the mass spectra¹⁸⁸. The results aligned well with western blot conclusions, demonstrating that TDP can offer an antibody-independent approach to quantify intact proteins and modified proteoforms¹⁸⁸.

Label-free quantification involves identifying mass features, calculating intensities and making relative comparisons. Online

a Label-free quantification



b Metabolic labelling



c Chemical labelling

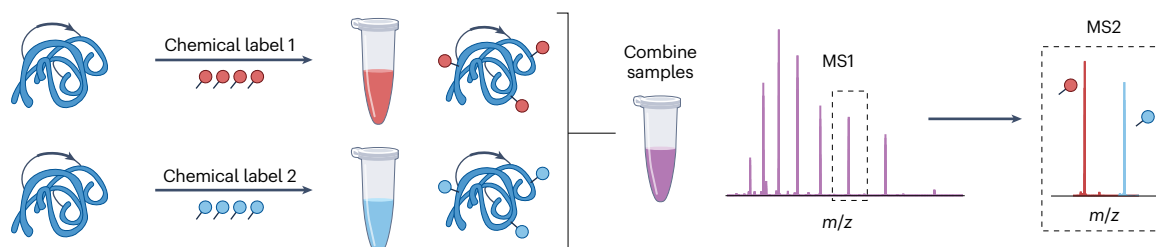


Fig. 6 | Overview of top-down proteomics quantification methods.

a, Label-free quantification, which relatively compares the mass spectral signal abundance of various proteoforms between individual liquid chromatography–mass spectrometry (MS) runs. **b**, Metabolic labelling, including isotopic labelling of proteins in vitro, for comparative MS1 quantification of proteoforms

expressed by cells cultured under various conditions. **c**, Chemical labelling strategies, which involve covalently modifying proteins at specific amino acid residues, generally Lys residues, and the N-terminal domain. Typically, tandem mass tag labelling is used and quantification is performed at the MS2 level. The red p represents protein phosphorylation. PTM, post-translational modification.

LC–MS/MS typically has a low duty cycle. Common practice is to generate proteoform libraries by combining all LC–MS/MS analyses or conducting additional experiments to maximize the identification of quantifiable proteoforms. The identification of mass features is performed by comparing to a proteoform library using mass measurement accuracy and LC retention time. In TDP with ESI, intact proteoforms will often contain multiple charge states⁷⁷. As a result, combining the ion intensities of multiple charge states can enhance the accuracy of intact proteoform quantification^{175,180}. This process can be accomplished through various deconvolution algorithms, including open-source – MS-Deconv+ (ref. 134), TopFD¹³⁵, THRASH¹²⁹, ProMex¹⁸⁹, Xtract¹⁹⁰, Mesh¹⁹¹, ICR-2LS¹⁹², Mascot¹⁹³ and FLASHDeconv¹³³ – and commercially available software. To minimize variation between runs, intensities are normalized based on the total ion current levels of each LC–MS run¹⁹⁴. Quality control and sample blank runs are also included in TDP workflows to ensure that variations in the detected features are not due to the system. Label-free TDP has been widely applied to quantify proteins from several or single cells^{195–197}.

Although label-free quantification is the most applied quantification method in TDP, isobaric chemical tag labelling is the gold standard for BUP, as it enables multiplexing for improved throughput and lower run-to-run variation. Previously, isobaric chemical tag labelling of intact proteins was limited to individually purified proteins and simple protein mixtures^{198–200}, and application to complex protein mixtures, such as whole cell lysates, was challenging owing to protein aggregation and insufficient labelling. However, recent optimizations enable better labelling of complex protein lysates. For example, tandem mass tag labelling of intact complex protein mixtures can be achieved by enrichment of low-molecular-mass proteins (<30 kDa)²⁰¹, optimization of chemical labelling parameters¹⁷⁷ and optimization of CID and high-energy collisional dissociation fragmentation energies²⁰².

Other labelling techniques – such as stable isotope labelling by amino acids (SILAC)²⁰³, isobaric and pseudoisobaric tags^{199,204,205} and NeuCode SILAC²⁰⁶ – have shown potential for quantitative TDP. For example, an intact-mass strategy with NeuCode SILAC was used to determine lysine count in the elucidation of proteoform families^{207,208}. Isobaric chemical tag labelling enables relative quantification by measuring reporter ions that are fragmented during MS2. However, as mass feature identification is also performed at the MS2 level, the fragmentation energy required for quantification and identification often requires careful optimization.

Statistical analysis and error calculations

TDP software tools evaluate the similarity between a tandem mass spectrum and a candidate proteoform by assigning a numeric score to reflect the degree of matching, a measure of how well the fragment data match the identified protein sequence. Typically, a *P* value – the probability that an annotated PrSM between a mass spectrum and protein sequence from a randomized database is within a specified threshold – or *E* value – the expected number of PrSMs in a specified threshold between a mass spectrum and protein sequence from a randomized database – is provided. These values indicate the probability of randomly obtaining the observed number of matching fragment ions by chance, considering the total number of proteoforms interrogated for PrSMs²⁰⁹. Poisson models¹⁵⁴, generating function approach^{131,161} and Markov chain Monte Carlo²¹⁰ methods have been used to compute *E* values of proteoform identifications. FDRs of identified PrSMs – the ratio of false positives to the number of total positive PrSM identifications – are usually estimated using

the target-decoy approach, which determines the ratio of identified decoy hits from a shuffled decoy database to target hits from a target database²¹¹. A shuffled database is appended to the target database to estimate the *Q* value, an alternative to the *P* value that incorporates FDR control and represents the minimum FDR in which a result may be considered statistically significant, which are computed at the PrSM level, proteoform level and intact protein level¹²². For quantitative TDP analysis, one-way analysis of variance and Student's *t*-tests (two-tailed) are commonly used for statistical analysis^{166,187,213}. Multiple testing adjustment is usually performed using the Benjamini–Hochberg method¹⁶⁶. If necessary, non-parametric Kruskal–Wallis one-way analysis of variance and Wilcoxon rank-sum test can be used for group comparisons²¹⁴. For quantitative TDP of human clinical samples, a linear mixed effects model with random intercept can further characterize heterogeneity among human individuals¹⁸⁵.

