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Review Article

Emerging mass spectrometry-based proteomics methodologies for novel biomedical applications

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Research into the basic biology of human health and disease, as well as translational human research and clinical applications, all benefit from the growing accessibility and versatility of mass spectrometry (MS)-based proteomics. Although once limited in throughput and sensitivity, proteomic studies have quickly grown in scope and scale over the last decade due to significant advances in instrumentation, computational approaches, and bio-sample preparation. Here, we review these latest developments in MS and highlight how these techniques are used to study the mechanisms, diagnosis, and treatment of human diseases. We first describe recent groundbreaking technological advancements for MS-based proteomics, including novel data acquisition techniques and protein quantification approaches. Next, we describe innovations that enable the unprecedented depth of coverage in protein signaling and spatiotemporal protein distributions, including studies of post-translational modifications, protein turnover, and single-cell proteomics. Finally, we explore new workflows to investigate protein complexes and structures, and we present new approaches for protein–protein interaction studies and intact protein or top-down MS. While these approaches are only recently incipient, we anticipate that their use in biomedical MS proteomics research will offer actionable discoveries for the improvement of human health.

Introduction

Developments in both mass spectrometry (MS) hardware and software within the last decade are opening new avenues for the quantitative investigation of proteins involved in human health and disease. Both absolute and relative quantitative measurements, reviewed in detail elsewhere [1], are possible using commonly applied MS acquisition methods. The choice of MS acquisition method influences selectivity, reproducibility, repeatability, limit of detection, dynamic range, and data density [2]. Additionally, the variety of acquisition types places specific requirements on experimental design and strongly influences the computational strategy for analyzing data. A comparison of these different workflows is presented in Figure 1 featuring some of their unique strengths as discussed in detail below.

Generally, MS acquisitions fall into two categories defined by how the mass spectrometer acquires precursor ion scans (MS1) and fragment ion scans (MS/MS or MS2), resulting in either workflows that require no prior knowledge about the proteins in the sample (discovery-based proteomics, sometimes referred to as 'shotgun' proteomics) or workflows that rely on a hypothesis to guide the acquisition (targeted proteomics). The discovery acquisition workflow, also referred to as 'data-dependent acquisition' (DDA), is defined by how the instrument selects the top N most abundant precursor ions (MS1) for fragmentation and MS/MS analysis during each scan cycle. It typically acquires just one MS/MS spectrum for each precursor ion, as precursor ions are usually dynamically excluded after selection for MS/MS. This results in an analyte-dependent 'sampling' and analysis of the peptides in a proteome. These DDA protein discovery experiments can also quantify proteins, when taking advantage of the quantitative MS1-scan signal by extracting the MS1 precursor ion chromatograms that some studies refer to as

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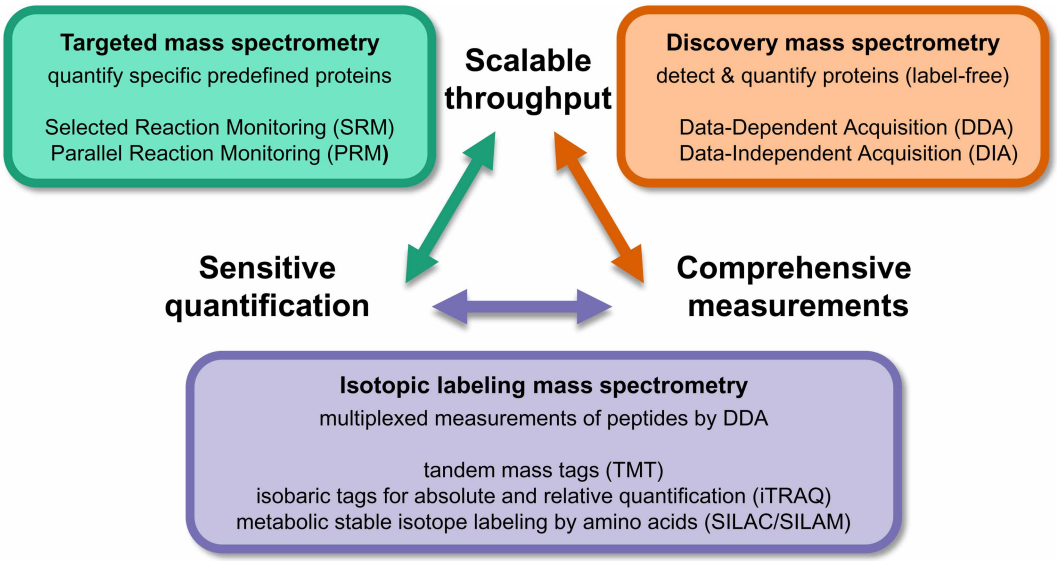

Figure 1. Comparison of MS acquisition methods for proteomics.

Protein samples can be analyzed by mass spectrometry using conceptually different methodologies which each require their own unique experimental design. These workflows feature differences in the scalability of sample throughput (the number of samples in a given experiment), in the comprehensiveness of protein and peptide measurements (the number of proteins/peptides that can be detected and quantified), and finally the sensitivity of quantification (the lowest amount of protein/peptide that the method can reliably measure). Targeted mass spectrometry workflows include SRM and PRM (green box) and can measure many samples using optimized assays with deep quantitative sensitivity and accuracy. Label-free discovery mass spectrometry workflows (orange box), such as DDA and DIA may feature overall lower sensitivity for quantification, however, allow to measure proteins more comprehensively in an unbiased approach. DDA workflows are often augmented by sample preparation methods referred to as 'isotopic labeling' which feature good quantitative sensitivity and preserve comprehensive acquisitions but are challenged by the maximum number of samples easily achievable in a quantitative experiment.

MS1 filtering [3] or label-free quantification [4]. Alternatively, DDA workflows are often combined with stable isotope labeling techniques to add a quantitative dimension. For example, stable isotope labeling with amino acids in cell culture (SILAC) incorporates heavy isotope lysine and arginine amino acids during cell culture [5]. Isobaric tagging methods, such as tandem mass tagging (TMT) [6,7] (discussed in more detail below) and isobaric tag for relative and absolute quantitation (iTRAQ) [8], are popular for their ease of implementation and amenity to multiplexing samples. Although DDA methods are straightforward to implement and a wealth of analytic software is available for data processing, the stochastic sampling characteristic of DDA often fails to trigger MS/MS spectra acquisition reproducibly from sample to sample, even comparing technical replicate acquisitions, posing challenges for quantification and missing some analytes entirely. On the other hand, several hypothesis-driven, targeted acquisition methods are available. Selected reaction monitoring (SRM) [9], also known as multiple reaction monitoring (MRM) [10], is performed on triple quadrupole-type mass spectrometers. More recently, parallel reaction monitoring (PRM) [11–13] involves high-resolution instruments, such as quadrupole orbitrap MS or quadrupole time of flight (QTOF) instruments. SRM/MRM and PRM methods acquire peptides based on a pre-programmed list of analytes that subsequently allow the use of fragment ions (MS2) for accurate quantification and often results in higher specificity and selectivity. A large variety of informatics tools have been developed to aid in assay development and to process the data collected by various acquisition types [14,15]. Novel bioinformatics tools include quality control algorithms [16], *de novo* peptide sequencing [17], and web-based resources for dissemination of results [18].