There are more proteoforms than corresponding genes⁴, making the search space of potential proteoforms vast. Automated proteoform identification solutions are prone to errors. They frequently mislocalize PTMs, report false cleavages and incorrectly calculate the precursor mass^{131,211}. As a result, users often need to manually validate and refine software results. Modern TDP software solutions, both open source and commercial, are continuously developing rigorous and sophisticated statistical approaches to improve accuracy in the TDP analysis. Accurate proteoform matching involves spectral alignment²¹⁵ with possible PTMs – TopPIC¹⁶¹, MSPathFinder¹³¹, TopMG¹⁵⁵ and pTop²¹⁶ – and statistical significance computed as a *P* value – for example, in MS-GF+, MS-Align+, TopPIC and MSPathFinder – *E* value and FDR²¹⁷. Newer characterization methods, such as C-score and MIscore^{218,219}, have integrated Bayesian approaches to improve proteoform identification and provide a more accurate scoring system. Additionally, there are several emerging TDP software packages for simpler statistical analysis workflows, such as TopPICR²²⁰ and Informed-Proteomics¹³¹. Visualization of deconvolved TDP data, peak lists and sequence coverage maps is essential for validation and refinement of the TDP analysis and can be achieved with open-source software, including ProSight, LcMSspectator, TopMSV and MASH Explorer/MASH Native^{146–148,154,221,222}. The identification of differentially expressed proteoforms in TDMS is similar to the identification of differentially expressed genes in the RNA-Seq data analysis. Consequently, many statistical methods developed in transcriptomics – based on Poisson, negative binomial, linear and non-parametric models – can be applied to TDMS, such as Limma, EdgeR and DESeq2 (ref. 223). Similarly, statistical methods developed for BUP, such as MsStats and MaxQuant, can be extended to identify differentially expressed proteoforms in TDP^{224,225}.

Applications

Global proteoform discovery

Improved sample prefractionation methods and robust LC–MS/MS workflows have expanded the application of label-free TDP, enabling the global proteoform analysis of biological samples²²⁶. The first discovery-mode global TDP study that mapped intact proteoforms used a 4D separation system²⁸. Recently, proteoform landscapes from five human tissues – lungs, heart, spleen, small intestine and kidneys – were comparatively mapped using a combination of capillary zone electrophoresis (CZE)-MS and RPLC-MS²²⁷. Over 11,000 proteoforms were identified, 64% of which were not previously reported²²⁷. In another example, the Blood Proteoform Atlas revealed approximately 30,000 unique proteoforms, offering a nuanced understanding of cellular differentiation and demonstrating the clinical potential of TDP³¹.

Advanced global proteoform platforms and instrumentation are increasingly able to discover and characterize proteoforms²²⁸, reinforcing the importance of proteoform-level knowledge⁴. CTDP has begun an effort analogous to the 2002 Human Genome Project, called the Human Proteoform Project & Atlas, which seeks to construct the first Human Proteoform Atlas³. The goal of this initiative is to map the entire human proteome, an effort that will require technical leaps in the discovery and characterization of proteoforms in health and disease. It is anticipated that the next generation of human proteomics will be structured around ~20,000 proteoform families¹, each corresponding to a specific gene. Extensive proteoform repositories assembled for key model organisms and thoroughly characterized mammalian cell lines are expected to provide foundational knowledge of the global proteome. This will likely serve as an essential cornerstone in modern biology.

Biomedical applications

Mass spectrometry-based proteomics has become an indispensable technique for biomedical research (Fig. 7a), playing a crucial role in uncovering novel disease biomarkers and unravelling the mechanisms underlying human disease^{185,229,230}. Large-scale, discovery-mode, global profiling of proteoforms has provided critical knowledge to map the overall proteoform landscape. However, hypothesis-driven, targeted TDP at the sub-proteome level can offer novel molecular insights to understand structure–function relationships and underlying disease mechanisms^{34,231,232}. TDP has analysed many clinically relevant sample types, including serum, biofluids or biopsy tissue, to identify specific proteoform biomarkers^{233–235}. This section illustrates four important human disease areas, showcasing instances in which proteoforms were recognized by TDP and associated with disease development.

Cancer. Understanding cancer biology involves studying proteins and their PTMs, especially in signalling pathways governed by intracellular phosphorylation³. As TDP can detect the entire proteoform landscape, it has the potential to discern oncoproteoforms, particularly those arising from combinations of driver mutations, PTMs and RNA splice variants. This capability is exemplified in the context of rat sarcoma (RAS) biology, in which TDP has precisely distinguished PTMs in four isoforms derived from RAS family genes and established driver mutation/PTM crosstalk in human colorectal cells and tumours²³⁶. Gene mutations in the RAS family, which encode small GTPases, are responsible for more than 40% of all cancers, with a particularly high incidence exceeding 90% in pancreatic tumours. The complex RAS isoforms are derived from three genes, yielding four isoforms with a high sequence homology in the initial 165 residues. The PTMs of these isoforms can be precisely characterized by TDP after immunoprecipitation²³⁶. The proteoform-level study offers a thorough molecular definition and abundance comparison between wild-type and mutant RAS proteoforms, providing insights not accessible with conventional BUP. Large-scale global TDP has helped advance cancer research. For instance, a global TDP study identified more than 23,000 proteoforms from 2,332 proteins in colorectal cancer cells and revealed substantial proteoform-level differences between metastatic and non-metastatic cells²³⁷. The study was limited owing to the majority of identified proteoforms having a low-molecular mass (<20 kDa). More work is needed for global identification and quantification of larger proteoforms (>30 kDa).