In this review, we examine the latest advances in the field so that biomedical researchers can quickly familiarize themselves with the newest proteomics workflows (Figure 2) that may be best suited for a given biological project.

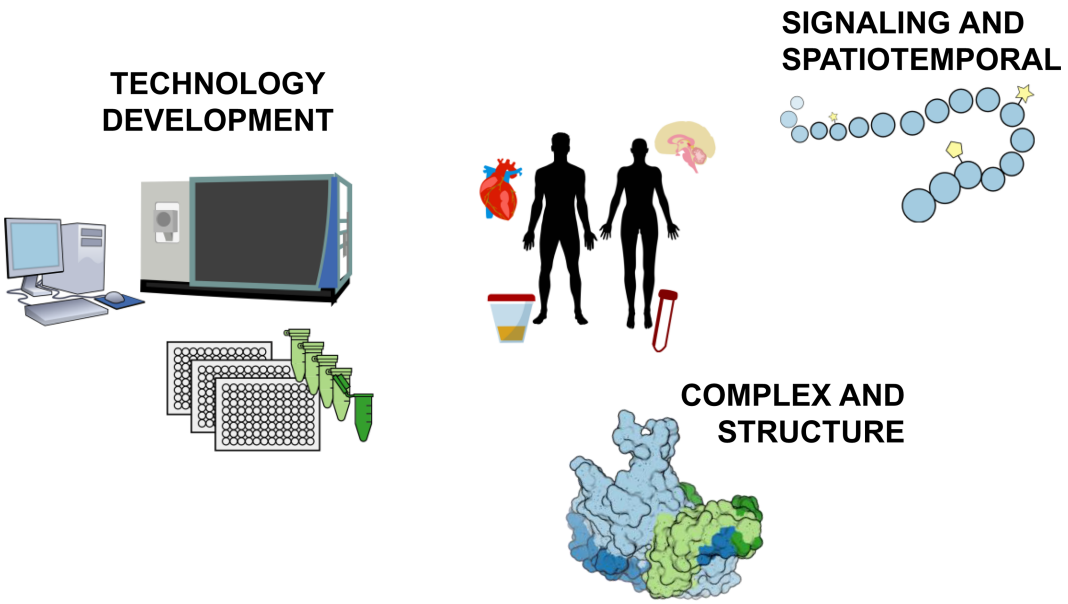

Figure 2. Biomedical research applications supported by recent MS-based proteomics technological advances.
(clockwise from left) Technological advances in data acquisition and throughput have improved the ability to profile the proteomes of biological samples deeply and accurately, including clinical applications. New approaches for signaling and spaciotemporal protein dynamics improve the detection and quantification of post-translational modifications, proteostasis, and even enable single-cell proteomics. Finally, methods for studying protein complexes and structure facilitate studies of protein–protein and protein–drug interactions.

Technological developments in mass spectrometry

New data acquisition innovation

Data-independent acquisitions (DIA) [19] or SWATH [20,21] is a systematic MS acquisition method that uses wide, nonspecific precursor isolation windows to activate all ions for collision in a given *m/z* range. Detailed reviews of DIA methodology can be found elsewhere [22,23], including peptide-centric approaches to DIA [24].

One advantage of DIA/SWATH MS over other methods is the resulting comprehensive and reproducible quantification of proteomes, making DIA/SWATH an attractive choice for broad quantitative studies of basic disease biology [22–25]. Machine learning models have alleviated earlier data analysis reliance on prior knowledge, specifically spectral libraries, by predicting MS/MS fragmentation spectra that allow researchers to build spectral libraries *in silico* [26,27] and the 'chromatogram library' approach for refining those predicted spectral libraries [28]. While there are several popular software options for analyzing DIA data [28–32], benchmark comparisons have thus far found the results comparable [33], freeing researchers to use whichever software they find most convenient. DIA/SWATH applications have been applied broadly wherever high-dimensional proteome quantification is interesting, including cancer biology [34], trisomy 21 Down Syndrome [35], differentiation [36], and stem cell biology [37].

Recently, an additional gas-phase separation methodology 'trapped ion mobility spectrometry' (tims) was combined with a fast scanning QTOF mass spectrometer, and the timsTOF PRO platform allows for novel acquisition methods called 'parallel accumulation–serial fragmentation' or PASEF [38] where the release of precursor ions can be synchronized with the quadrupole selection for fragmentation. Combining PASEF with DIA, diaPASEF [39] subsequently reduces complexity and depth of coverage as well as maintaining quantitative accuracy. Unlike typical DIA approaches that are based predominantly on chromatographic separation, diaPASEF suggests extending the comprehensive, systematic sampling of precursor ions by additional and orthogonal peptide separation applying ion mobility.

Advanced isotope labeling strategies

In addition to the label-free workflows described above, stable isotope labeling techniques have also improved, specifically allowing for increased multiplexing. Metabolic labeling such as stable isotope labeling with amino acids in cell culture/mammals (SILAC/SILAM) [5,40] and isobaric tagging methods such as TMT [6,7] and

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iTRAQ [8] are commonly used workflows. Label-based methodologies are useful as a multiplexing technique as shown in Figure 3. It allows the pooling of individually tagged/labeled samples to provide quantitative information by using isotope mass shifts or reporter ion signals that reflect the relative abundance of the original individual samples [41]. This process mitigates nonspecific and acquisition-specific artifacts that may occur with multiple acquisitions. SILAC/SILAM methods use metabolic labeling (typically heavy lysine and/or heavy arginine residues), where isotopes are incorporated into proteins *in vivo*, then protein lysates are mixed at equimolar ratios prior to downstream sample processing. Thus any downstream processing variability does not affect the ratios between the investigated conditions/samples. For isobaric labeling approaches typically 10–11 samples are lysed and digested individually, subsequently reacted with the labeling reagents (e.g. TMT/iTRAQ), then mixed at equimolar ratios (Figure 3).