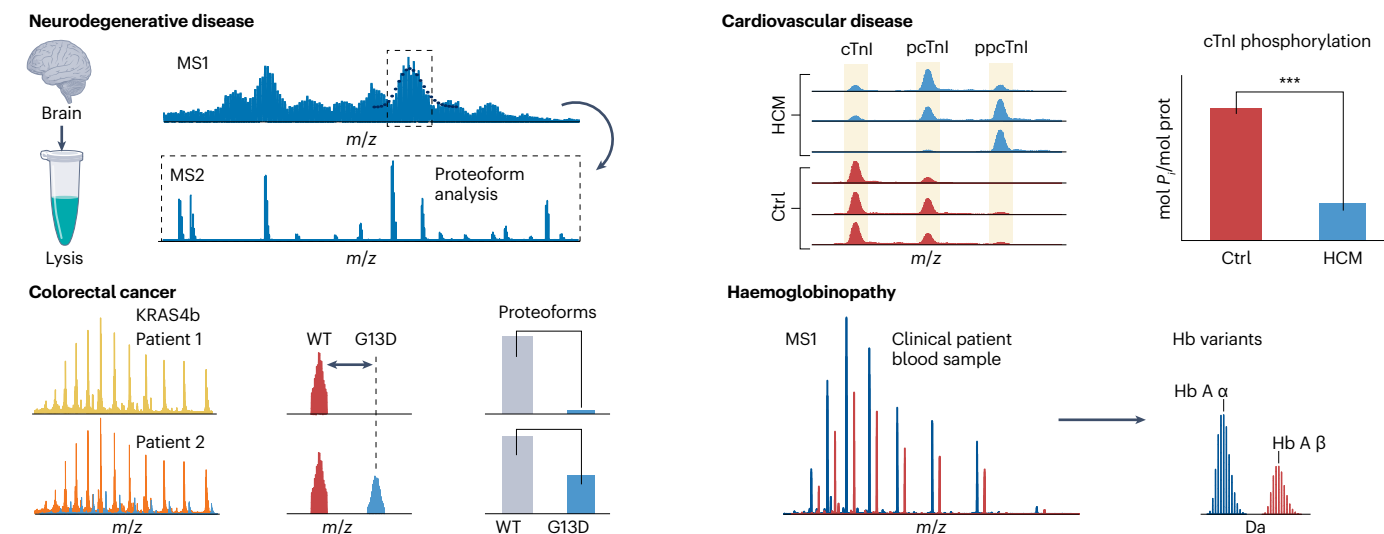
Cardiovascular disease. Cardiovascular diseases are the primary global cause of death and the affected population is projected to rise

as demographics shift towards an ageing population²³⁸. Efforts have been made to use proteomics with cardiac biology and clinical diagnosis^{239,240}. For instance, TDP analysed paired serum samples in the CARDIA study, revealing proteoform-specific association between apolipoproteins AI and AII with cardiometabolic indices²⁴¹. Several TDP studies have associated changes in cardiac proteoforms with disease phenotypes, in both human clinical samples and animal models of heart diseases^{32,240}. A quantitative TDP study identified phosphorylated proteoforms of cardiac troponin I (cTnI) as potential biomarkers for chronic heart failure, the first TDP study discovering biomarkers from tissues¹⁸⁵. An enrichment strategy using peptide functionalized nanoparticles was integrated with TDP to capture cTnI directly from human serum. This unveiled molecular fingerprints of various cTnI proteoforms, underscoring their potential for disease diagnosis in serum at the proteoform level⁶⁵. TDP has also identified actin proteoforms as potential cardiac disease markers²⁴² and uncovered newly identified phosphorylation of a pivotal Z-disc protein, *enigma* homologue isoform 2, in a swine model of acute myocardial infarction²²⁹. Given the critical role of PTMs and alternative splicing during maturation of human pluripotent stem-cell-derived cardiomyocytes, identifying and quantifying proteoforms and splicing isoforms enables unambiguous assessment of the maturation stages¹⁶⁶. TDP was used to analyse heart tissue samples from septal myectomy surgery in patients with hypertrophic cardiomyopathy, the most common heritable heart disease. The genetic cause of hypertrophic cardiomyopathy is linked to mutations in genes encoding sarcomeric protein²¹⁴. The TDP study uncovered unexpected results and demonstrated the capacity of proteoforms to more accurately reflect the clinical manifestation of a patient. Most identified cardiovascular proteoforms are from the cardiac sarcomere and further efforts will be needed to expand coverage to the broader cardiac proteome.

Neurodegenerative diseases. More than 47 million people globally are affected by dementia and this number is expected to reach 135 million by 2050 (ref. 243). Dysregulated PTMs can impact protein aggregation in neurodegenerative disease (Fig. 7a) and many PTMs are modulators of proteinopathy in neurodegenerative conditions. For instance, Alzheimer disease is impacted by phosphorylation of amyloid- β or tau and isoaspartate formation in amyloid- β ; Parkinson disease is related to deacetylation, 4-hydroxy-2-neonal modification, O-GlcNAcylation or phosphorylation of α -synuclein; amyotrophic lateral sclerosis is influenced by acetylation or phosphorylation of transactive response DNA-binding protein-43 and SUMOylation of superoxide dismutase 1; and Huntington's disease by phosphorylation of huntingtin²⁴⁴. Studies with superoxide dismutase 1 emphasize the importance of TDP to understand relationships among PTMs, sequence variants and protein complexes involved in proteinopathies²⁴⁵. This knowledge is vital to understand the mechanisms underlying neurodegenerative diseases and help develop innovative diagnostic and therapeutic treatment methods¹⁰⁹. However, the complexity of proteoforms in neurodegenerative diseases, such as tau proteins in Alzheimer disease²⁴⁶, means that there is need for improved instrumentation to resolve large and highly modified proteins, and data analysis methods to resolve combinatorial PTMs. A proteoform imaging mass spectrometry method, which combines individual ion mass spectrometry for TDMS of brain cells, could help address these challenges²⁴⁷.

Infectious diseases. Severe infectious disease outbreaks, such as the COVID-19 pandemic, can have a large impact on lives of people

a Biomedical applications for TDP



b Biopharmaceutical applications for TDP

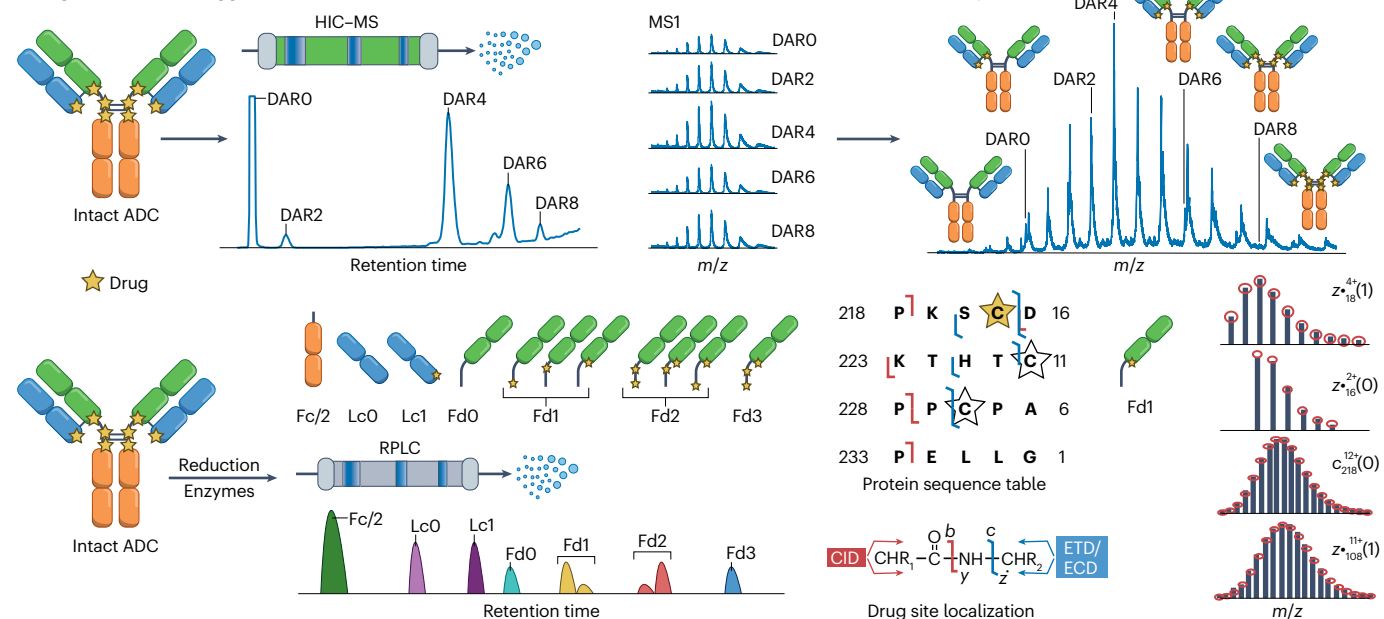


Fig. 7 | Biological applications for top-down proteomics. a, Schematic depiction of various human organ systems and representative examples of biomedical top-down proteomics (TDP) applications. Four major human disease applications are shown. Neurodegenerative disease involving TDP analysis of hypermodified brain proteins linked to Alzheimer disease. Cardiovascular disease showing the top-down label-free quantification of cardiac troponin I (cTnI) phosphorylation state, which can serve as a biomarker for major cardiac diseases, such as ischaemic cardiomyopathy or hypertrophic cardiomyopathy (HCM). In clinical applications of TDP, haemoglobinopathy involves the top-down mass spectrometry analysis of haemoglobin (Hb) variant characterization from various human clinical blood samples. Colorectal cancer showing the top-down mass spectrometry analysis of various KRAS4b proteoforms to inform disease state. The p and pp represent phosphorylation and bisphosphorylation, respectively. **b**, Illustration of major biopharmaceutical analysis of antibody–drug conjugates (ADCs). Here, a Cys-based ADC is shown.

The top-down approach is ideal for determining the drug-to-antibody (DAR) ratio of ADCs by direct infusion analysis of intact ADCs. Site-specific localization of covalent drug attachment can be achieved through an online top-down liquid chromatography–mass spectrometry (LC–MS) approach. Disulfide reduction and enzymatic treatment can result in a total of seven separated subunits including Fc/2, Lc without drug (Lc0), Lc with 1 drug (Lc1), Fd without drug (Fd0) and Fd with 1–3 drugs (Fd1–3). Electron-transfer dissociation (ETD) and collision-induced dissociation (CID) tandem mass spectrometry characterization of reduced Fd1 isomer of brentuximab vedotin after IdeS digestion are shown, with a corresponding truncated protein sequence table as an example. The stars represent possible conjugation site, with Cys220 (yellow star) the confidently localized Fd1 drug-bound isomer that was identified. Theoretical ion distributions are indicated by the red dots. ECD, electron capture dissociation; HIC–MS, hydrophobic interaction chromatography–mass spectrometry; RPLC, reversed-phase liquid chromatography; WT, wild type.

Glossary

Bottom-up proteomics

A technique used to analyse peptide fragments from the proteolytic digestion of intact proteins by mass spectrometry, enabling sensitive and high-throughput identification of proteins.

Convoluting mass spectra

Refers to the potential overlap of two or more peaks with similar mass-to-charge (m/z) ratios. This can lead to incomplete separation of two or more mass spectral peaks owing to resolution limits and complicated mass spectral identification.

Data-dependent acquisition

Refers to the tandem mass spectrometry technique that involves specific selection of precursor ions before MS2 fragmentation. This technique commonly selects several of the most intense peaks observed in a single MS1 survey scan for fragmentation and only fragmenting a small subset of the total ions present.

Data-independent acquisition

Refers to the tandem mass spectrometry technique that forgoes specific selection of precursor ions and instead fragments all ions present in an MS1 survey scan.

Monoisotopic peak

The exact mass of a molecule, represented by the sum of the masses of the atoms in the molecule using the principal (most abundant) isotope for each element.