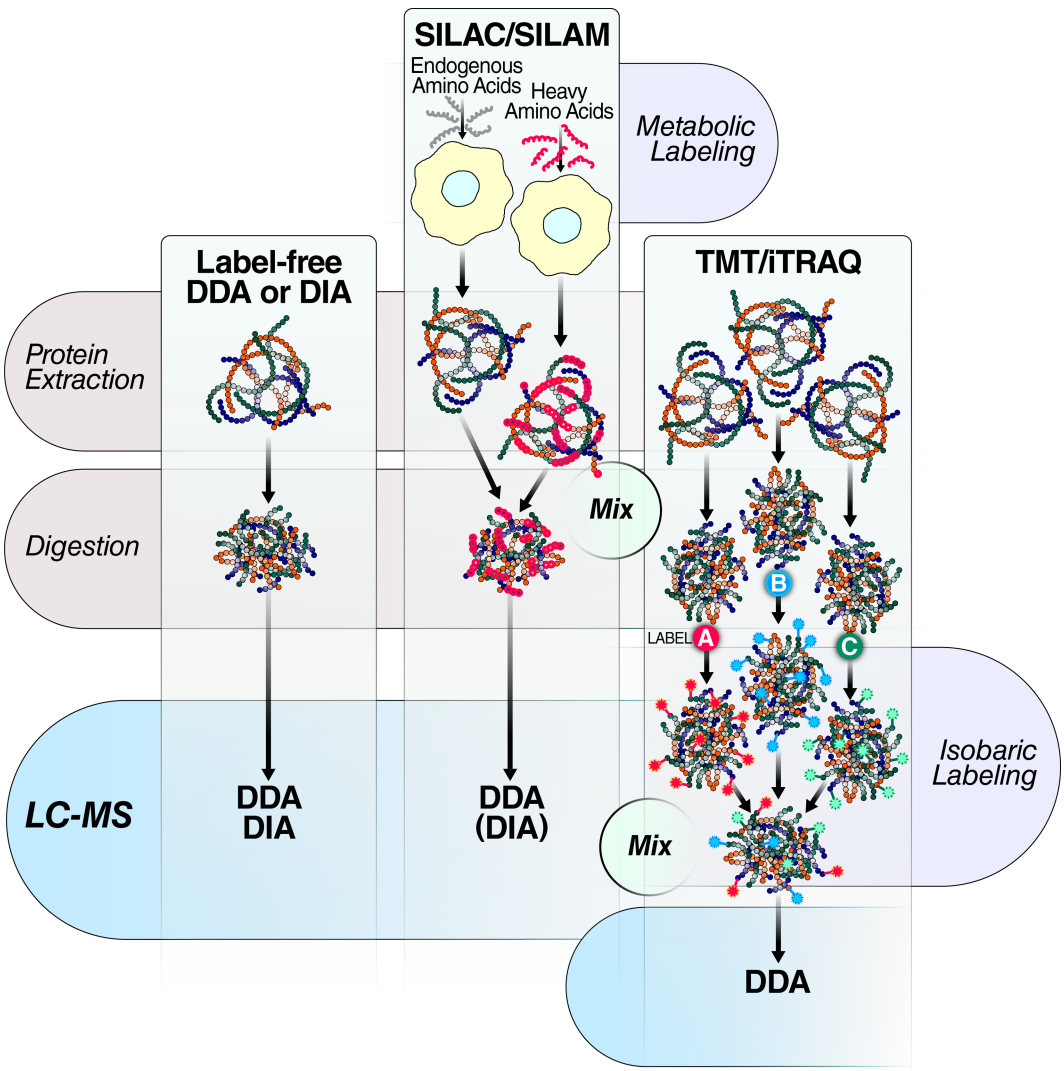

Figure 3. Overview of various comprehensive quantitative proteomics workflows.

The shared workflow for most quantitative proteomics experiments typically involve protein extraction, proteolytic digestion, and finally analysis by LC–MS using either DDA or DIA acquisitions. The most general workflow, *label-free DDA or DIA*, requires no additional sample processing (*left*). *Metabolic labeling strategies* like SILAC/SILAM require that the heavy isotope label is incorporated into the metabolically active cells or organisms. After harvesting and lysis cells different samples are mixed and then digested and processed together prior to DDA or in some emerging cases DIA analysis (*middle*). *Isobaric labeling strategies*, such as TMT and iTRAQ, require each sample in the multiplex to be individually digested and subsequently be reacted with the specific chemical label, prior to mixing all samples and DDA analysis *(right)*.

With the latest advances for TMT, researchers can now even label up to 16 distinct samples with different TMT tags [42], pool them, and subsequently analyze them all in a single acquisition. Multiplexing can be increased to even higher levels [43] in anticipation of newer reagents in the future. Isobaric labeling strategies TMT workflows and the various improved generations of reagents have greatly improved MS multiplexing capabilities for relative quantification of different experimental conditions [44]. Proteomic depth of coverage is often achieved by off-line separation before MS analysis, at the cost of slower throughput.

However, one challenge to using TMT has been recognized and is referred to as ratio compression. To overcome these problems, additional elegant MS methodologies and scan sequences were developed. For example, interference in the TMT reporter ion region can be reduced by TMT-MS3 workflows in which TMT-labeled MS2 fragment ions are selected for further fragmentation in the ion trap (MS3) [45]. This workflow was further improved by a MultiNotch MS3 method that uses isolation waveforms with multiple frequency notches (i.e. synchronous precursor selection, SPS) to co-isolate and co-fragment multiple MS2 fragment ions, thereby increasing the number and intensity of reporter ions in the MS3 spectrum [46]. The TMT-SPS-MS3 workflow has gained interest, and several high-impact biological studies recently used this technology [47–50]. The throughput and depth in proteome coverage were showcased in a recent murine tissue-based study quantifying over 11 000 proteins [49]. TMT-SPS-MS3 analysis workflows have also been applied to large-scale phosphoproteomics projects (also called SL-TMT for streamlined TMT) [49], and a recent large analysis of mouse proteome tissue specificity [51]. Notably, modifications to isobaric labeling improved its efficiency so that it requires significantly less reagent. The significant reduction in the cost per acquisition make it more likely that TMT will be adopted in clinical tests or patient-specific oncological mapping [52].

Improvements in sample separations and throughput

MS proteomics is usually paired with chromatographic separation, which reduces sample complexity and therefore improves the depth of proteome coverage. Liquid chromatography (LC) can be performed 'off-line' prior to analysis by mass spectrometry or 'on-line' because it is relatively straightforward to connect a LC to a mass spectrometer and ionize the eluting analytes in the ion source via electrospray ionization (ESI). Off-line fractionation is commonly performed for peptides, especially for multiplexed isotopically labeled samples (discussed in more detail above), using basic reverse phase (BRP; also called high pH reverse phase, HPRP) fractionation to separate peptides by hydrophobicity prior to analysis by on-line LC and MS [53]. While on-line LC at nanoliter flow rates (nanoLC) is commonly used because it requires less input material and may be more sensitive than higher flow rates (e.g. microflow LC), nanoflow is often considered less robust and potentially less reproducible. Modern LC systems combine the sensitivity of nanoLC with the robustness of microLC [54]. In the search for new biomarkers, the scale of proteomics experiments has grown exponentially and the robustness of microLC has been increasingly applied to improve throughput to thousands of biomedical samples [55,56].

While high-performance LC (HPLC) has been the separation method of choice for MS workflows, other separations developments are also improving the depth and breadth of peptide and protein identification from complex biosamples. A new data acquisition technique, BoxCar [57], improves precursor ion dynamic range and signal-to-noise ratios by first profiling the peptide precursor ions in a sample and then filtering the ions into segments of m/z ranges ('boxes') so that each MS1 segment shows an equal representation of ions across the full precursor range. With this methodology, Meier et al. [57] detected more than 10 000 proteins in only 100 min with sensitivity into the low-attomolar range. While BoxCar approaches were originally developed to augment DDA scan types, the principle can also be applied to DIA [58].