Post-translational modifications

All covalent processing events and modifications to the amino acid sequence of a given protein occurring after protein biosynthesis.

Proteoforms

A term used to describe all the different molecular forms of a protein product from a single gene. This includes changes from genetic variations, alternatively spliced RNA transcripts and post-translational modifications such as protein phosphorylation, glycosylation and protein truncations.

Tandem mass spectrometry

A technique performed using one or more mass analysers, involving multiple consecutive stages of mass spectrometry analysis — typically two, MS/MS, also known as MS2 — to fragment selected precursor ions in the MS1 spectrum and generate product ions that can elucidate the structure and chemical composition of a molecule.

specific O-glycosylated proteoforms of the spike protein were associated with the omicron variant, which could provide information about how the variant escapes immunological protection²⁵⁴.

Biopharmaceutical applications

Protein-based pharmaceuticals represent an increasingly large share of total drug sales, currently more than 50% of ongoing drug development pipelines and FDA approvals²⁵⁵. Biotherapeutics cover a broad spectrum of masses, ranging from 5.8 kDa for human insulin to approximately 150 kDa for monoclonal antibodies (mAbs) and antibody–drug conjugates (ADCs). Additionally, fusion proteins exceeding 150 kDa were created as innovative treatments for cancer, autoimmunity, inflammation and genetic disorders²⁵⁶. In both academic and industrial laboratories, TDP is increasingly used to analyse the structure of biotherapeutic mAbs and advanced modalities^{256–261} (Fig. 7b). Most ADCs currently available or in clinical trials use either Lys or Cys conjugation. Both conjugation methods lead to multiple positional isomers for a specific drug-to-antibody ratio species. These isomers play a crucial role in influencing the efficacy, stability and safety of the ADCs, making the drug-to-antibody ratio analysis highly important in quality control²⁶². Importantly, TDP reduces the risk of introducing artefactual modifications by minimizing sample preparation and providing complementary structural information to conventional BUP²⁶³. Coupling with front-end separation approaches — such as HIC, RPLC, SEC or CE — is promising for ADC separation and drug conjugation site localization^{264–268}. The original TDP approaches applied to intact ~150 kDa mAbs analysis were based on CID and provided limited total sequence coverage (~10%)^{269–271}. A significant increase in the sequence coverage, up to 35%, was achieved by applying ETD to the intact murine and human IgG1 species²⁷². This advance motivated new developments in the mAbs TDP analysis, followed by application of ETD on other mass spectrometry platforms²⁷³, and alternative MS/MS approaches²⁵⁶.

Methods to enhance sequence coverage use a middle-down mass spectrometry approach, using a limited digestion of intact biomolecules to simplify the analytical challenges of characterizing large proteins^{274,275}. Compared with the intact mAbs analysis, middle-down approaches characterizing ~25 kDa antibody subunits — for example, Fd, Fc/2 and light chain — show substantially improved separation performance by RPLC, CE and CZE, yielding higher fragmentation efficiency and better product ion detection^{276–278}. Various MS/MS methods coupled with ion activation, either before or after the electron transfer/capture or ion–ion reaction, can enhance protein sequence characterization²⁵⁶. Including assignment of internal fragments also enhances TDP-derived mAb sequence coverage, as demonstrated by the analysis of intact NIST mAb, in which a sequence coverage of >75% was reported²⁷⁹. Including internal product ions also helps provide information about PTMs, intrachain disulfide bond connectivity, N-glycosylation sites and chain pairing²⁸⁰. Although IgG1 is the most frequently studied mAb in TDP applications, several works describe the analysis of IgG2, IgA and the MDa molecular mass IgM species²⁵⁹. These results suggest that TDP may be useful for de novo sequencing of mAbs, such as IgA1s from milk, saliva or serum.

Currently, TDP requires multiple targeted experiments on selected biopharmaceuticals using a combination of fragmentation methods and experimental parameters. When performing large-scale, proteomics-grade TDP analysis on biopharmaceutical, constraints of time, sample quantity and protein structure can substantially reduce spectral data quality and limit the obtainable sequence coverage²⁹. As a result, crucial information typically found at low abundance levels

worldwide. Alongside pandemics, antimicrobial resistance is continuing to spread. Alternative strategies to better detect, characterize and treat infectious diseases are urgently needed. Assessing proteoforms is a promising approach. The cause of cerebrospinal meningitis, *Neisseria meningitidis*, was found to have a specific Pile proteoform that is tightly associated with crossing the epithelial barrier and accessing the bloodstream²⁴⁸. Highly glycosylated Pile proteoforms are linked to immune escape²⁴⁹. For *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*, the most common foodborne pathogen, specific S-cysteinylation proteoforms were reported in response to infection-like conditions²⁵⁰. The large-scale analysis of bacterial proteoforms using TDP can also overcome the limitations of MALDI-TOF-MS, the method used in hospitals to rapidly identify bacterial pathogens and discriminate closely related bacteria²⁵¹. In a more straightforward approach, liquid extraction surface analysis mass spectrometry can identify ESKAPE pathogens directly from live cultures²⁵². For SARS-CoV-2, specific proteoforms of the nucleocapsid protein were found to bind viral RNA and exhibit significantly different interactions with IgM, IgG and IgA antibodies from convalescent plasma and could be candidates for immune-directed therapies²⁵³. For the same virus,

is not achievable in LC timescales. Developments to TDP methodologies, techniques, automation and data analysis are needed for broader adoption of TDP. In biopharmaceutical applications, examples in which TDP complements and exceeds the capabilities of the current gold standard – BUP, subunit and intact mass spectrometry – are needed for it to be used more widely.