High-field asymmetric waveform ion mobility spectrometry (FAIMS; also called differential ion mobility spectrometry, DMS) can replace LC prior to introduction to the MS. FAIMS/DMS often separates analyte peptides or PTM isoforms that otherwise would co-elute and improves MS quantification by decreasing interference. FAIMS has been used to extend the dynamic range and accuracy of TMT-labeling quantification [59] and to reduce the length of LC gradients without sacrificing proteome coverage [60]. Another type of ion mobility mentioned above in the context of DIA, trapped ion mobility spectrometry (TIMS), provides capabilities to trap and release ions in a mobility-selective manner, thus categorized as a 'Confinement and Selective Release' methodology, while FAIMS and DMS approaches are considered and categorized as 'Spatially-Dispersive' ion mobility separation techniques. Briefly, a parallelizable 4D feature detection algorithm extracts peaks efficiently, and a new algorithm implemented in the free-to-use software MaxQuant called matching between runs (MBR) utilizes collisional cross section (CCS) values of MS1 features which significantly gains specificity from the extra separation dimension [61].

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Advances in signaling and spatiotemporal proteomics

Post-Translational modifications

Post-translational modifications (PTMs), such as acetylation and phosphorylation, are widely used to regulate biological processes as recently comprehensively reviewed by Doll and Burlingame [62]. However, PTM analysis is challenging because the endogenous abundance PTM-containing peptides is typically much lower than that of unmodified peptides. Therefore, the modified peptides must be enriched from cells or tissues before MS analysis. Many PTM enrichment strategies involve antibody-based affinity purification (discussed below), which typically requires large amounts of starting material. Isobaric chemical tags, particularly the TMT method discussed above, are highly sensitive. They require less input material than conventional approaches for PTM analysis and have become a popular choice for studying modifications [7]. However, there are challenges. For example, ubiquitin remnants are chemically altered by the reagents, and thus, combining these two approaches for these types of studies has been difficult. One solution is the ubiquitylation enrichment protocol UbiFast. It labels the ubiquitin remnants (K-ε-GG) while they are bound to the anti-ubiquitin antibody and not exposed to the solvent. This method requires half the starting material and quantifies thousands more ubiquitylation sites than conventional approaches [63].

In addition, computationally detecting PTMs in mass spectrometry data is challenging. To solve this problem, researchers often perform an initial analysis with synthetic peptides. For example, the ProteomeTools project [64] aims to systematically characterize the human proteome by synthesizing over a million peptides, with initial data for 5000 peptides carrying 21 different modifications. Similarly, methods utilizing prior knowledge of the glycan structures released from glycoproteins are supporting glycomics studies [65]. A new informatic approaches called 'open modification searching' (OMS) focus not on specific predefined PTMs, but rather it detects shifts in peptide and fragment ion masses, allowing for the discovery of any possible PTM present in the data [66–68]. Although a powerful approach, OMS analyses often remain difficult to interpret biologically.

As these advances make PTM-centric experiments easier, efforts to describe the biomedical causes and consequences of protein modifications are increasing. For example, recent applications of PTM-centric mass spectrometry studies include the acetylome effects of deacetylase SIRT5, implicated in maintaining mitochondrial function during acute kidney injury [69]; malonylation and crotonylation have functions in inflammatory signaling [70] and regulation of chromatin remodeling [52], respectively.

Approaches for studying proteostasis by protein turnover

Healthy proteostasis requires that proteins are synthesized and degraded at appropriate rates [71]. Newly synthesized proteins are measured by incorporating uncommon but metabolically viable amino acids, allowing researchers to elucidate how often specific proteins are synthesized and degraded. The most conventional experimental design for cell culture, commonly called pulse SILAC (pSILAC), uses media containing stable isotope-labeled amino acids, such as heavy arginine and heavy lysine. Interest in protein turnover for more complex model systems has reintroduced stable isotope labeling of amino acids in mammals [72]. Commercially available mouse chow containing the isotopes has been used with a focus on proteostasis in the brain [73,74].

In addition to pSILAC, non-canonical amino acid incorporation has emerged as a tool to elucidate tissue-specific protein turnover by coupling a mutant tRNA synthetase engineered to incorporate a specific non-canonical amino acid with a cell-type-specific driver [75]. Notably, when designing experiments with non-canonical amino acids, it is important to determine if the introduction of those amino acids causes unintended changes to the model system being studied [76].

Single-cell proteomics

The amount of input material required for MS-based proteomics has made it unsurprisingly challenging to perform single-cell proteomics with MS. Perhaps the most straightforward single-cell approach to conventional bottom-up MS proteomics is to simply limit sample exposure to plastics. Coupling microfluidic chips to MS is not a recent innovation; in fact, the first microfluidics approaches for MS were introduced over two decades ago [77,78]. The rise of droplet-based microfluidics has emerged as a powerful tool in single-cell proteomics. Among the first nanodroplet-based devices for MS proteomics, nanodroplet processing in one pot for trace samples (nanoPOTS) measured over 3000 proteins from 10 cells using specially fabricated devices but conventional reagents and data analysis software [79]. Despite the impressive detection and quantification metrics reported by nanoPOTS, the lack of a commercially available nanoPOTS chip has reduced the adoption of the

technique. nanoPOTS was followed up with an alternative approach using commercially available consumables, termed microPOTS [80], which can profile proteomes from as few as 100 cells but does not reach the same sensitivity as the nanoPOTS protocol. In contrast, another single-cell proteomics approach, Single Cell ProtEomics by Mass Spectrometry (ScOPE-MS) [81], employs TMT labeling to achieve single-cell sensitivity. ScOPE-MS creatively addresses two challenges. It reduces input loss by mixing the single-cell peptides of interest with so-called 'carrier peptides', and it uses acquisition methods that maximize ion transmission to reduce the limitations of instrument sampling [82]. Yet another single-cell method combines these two approaches: nanoPOTs droplet-based sample preparation and ScOPE-MS's isobaric tagging signal boosting [83].

There has not yet been a study to benchmark the detection sensitivity, precision, or quantitative accuracy of the various single-cell proteomics techniques, and so it is difficult to draw comparisons. As of this review, despite the functional importance of proteins, no single-cell proteomics technique is yet able to match the reproducibility or scale of sequencing-based analyses, such as sc-RNA-Seq, and adoptions of these approaches have been limited in part due to the high specialization and cost associated with droplet-based sample preparation and TMT tagging. However, beyond single-cell proteomics, methods to restrict MS analysis to specific parts of a cell or tissue are incredibly valuable and provide more accurate descriptions of cell function [84]. Subsampling techniques, such as fluorescence-activated cell sorting (FACS) [85,86] or laser-capture microdissection [87], are used to decrease the total input amount while simultaneously selecting for homogeneous cell populations. For example, the combination of precise dissections, FACS, and proteomic analysis has been used to garner the most accurate and delineated proteomes in the brain [88].

Imaging mass spectrometry

Imaging mass spectrometry (IMS) is a robust label-free technique that enables users to visualize and characterize a broad spectrum of biomolecules, ranging from metabolites to complex peptides and protein formations and based on their spatial molecular distribution. Unlike traditional imaging methods, it allows the study of complex peptides and proteins without the need for existing knowledge of analytes [89–91]. The ionization technique for IMS is different than that of the ESI method described earlier. While multiple ionization technologies are compatible with IMS, matrix-assisted laser desorption/ionization-mass spectrometry is a popular choice. It allows multiplex analysis and can be used to image a variety of biomolecules in the same tissue section simultaneously [92]. Technological advances in IMS now allow comprehensive study of complex 3D cell-culture models, providing scientists the ability to effectively measure the efficacy of drugs before treatment and to observe the time-dependent penetration of chemotherapy drugs into tumor tissues [90]. An area of rapid development in IMS is data processing and statistical analysis [93–95], drawing newcomers to the IMS field by easing the burden of data analysis. Despite these computational advances, in-depth sample preparation protocols are needed for IMS to be utilized in more laboratories [91].

Innovations in mass spectrometry-based protein complex and structure studies

Several general approaches detect and quantify protein–protein interactions, including size-exclusion chromatography (SEC), affinity purification, and proximity labeling (PL). A foundational review of these high-throughput technologies for protein interaction networks and others is found elsewhere [96]. Below, we focus on the most recent advances in these three approaches and highlight their differences.

Size-exclusion chromatography

SEC itself was introduced half a century ago [97], but coupling SEC with MS for high-throughput analysis of protein complex components has only emerged in the past decade [98–100]. These approaches typically relied on label-based quantification strategies due to MS limitations; however, even more recently, advancements in computational proteomics have improved SEC-MS. One such workflow, hyperLOPIT, incorporates advances in labeling technologies discussed above to improve the spatial resolution of organelle proteomes [101]. Another computational analysis employs hierarchical clustering to group proteins with similar SEC elution profiles, so that identified proteins form the leaves of a dendrogram and protein complexes are determined by cutting the dendrogram at different levels [102]. A third method uses a semi-supervised support vector machine classification to determine protein complexes, taking as input co-fractionation data and additional information, such as published PPI datasets, co-evolution based on the assumption that true protein complexes will have conserved

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sequence and function, and other metrics that describe protein complexes [103]. A third example, CCprofiler [104], introduces a novel 'complex centric' analysis, which is the first to control error rates for protein complex analysis by using a statistical target-decoy method and also makes use of SWATH acquisition (discussed above) to improve the quantitative accuracy of SEC-MS.

Affinity-purification mass spectrometry

Affinity-purification mass spectrometry (AP-MS) is another well-established method for probing protein–protein interactions, in which antibodies raised against a protein of interest to co-immunopurify the protein and its interactors then analyze the enriched proteins by MS (Figure 4). Alternatively, epitope tagging can be used [105]. A monumental effort by the BioPlex project built tagged versions of, performed affinity purification for, and reported the PPI for nearly every protein in two cell lines [106]. Another application of AP-MS is in immunopeptidomics for novel vaccine development, where researchers seek to identify peptide antigens by purifying naturally processed peptide complexes with the major histocompatibility complex and analyzing the released peptide antigens by MS [107,108].

Proximity labeling mass spectrometry

Finally, protein engineers have developed complementary means for localizing proteins and their interactors by fusing proteins of interest with PL enzymes (Figure 4). These enzymes chemically modify nearby proteins within a certain labeling radius with moieties, such as biotin, which is enriched for using streptavidin and analyzed by MS. The PL enzymes BioID [109], BioID2 [110], and APEX [111] have been successful in a variety of subcellular localization experiments, reviewed in detail elsewhere [112–114]. To address labeling efficiency limitations, recent

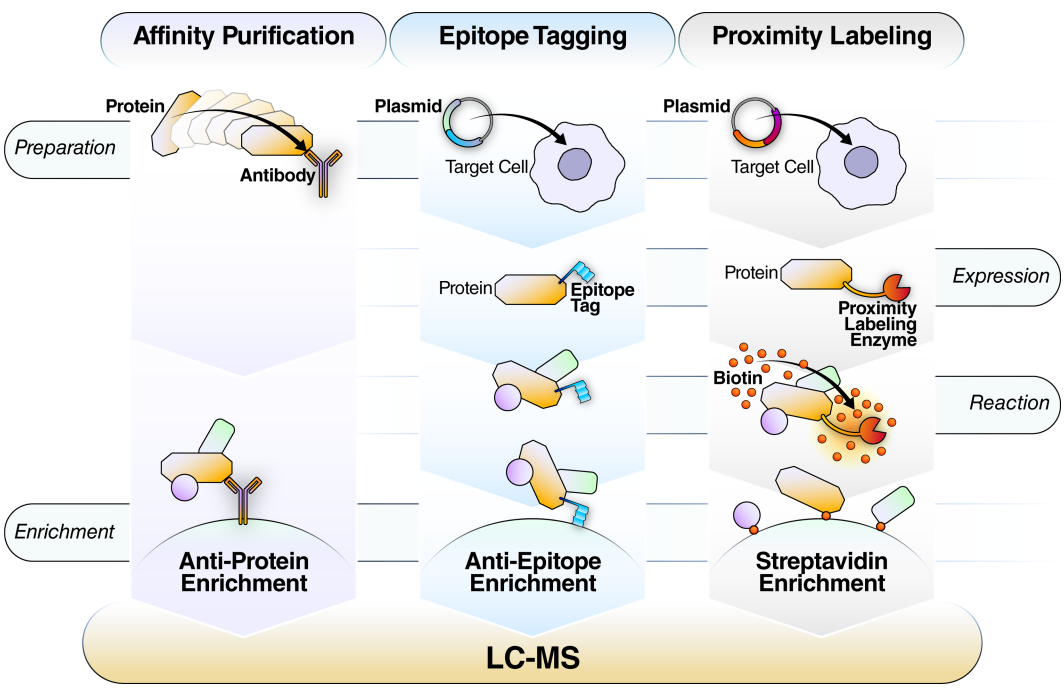

Figure 4. Strategies for assessing protein–protein interactions by mass spectrometry.

The common goal for protein–protein interaction studies involves enriching and purifying a target protein of interest (the bait protein is indicated in yellow) together with its protein network/protein complex prior to identification by LC–MS. *Affinity Purification*: antibodies specific to the protein of interest typically are coupled to beads and are used to enrich the protein complex directly (*left*). *Epitope Tagging*: a plasmid construct with the gene of interest tagged with a common epitope is engineered, subsequently, the tagged protein is expressed in the model system, enabling the use of common affinity enrichment systems like streptavidin, anti-HA, or anti-FLAG (*middle*). *Proximity labeling*: a plasmid construct with the gene of interest fused to a proximity labeling enzyme such as APEX or BioID is engineered allowing for the covalent biotin labeling of any protein within a predetermined vicinity of the expressed fusion construct, followed by enrichment with streptavidin beads (*right*).

work in protein engineering produced the more efficient PL enzymes TurboID and miniTurbo [115]. Adoption of PL into the community is supported by plasmid and lentiviral tool kits [116]. PL-MS studies primarily employ DDA techniques to identify proteins and typically use label-free spectral counting for protein quantification, although SILAC [117,118] and targeted approaches [119] have been used to improve quantification.