Clinical TDP

Clinical TDP analysis at the proteoform level has been effectively implemented in many clinical laboratories, particularly to identify pathogens with MALDI-TOF-MS, which can rapidly detect proteoform profiles directly from an intact bacterial cell surface²⁸¹. This has resulted in commercialization of specialized MALDI-TOF-MS technologies, such as the Bruker biotyper and VITEK mass spectrometer, to establish a public health reference laboratory for identifying microorganisms with high throughput, accuracy and low cost²⁸². A large number of protein markers are tested in clinical laboratories, and proteoforms, which are influenced by pathophysiological conditions, are increasingly being recognized as holding important clinical diagnostic value²⁸³. In most cases, conventional clinical tests cannot resolve proteoforms as few clinical analytical platforms are compatible with molecular characterization of intact proteins. The promise of TDP in clinical diagnosis is shown by the identification of haemoglobin variants for haemoglobinopathy²⁸⁴ and the detection of monoclonal immunoglobulins for monoclonal gammopathy²⁸⁵. Specifically, TDP can accurately identify and characterize haemoglobin variants from clinical patient blood²⁸⁶, presenting advantages for diabetes diagnosis compared with conventional methods and next-generation gene sequencing²⁸⁴. TDMS was successfully applied to detect and characterize immunoglobulins (M-proteins) for plasma cell disorder diagnosis²⁸⁷. Additionally, TDMS can differentiate endogenous M-proteins from therapeutic mAbs in serum for accurate diagnosis, potentially replacing traditional methods of serum protein electrophoresis and immunofixation. The traditional techniques have limited resolution and cannot accurately monitor therapeutic response when the M-protein co-migrates with therapeutic mAbs²⁸⁸.

Proteoforms are important to understand disease and as prognostic biomarkers. This is illustrated in a report showing that monoclonal gammopathy of uncertain significance patients with glycosylated light chains has significantly increased risk of progressing to plasma cell dyscrasias in clinical pathologies²⁸⁹. As the role of proteoforms is better understood, the more TDP is expected to impact the clinical arena²⁹⁰. Although TDP technology is rapidly advancing in clinical settings, there is a limit to what can be achieved in clinical laboratories, even with advanced instrumentation. Efforts to improve TDP proteome depth and sensitivity will be needed to analyse low-abundance proteoforms and biomarkers from clinical samples. Automation and streamlining informatics are also required for TDP to be widely adopted in the clinic.

Reproducibility and data deposition

Reproducibility

Reproducibility of TDP data is critically important to ensure reliable, accurate proteoform annotations and for broader adoption of TDP in academia and industry. TDP is a relatively new field and, unlike the mature BUP approach, universally accepted experimental methods and data reporting standards have yet to be developed. Standardization efforts led by the CTDIP push for inter-laboratory comparisons to better understand challenges and improve reproducibility^{29,33}.

Proteoforms are susceptible to variations in sample handling and instrumentation methodologies, making scientific rigour and sufficient data reporting practices important. Appropriately detailed descriptions of sample preparation, separation methods and instrumentation parameters need to be given for reliable proteoform and PTM reporting. This is especially critical when reporting PTMs that are easily artefactually produced by variations in experimental design or instrument settings, such as oxidation²⁴⁰, non-enzymatic glycation²⁹¹ or labile PTMs, for instance, phosphorylation²⁹², palmitoylation²⁹³ and glycosylation^{112,254}. Standards for proteoform annotation and data reporting are continuously improving. Efforts to formally define a proteoform-level classification system²¹² develop a standardized lexicon for enhanced data reporting clarity¹², and multi-software tool comparisons²⁹ can define best practice in collection, reducibility and analysis.

Data deposition

All TDP data should be made publicly available. Many journals have implemented this requirement, but it will require a community effort to ensure proper data handling and reporting practices are enforced. As the TDP field is relatively new, there are few dedicated top-down data repositories. Instead, TDP data are often deposited in general proteomics repositories that are mainly formulated for BUP data sets: PRIDE (EMBL-EBI, Cambridge, UK)²⁹⁴, PeptideAtlas (ISB, Seattle, WA, USA)²⁹⁵, MassIVE (UCSD, San Diego, CA, USA)²⁹⁶, jPOST (various institutions, Japan)²⁹⁷, iProX (National Center for Protein Sciences, Beijing, China)²⁹⁸ and Panorama Public (University of Washington, Seattle, WA, USA)²⁹⁹. The [Proteoform Repository](#) at the CTDIP represents a unique hub for scientists to browse deposited proteoforms and contribute TDP data sets³⁰⁰. Data repositories are essential for TDP data to comply with the FAIR data deposition standards³⁰¹. New avenues and initiatives to platform TDP data sets and serve as central repositories will be extremely valuable to advance the accessibility and sharing of TDP data, which will in turn benefit the TDP field³⁰⁰.

Limitations and optimizations

TDP has grown rapidly owing to many new technologies and methods. Techniques are continuing to emerge, aiding analysis of complex protein mixtures, basic scientific research, new biomarker discovery and novel biological insights^{166,214,236,248,250,302–304}. However, challenges remain⁵, including protein solubility, proteome complexity, data analysis, connecting and establishing proteoform-to-function relationships and analytical throughput⁵. Although solutions are being developed, this section highlights limitations to demonstrate the assumptions underpinning TDP workflows, with strategies suggested to overcome current limitations.

High sensitivity

High analytical sensitivity is needed to analyse proteoforms from sample-limited biological systems. However, achieving high sensitivity is a major challenge in TDP. Conventional TDP workflows require a relatively large amount of starting sample – micrograms of total protein or millions of cells – for high-quality data and sufficient analyte signals for MS/MS¹²⁴. By contrast, the well-established BUP approach enables deep proteome coverage across many biological samples and can be performed with relatively low sample amounts (<200 ng)^{20,305,306}. The need for relatively large protein quantities is a major barrier when applying TDP in sample-limited biological settings, such as clinical samples and single cells. To address this, a high-sensitivity TDP method was

developed and used to identify proteoform variations in large proteins in individual muscle cells. This high-sensitivity approach enabled proteomics to be integrated with functional properties¹⁶⁵. Initially, CE-MS showed potential for high-sensitivity TDP analysis of single cells using an on-capillary cell lysis approach³⁰⁷. The nanoPOTS – nanodroplet processing in one pot for trace samples¹⁹⁶ – technology was originally developed for single-cell BUP and can be used for high-sensitivity TDP. Protein extraction can be enhanced with a combination of *n*-dodecyl- β -D-maltoside surfactant and urea³⁰⁸. This approach relies on specialized devices that are in the early stages of development. Despite this, high-sensitivity platforms have the potential to accelerate highly sensitive TDP applications, enabling routine single-cell TDP.

Large proteoform identification

High-molecular mass proteoforms are often under-represented in top-down data sets³⁰⁹. TDP has major limitations in the effective depth of proteome coverage owing to the large range of protein molecular masses within a proteome³¹⁰ and difficulties in effectively separating intact proteins before mass spectrometry. This challenge is compounded by the high dynamic range of the proteome, the exponential decay S/N of large proteoforms owing to increasing charge states from ESI, greater contribution of heavy isotopes at higher precursor mass and detrimental presence of smaller, coeluting proteoforms during the large proteoform analysis. In general, larger proteoforms (>30 kDa) tend to generate larger MS/MS product ions (>10 kDa), exacerbating the already high instrumentation burden of TDP. To analyse larger ions, ultrahigh resolution platforms, such as FTICR mass spectrometers³¹¹, may be required. Size-based fractionation methods with SEC or gel-based techniques, for instance, the integrative proteomics approach or PEPPI-MS, before mass spectrometry could address the challenge of large ion analysis^{81,83–85}. However, broad use of size-based fractionation is hindered by time-consuming sample processing and large sample requirements (typically >100 μ g). Advanced sheath-flow and sheathless interfaces have enabled wider application of CZE in TDP^{312–316}. Limited sample loading quantities constrain the total number of identifications attainable from the CZE analysis of protein mixtures³¹⁴. Obtaining sufficient fragmentation for large proteoform identification is also a challenge, especially in the chromatographic timescale of an LC-MS/MS experiment. Currently, no single separation strategy or MS/MS configuration can comprehensively resolve the entire proteome. Increasingly sophisticated instrumentation, new method development and improved informatics tools will be needed to address this challenge.

Tandem mass spectrometry of proteins

Protein fragmentation typically yields protein products with an N or C terminus^{317,318}. In general, protein fragmentation efficiency is higher towards either end of the sequence termini, whereas fragmentation coverage in the middle is limited^{319,320}. This discrepancy is more evident in larger proteins and is believed to arise from residual higher-order protein structures (secondary and tertiary) that persist even under denaturing conditions, restricting accessibility^{25,320}. Fragmentation depth in TDP is also constrained by the network of protein disulfide bonds²⁵⁶. For example, disulfide bond reduction before TDP facilitates protein unfolding and increases sequence coverage from regions previously shielded by disulfide bonds, such as the middle region³²¹. Cleavage events from the middle region often result in large product ions with lower S/N and isotopic resolution, complicating identification and characterization³²⁰. Secondary gas-phase dissociation of large fragment ions can hinder fragment ion identification but, in

certain cases, can also help uncover the restricted middle region³¹⁹. Internal fragments, which result from at least two backbone cleavages and do not have N or C terminus, are being increasingly considered in top-down fragmentation³²². As the molecular size of a protein increases, the total number of unique internal ions that can form increases markedly³²³. New methods and data analysis workflows that can accurately integrate internal fragmentation could enhance protein sequence characterization and proteoform annotation³²⁴. The recently developed TDP software, ClipsMS, can assign internal fragment masses to protein sequences, improving overall coverage depth^{325,326}. However, limitations remain, such as duplicated fragment assignment from identically matching fragment masses, lower statistical confidence in matching smaller internal fragments, no neutral losses assignments and lack of annotation for more diverse fragment types, namely, *c* + 1, *z* + 1 and *z* $\bullet$ fragments. Internal fragmentation assignments and new protein dissociation technologies³²⁷ are likely to improve the understanding of top-down fragmentation mechanisms and lead to new data analysis pipelines that can handle multiple fragmentation types.

Localization of specific modification sites

The TDP approach is the most practical method for characterizing proteoforms. Unlike BUP, which benefits from primary sequence simplification using enzymes and other cleavage methods²⁰, TDP lacks a straightforward and reliable way to resolve proteoform complexity. Experimental localization of PTMs and precise characterization of proteoform chemical composition are challenging²¹². Low-abundance proteoforms, such as those with phosphorylation, are often hindered by low sensitivity and limited retention of covalent phosphate linkages owing to labile PTMs³²⁸. Enrichment strategies can boost low stoichiometric or low abundance signals; however, addressing labile PTMs often requires optimization of the specific fragmentation method, for instance, by using a gentler electron-based method such as ETD or ECD³²⁹. Intact protein ions are less susceptible to cleavage of labile PTMs by CID, in contrast to peptides, potentially owing to protein ions retaining a degree of high-order structure in the gas phase²⁵. Studies have shown that targeted TDMS by ECD and ETD can effectively elucidate the primary sequences of biologically relevant proteoforms, particularly those with labile PTMs^{330–333}. A five-level proteoform classification system was proposed to clarify ambiguities in proteoform identification and compare results from different laboratories and techniques²¹². Beyond classification, localization of specific PTMs is also limited by the robustness of MS/MS spectra obtained for a given proteoform. It can be laborious, often requiring multiple fragmentation methods or internal fragment ion products, to achieve sufficient fragment ion complementarity for accurate PTM assignment, localization and sufficient protein sequence coverage^{279,334}.