Hydrogen-deuterium exchange mass spectrometry

In addition to PPI analyses, purified proteins obtained through any of the above techniques can themselves also be analyzed by MS to determine their combinatorial PTMs and their structures using hydrogen-deuterium exchange mass spectrometry (HDX-MS) [120]. In this highly technical procedure, the purified protein is exposed to deuterated water to exchange solvent-accessible hydrogens with deuterium, causing a mass-shift that is detected by MS. Developments in microfluidics [121] and nano-electrospray theta-capillary technologies [122] improved upon back-exchange issues. Although HDX-MS is relatively challenging, it has useful biomedical applications in pharmacological drug discovery and development to determine structure-activity relationships of small molecules and their protein targets and to classify ligands by their functional selectivity [123–125].

Top-down mass spectrometry

Although the approaches described above all employ 'bottom-up' MS methods in which proteins are enzymatically digested prior to analysis, 'top-down' MS [126] is also used in protein interaction studies, especially in the context of determining proteoforms [127] and complex stoichiometry. Unlike the bottom-up approach that measures peptides not proteins, top-down mass spectrometry preserves all the structural information at the protein level, such as allelic variance, transcript splicing, and combinatorial PTMs. While top-down proteomics relies on the prefractionation of complex samples to purify the protein or complex of interest, new separations approaches, such as modified SEC purification techniques [128] and GELFrEE [129], preserve proteins in their native form. Native mass spectrometry, in contrast with most denaturing top-down proteomics approaches, is performed under native conditions, specifically at pH 7, to preserve protein complexes, capturing information about component stoichiometry and the spatial organization of those components [130]. Advances in instrumentation [131,132] and informatic analysis [133] eased the entry barrier for performing top-down and native proteomics.

Conclusion

With modern advances in mass spectrometry and new instrumentation features, novel workflows now enable previously impossible biomedical applications. Even a few years ago, PL or single-cell proteomics seemed distant future but are now becoming much more streamlined. We also anticipate that many new technological innovations will be implemented in new generation mass spectrometers in the next coming years.

Perspectives

- MS-based proteomics fills the need to reliably ascertain information about a sample; the desired information guides the selection of the methodology used. Now more than ever, MS methods have a range of capabilities broad enough to support just about any proteomics investigation.
- A common theme in the efforts to improve MS techniques is to combine two or three methods and optimize them so they can provide better results in tandem than either one can provide separately. Another theme is to push the limits of a single method in terms of its reagents, machines, and technique capabilities.
- Ultimately, in the future, we envision widespread implementation clinical MS and the development of personalized medicine employing these MS-based proteomics technologies.

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Competing Interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Abbreviations

DDA, data-dependent acquisition; DIA, data-independent acquisitions; DMS, differential ion mobility spectrometry; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FAIMS, high-field asymmetric waveform ion mobility spectrometry; HDX-MS, hydrogen-deuterium exchange mass spectrometry; IMS, imaging mass spectrometry; LC, liquid chromatography; MS, mass spectrometry; nanoPOTS, nanodroplet processing in one pot for trace samples; PASEF, parallel accumulation–serial fragmentation; PL, proximity labeling; PRM, parallel reaction monitoring; pSILAC, pulse SILAC; PTMs, post-translational modifications; QTOF, quadrupole time of flight; ScOPE-MS, Single-cell ProtEomics by mass spectrometry; SEC, size-exclusion chromatography; SILAC, stable isotope labeling with amino acids in cell culture; SPS, synchronous precursor selection; SRM, selected reaction monitoring; TIMS, trapped ion mobility spectrometry; TMT, tandem mass tagging.

References

1 Ong, S.-E. and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* **1**, 252–262 https://doi.org/10.1038/nchembio736
2 Domon, B. and Aebersold, R. (2010) Options and considerations when selecting a quantitative proteomics strategy. *Nat. Biotechnol.* **28**, 710–721 https://doi.org/10.1038/nbt.1661
3 Schilling, B., Rardin, M.J., MacLean, B.X., Zawadzka, A.M., Frewen, B.E., Cusack, M.P. et al. (2012) Platform-independent and label-free quantitation of proteomic data using MS1 extracted ion chromatograms in skyline: application to protein acetylation and phosphorylation. *Mol. Cell. Proteomics* **11**, 202–214 https://doi.org/10.1074/mcp.M112.017707
4 Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N. and Mann, M. (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell. Proteomics* **13**, 2513–2526 https://doi.org/10.1074/mcp.M113.031591
5 Ong, S.-E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A. et al. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **1**, 376–386 https://doi.org/10.1074/mcp.M200025-MCP200
6 Thompson, A., Schäfer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G. et al. (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* **75**, 1895–1904 https://doi.org/10.1021/ac0262560
7 Zecha, J., Satpathy, S., Kanashova, T., Avanessian, S.C., Kane, M.H., Clauser, K.R. et al. (2019) TMT labeling for the masses: a robust and cost-efficient, in-solution labeling approach. *Mol. Cell. Proteomics* **18**, 1468–1478 https://doi.org/10.1074/mcp.TIR119.001385
8 Wiese, S., Reidegeld, K.A., Meyer, H.E. and Warscheid, B. (2007) Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics* **7**, 340–350 https://doi.org/10.1002/pmic.200600422
9 Picotti, P. and Aebersold, R. (2012) Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat. Methods* **9**, 555–566 https://doi.org/10.1038/nmeth.2015
10 Zhang, H., Liu, Q., Zimmerman, L.J., Ham, A.-J.L., Slebos, R.J.C., Rahman, J. et al. (2011) Methods for peptide and protein quantitation by liquid chromatography-multiple reaction monitoring mass spectrometry. *Mol. Cell. Proteomics* **10**, M110.006593 https://doi.org/10.1074/mcp.M110.006593
11 Peterson, A.C., Russell, J.D., Bailey, D.J., Westphall, M.S. and Coon, J.J. (2012) Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol. Cell. Proteomics* **11**, 1475–1488 https://doi.org/10.1074/mcp.O112.020131
12 Gallien, S., Duriez, E., Crone, C., Kellmann, M., Moehring, T. and Domon, B. (2012) Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. *Mol. Cell. Proteomics* **11**, 1709–1723 https://doi.org/10.1074/mcp.O112.019802
13 Schilling, B., MacLean, B., Held, J.M., Sahu, A.K., Rardin, M.J., Sorensen, D.J. et al. (2015) Multiplexed, scheduled, high-resolution parallel reaction monitoring on a full scan QqTOF instrument with integrated data-dependent and targeted mass spectrometric workflows. *Anal. Chem.* **87**, 10222–10229 https://doi.org/10.1021/acs.analchem.5b02983
14 Colangelo, C.M., Chung, L., Bruce, C. and Cheung, K.-H. (2013) Review of software tools for design and analysis of large scale MRM proteomic datasets. *Methods* **61**, 287–298 https://doi.org/10.1016/j.ymeth.2013.05.004