Throughput and ease of analysis

The relatively low throughput and high data complexity of TDP are major barriers for both new and experienced users^{32,127}. With the exception of MALDI-TOF-based intact protein assays³³⁵, label-free discovery-based TDP has low measurement throughput, requiring time-consuming optimization of the whole workflow, particularly for sample preparation, separation and data analysis⁵. Detailed characterization of low-abundance membrane proteins³³⁶ and whole protein complexes³³⁷ is possible using a direct infusion approach. However, these direct infusion or injection methods require sophisticated hardware and robust analytical methods, limiting general applicability. Automated sample

preparation³³⁸ and separation systems would enhance the throughput, enabling broader application to complex biological systems^{339,340}. Currently, discovery-based TDP data processing includes deconvolution and database searches, which can take several hours to several days, depending on software performance and search parameters^{146,147}. This underscores the need for continuous benchmarking to systematically monitor and compare informatics across laboratories²⁹. Future developments in software and hardware are expected to enhance the throughput of TDP analyses. This will streamline experiments and alleviate computational burden associated with the data analysis. For example, by omitting the step of spectral deconvolution and directly matching expected features in experimental and simulated data, sensitivity and specificity of precursor and product ion analysis can be improved²⁸⁰. Continual developments in computational resources and programming capabilities will enable acquisition and processing of increasingly large data sizes (10–100 GB per single TDP experiment). This will enhance the achievable analytical performance to improve proteoform coverage depth and analysis ease.

Outlook

TDP is currently the only technology able to determine proteoform identities and quantify their abundances. The fundamental importance of proteoforms and their potential role as markers of cellular, environmental or biosystem health means that TDP technologies are expected to continue rapidly developing.

Two key areas to address are improving deep characterization of complex proteoform mixtures and the identification and characterization of larger proteoforms. An exciting development is single ion measurements, which can be implemented on existing commercial instruments and on specialized prototypes^{341–343}. However, this approach is limited by the fundamental constraints of single-molecule methods when sampling complex proteomes that have a range of protein abundances³⁴⁴. Efficient proteoform separation is particularly critical but remains underdeveloped. It is often challenging to implement separation globally owing to the large diversity in proteoform physicochemical properties. Recent advances in integrative proteomics approaches, liquid chromatography stationary phases, implementation of CE modalities, development of IMS-based separations and integration into multidimensional approaches will continue to improve proteome-wide measurements^{5,345}.

Opportunities are available by integrating top-down data flows with other data types, including genome and transcriptome sequences, BUP and glycomics³. Genome sequences are fundamental to proteomics, providing the gene models that underlie database construction and search algorithms used for proteomic identifications. Transcriptome information enables searches to focus on subsets of genes and RNA splice variants expressed in the tissues under study. Bottom-up data can further specify which proteins are present and the PTMs they contain. Protein glycoforms are among the most challenging^{112,346,347} but also the most functionally important proteoforms. There is a trade-off between constructing and searching against vast proteomic databases that contain all possible sequences and the cost of false identifications arising from an expanded search space. Integrating and using multiple data types to restrict the size of search databases while increasing their relevance to the sample is an emerging area for TDP optimization³.

One of the largest barriers for new TDP users is data analysis. The complex nature of MS1 and MS2 spectra makes them virtually impossible to decipher manually. Software is required to parse the data into a comprehensible result, but the relatively small size of the field has

limited the development of these tools. Proteoform quantification is more difficult than peptide quantification owing to the presence of many charge states and isotopologues, which result in lower S/N values. Turn-key tools that are intuitive and easy to use are urgently needed. Statistical methods that give confidence metrics for proteoform identifications are not well developed. All proteomic analyses are statistical in nature, and metrics such as FDR and posterior error probability are essential to interpret results. Further development of these areas will provide a foundation for expanded applications of TDP into the clinical and biopharmaceutical arenas.

As technology improves, important new application areas are becoming accessible. Single-cell proteomics is in its infancy but is already producing important new insights^{165,196,348–350}. Spatial biology³⁵¹ is close to understanding the mechanisms responsible for tissue and cellular organization. As the primary effectors of function, proteoforms determine cellular identities. However, measuring proteins and proteoforms in single cells or with near-single-cell resolution is a major challenge. Current approaches typically rely on labels or antibodies, which are limited in availability and specificity⁵⁹. They also require a priori knowledge of protein targets and are only able to provide a restricted view of what is present. Although BUP has been demonstrated for proteome-wide spatial profiling of tissue sections when coupled with laser capture microdissection³⁵², the approach uses peptides as a proxy for proteins and cannot characterize proteoforms. Extending single-cell and spatial measurements to the proteoform analysis will become possible by combining advanced technologies, such as microfluidics, mass spectrometry imaging and single ion measurements^{165,247,308,353–355}.

New proteomic platforms have emerged, adopting concepts pioneered in next-generation sequencing of nucleic acids³⁵⁶. Nanopore sequencing of proteoforms is being developed³⁵⁷, and several companies are exploring how to fabricate and interrogate complex protein and peptide arrays for target proteomes. Once a proteoform database is constructed for a system of interest, data produced by next-generation platforms can be searched against the database, transforming proteoform identification from a discovery process to a scoring process.

Although proteoforms offer unique insights into cellular processes, alone they cannot provide a biological interpretation. Integration with other omics measurements is necessary to link proteoforms to related measurable outputs – for example, transcripts and metabolites – and decipher the basic principles of biology. One example is the recently introduced nanoSPLITS (nanodroplet SPLITting for Linked-multimodal Investigations of Trace Samples) technology, which enables parallel transcriptomics and BUP from the same single cell³⁵⁸. With single-cell proteoform measurements rapidly emerging, technologies will expand to give an unprecedented view of transcripts, proteins and proteoforms in single cells. These exciting multiomics developments promise to bring a new era of biological prediction and control.

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Author contributions

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Competing interests

J.A.L., J.C.-R., J.N.A., L.P.-T., L.M.S. and Y.G. are currently board members of Consortium for Top-down Proteomics. Y.O.T. is an employee of Spectroswiss, a company that develops data acquisition systems and data processing software for mass spectrometry. X.L. has a project contract with Bioinformatics Solutions Inc., a company that develops data processing software for mass spectrometry. D.S.R. and Y.G. are named as inventors for the patent application US Patent App. 17/786,482. L.P.-T. is named as an inventor for the US Patent App. 17/954,834. Y.G. is named as an inventor for the US Patent App. 18/069,005; US Patent App. 17/978,793; US Patent App. 18/451,614; and US Patent 11,567,085. S.W. declares no competing interests.

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