15 Cham, J.A., Bianco, L., Barton, C. and Bessant, C. (2010) MRMaid-DB: a repository of published SRM transitions. *J. Proteome Res.* **9**, 620–625 https://doi.org/10.1021/pr900713u
16 Stanfill, B.A., Nakayasu, E.S., Bramer, L.M., Thompson, A.M., Ansong, C.K., Clauss, T.R. et al. (2018) Quality control analysis in real-time (QC-ART): a tool for real-time quality control assessment of mass spectrometry-based proteomics data. *Mol. Cell. Proteomics* **17**, 1824–1836 https://doi.org/10.1074/mcp.RA118.000648
17 Yang, H., Li, Y.C., Zhao, M.Z., Wu, F.L., Wang, X., Xiao, W.D. et al. (2019) Precision de novo peptide sequencing using mirror proteases of Ac-LysargiNase and trypsin for large-scale proteomics. *Mol. Cell. Proteomics* **18**, 773–785 https://doi.org/10.1074/mcp.TIR118.000918
18 Sharma, V., Eckels, J., Schilling, B., Ludwig, C., Jaffe, J.D., MacCoss, M.J. et al. (2018) Panorama public: a public repository for quantitative data sets processed in skyline. *Mol. Cell. Proteomics* **17**, 1239–1244 https://doi.org/10.1074/mcp.RA117.000543
19 Venable, J.D., Dong, M.-Q., Wohlschlegel, J., Dillin, A. and Yates, J.R. (2004) Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. *Nat. Methods* **1**, 39–45 https://doi.org/10.1038/nmeth705
20 Gillet, L.C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L. et al. (2012) Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell. Proteomics* **11**, O111.016717 https://doi.org/10.1074/mcp.O111.016717
21 Collins, B.C., Hunter, C.L., Liu, Y., Schilling, B., Rosenberger, G., Bader, S.L. et al. (2017) Multi-laboratory assessment of reproducibility, qualitative and quantitative performance of SWATH-mass spectrometry. *Nat. Commun.* **8**, 291 https://doi.org/10.1038/s41467-017-00249-5
22 Chapman, J.D., Goodlett, D.R. and Masselon, C.D. (2014) Multiplexed and data-independent tandem mass spectrometry for global proteome profiling. *Mass Spectrom. Rev.* **33**, 452–470 https://doi.org/10.1002/mas.21400
23 Bilbao, A., Varesio, E., Luban, J., Strambio-De-Castillia, C., Hopfgartner, G., Müller, M. et al. (2015) Processing strategies and software solutions for data-independent acquisition in mass spectrometry. *Proteomics* **15**, 964–980 https://doi.org/10.1002/pmic.201400323
24 Ting, Y.S., Egertson, J.D., Payne, S.H., Kim, S., MacLean, B., Käll, L. et al. (2015) Peptide-Centric proteome analysis: an alternative strategy for the analysis of tandem mass spectrometry data. *Mol. Cell. Proteomics* **14**, 2301–2307 https://doi.org/10.1074/mcp.O114.047035
25 Ludwig, C., Gillet, L., Rosenberger, G., Amon, S., Collins, B.C. and Aebersold, R. (2018) Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* **14**, e8126 https://doi.org/10.15252/msb.20178126
26 Gessulat, S., Schmidt, T., Zolg, D.P., Samaras, P., Schnatbaum, K., Zerweck, J. et al. (2019) Prosit: proteome-wide prediction of peptide tandem mass spectra by deep learning. *Nat. Methods* **16**, 509–518 https://doi.org/10.1038/s41592-019-0426-7
27 Searle, B.C., Swearingen, K.E., Barnes, C.A., Schmidt, T., Gessulat, S., Küster, B. et al. (2020) Generating high quality libraries for DIA MS with empirically corrected peptide predictions. *Nat. Commun.* **11**, 1548 https://doi.org/10.1038/s41467-020-15346-1
28 Searle, B.C., Pino, L.K., Egertson, J.D., Ting, Y.S., Lawrence, R.T., MacLean, B.X. et al. (2018) Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry. *Nat. Commun.* **9**, 5128 https://doi.org/10.1038/s41467-018-07454-w
29 Röst, H.L., Rosenberger, G., Navarro, P., Gillet, L., Miladinović, S.M., Schubert, O.T. et al. (2014) OpenSWATH enables automated, targeted analysis of data-independent acquisition MS data. *Nat. Biotechnol.* **32**, 219–223 https://doi.org/10.1038/nbt.2841
30 Tsou, C.-C., Avtonomov, D., Larsen, B., Tucholska, M., Choi, H., Gingras, A.-C. et al. (2015) DIA-Umpire: comprehensive computational framework for data-independent acquisition proteomics. *Nat. Methods* **12**, 258–264 https://doi.org/10.1038/nmeth.3255
31 Bruderer, R., Bernhardt, O.M., Gandhi, T., Miladinović, S.M., Cheng, L.-Y., Messner, S. et al. (2015) Extending the limits of quantitative proteome profiling with data-independent acquisition and application to Acetaminophen-treated three-dimensional liver microtissues. *Mol. Cell. Proteomics* **14**, 1400–1410 https://doi.org/10.1074/mcp.M114.044305
32 MacLean,, B., Tomazela,, D.M., Shulman,, N., Chambers,, M., Finney,, G.L., Frewen,, B., et al. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **26**, 966–968 https://doi.org/10.1093/bioinformatics/btq054
33 Navarro, P., Kuharev, J., Gillet, L.C., Bernhardt, O.M., MacLean, B., Röst, H.L. et al. (2016) A multicenter study benchmarks software tools for label-free proteome quantification. *Nat. Biotechnol.* **34**, 1130–1136 https://doi.org/10.1038/nbt.3685
34 Kim, Y.J., Sweet, S.M.M., Egertson, J.D., Sedgewick, A.J., Woo, S., Liao, W.-L. et al. (2019) Data-Independent acquisition mass spectrometry to quantify protein levels in FFPE tumor biopsies for molecular diagnostics. *J. Proteome Res.* **18**, 426–435 https://doi.org/10.1021/acs.jproteome.8b00699
35 Liu, Y., Borel, C., Li, L., Müller, T., Williams, E.G. Germain, P.-L. et al. (2017) Systematic proteome and proteostasis profiling in human Trisomy 21 fibroblast cells. *Nat. Commun.* **8**, 1212 https://doi.org/10.1038/s41467-017-01422-6
36 Amon, S., Meier-Abt, F., Gillet, L.C., Dimitrieva, S., Theocharides, A.P.A., Manz, M.G. et al. (2019) Sensitive quantitative proteomics of human hematopoietic stem and progenitor cells by data-independent acquisition mass spectrometry. *Mol. Cell. Proteomics* **18**, 1454–1467 https://doi.org/10.1074/mcp.TIR119.001431
37 Merkle, F.T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C. et al. (2017) Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. *Nature* **545**, 229–233 https://doi.org/10.1038/nature22312
38 Meier, F., Brunner, A.D., Koch, S., Koch, H., Lubeck, M., Krause, M. et al. (2018) Online parallel accumulation-serial fragmentation (PASEF) with a novel trapped ion mobility mass spectrometer. *Mol. Cell. Proteomics* **17**, 2534–2545 https://doi.org/10.1074/mcp.TIR118.000900
39 Meier, F., Brunner, A.-D., Frank, M., Ha, A., Bludau, I., Voytik, E. et al. (2020) Parallel accumulation – serial fragmentation combined with data-independent acquisition (diaPASEF): bottom-up proteomics with near optimal ion usage. PREPRINT *bioRxiv* https://doi.org/10.1101/656207
40 Rauniyar, N., McClatchy, D.B. and Yates, J.R. (2013) 3rd. Stable isotope labeling of mammals (SILAM) for in vivo quantitative proteomic analysis. *Methods* **61**, 260–268 https://doi.org/10.1016/j.ymeth.2013.03.008
41 Baker, E.S., Burnum-Johnson, K.E., Ibrahim, Y.M., Orton, D.J., Monroe, M.E., Kelly, R.T. et al. (2015) Enhancing bottom-up and top-down proteomic measurements with ion mobility separations. *Proteomics* **15**, 2766–2776 https://doi.org/10.1002/pmic.201500048
42 Li, J., Van Vranken, J.G., Pontano Vaites, L., Schweppe, D.K., Huttlin, E.L., Etienne, C. et al. (2020) TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. *Nat. Methods* **17**, 399–404 https://doi.org/10.1038/s41592-020-0781-4
43 Merrill, A.E., Hebert, A.S., MacGilvray, M.E., Rose, C.M., Bailey, D.J., Bradley, J.C. et al. (2014) Neucode labels for relative protein quantification. *Mol. Cell. Proteomics* **13**, 2503 https://doi.org/10.1074/mcp.M114.040287
44 Rauniyar,, N. and Yates, III, J.R. (2014) Isobaric labeling-based relative quantification in shotgun proteomics. *J. Proteome Res.* **13**, 5293–5309 https://doi.org/10.1021/pr500880b

45 Ting, L., Rad, R., Gygi, S.P. and Haas, W. (2011) MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat. Methods* **8**, 937–940 https://doi.org/10.1038/nmeth.1714
46 McAlister, G.C., Nusinow, D.P., Jedrychowski, M.P., Wuhr, M., Huttlin, E.L., Erickson, B.K. et al. (2014) Multinotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* **86**, 7150–7158 https://doi.org/10.1021/ac502040v
47 Minajigi, A., Froberg, J., Wei, C., Sunwoo, H., Kesner, B., Colognori, D. et al. (2015) Chromosomes. A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. *Science* **349**, aab2276 https://doi.org/10.1126/science.aab2276
48 Christoforou, A., Mulvey, C.M., Breckels, L.M., Geladaki, A., Hurrell, T., Hayward, P.C. et al. (2016) A draft map of the mouse pluripotent stem cell spatial proteome. *Nat. Commun.* **7**, 8992 https://doi.org/10.1038/ncomms9992
49 Navarrete-Perea, J., Yu, Q., Gygi, S.P. and Paulo, J.A. (2018) Streamlined tandem mass Tag (SL-TMT) protocol: an efficient strategy for quantitative (phospho)proteome profiling using tandem mass tag-synchronous precursor selection-MS3. *J. Proteome Res.* **17**, 2226–2236 https://doi.org/10.1021/acs.jproteome.8b00217
50 Paulo, J.A., O'Connell, J.D., Everley, R.A., O'Brien, J., Gygi, M.A. and Gygi, S.P. (2016) Quantitative mass spectrometry-based multiplexing compares the abundance of 5000 S. cerevisiae proteins across 10 carbon sources. *J. Proteomics* **148**, 85–93 https://doi.org/10.1016/j.jprot.2016.07.005
51 Paulo, J.A., Jedrychowski, M.P., Chouchani, E.T., Kazak, L. and Gygi, S.P. (2018) Multiplexed isobaric tag-based profiling of seven murine tissues following in vivo nicotine treatment using a minimalistic proteomics strategy. *Proteomics* **18**, e1700326 https://doi.org/10.1002/pmic.201700326
52 Wan, J., Liu, H., Chu, J. and Zhang, H. (2019) Functions and mechanisms of lysine crotonylation. *J. Cell Mol. Med.* **23**, 7163–7169 https://doi.org/10.1111/jcmm.14650
53 Yang, F., Shen, Y. and Camp, III, D.G. (2012) Smith RD. High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis. *Exp. Rev. Proteomics* **9**, 129–134 https://doi.org/10.1586/epr.12.15
54 Bache, N., Geyer, P.E., Bekker-Jensen, D.B., Hoerning, O., Falkenby, L., Treit, P.V. et al. (2018) A novel LC system embeds analytes in pre-formed gradients for rapid, ultra-robust proteomics. *Mol. Cell. Proteomics* **17**, 2284–2296 https://doi.org/10.1074/mcp.TIR118.000853
55 Zhang, M., An, B., Qu, Y., Shen, S., Fu, W., Chen, Y.-J. et al. (2018) Sensitive, high-Throughput, and robust trapping-Micro-LC-MS strategy for the quantification of biomarkers and antibody biotherapeutics. *Anal. Chem.* **90**, 1870–1880 https://doi.org/10.1021/acs.analchem.7b03949
56 Bian, Y., Zheng, R., Bayer, F.P., Wong, C., Chang, Y.-C., Meng, C. et al. (2020) Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS. *Nat. Commun.* **11**, 157 https://doi.org/10.1038/s41467-019-13973-x
57 Meier, F., Geyer, P.E., Virreira Winter, S., Cox, J. and Mann, M. (2018) Boxcar acquisition method enables single-shot proteomics at a depth of 10,000 proteins in 100 minutes. *Nat. Methods* **15**, 440–448 https://doi.org/10.1038/s41592-018-0003-5
58 Huang, T., Bruderer, R., Muntel, J., Xuan, Y., Vitek, O. and Reiter, L. (2019) Combining precursor and fragment information for improved detection of differential abundance in data independent acquisition. *Mol. Cell. Proteomics* **19**, 421–430 https://doi.org/10.1074/mcp.RA119.001705
59 Schweppe, D.K., Prasad, S., Belford, M.W., Navarrete-Perea, J., Bailey, D.J., Huguet, R. et al. (2019) Characterization and optimization of multiplexed quantitative analyses using high-Field asymmetric-waveform ion mobility mass spectrometry. *Anal. Chem.* **91**, 4010–4016 https://doi.org/10.1021/acs.analchem.8b05399
60 Bekker-Jensen, D.B., Martinez-Val, A., Steigerwald, S., Rüther, P.L., Fort, K.L., Arrey, T.N. et al. (2020) A compact quadrupole-orbitrap mass spectrometer with FAIMS interface improves proteome coverage in short LC gradients. *Mol. Cell. Proteomics* **19**, 716–729 https://doi.org/10.1074/mcp.TIR119.001906
61 Prianichnikov, N., Koch, H., Koch, S., Lubeck, M., Heilig, R., Brehmer, S. et al. (2020) Maxquant software for ion mobility enhanced shotgun proteomics. *Mol. Cell. Proteomics* **19**, 1058 https://doi.org/10.1074/mcp.TIR119.001720
62 Doll, S. and Burlingame, A.L. (2015) Mass spectrometry-based detection and assignment of protein posttranslational modifications. *ACS Chem. Biol.* **10**, 63–71 https://doi.org/10.1021/cb500904b
63 Udeshi, N.D., Mani, D.C., Satpathy, S., Fereshetian, S., Gasser, J.A., Svinkina, T. et al. (2020) Rapid and deep-scale ubiquitylation profiling for biology and translational research. *Nat. Commun.* **11**, 359 https://doi.org/10.1038/s41467-019-14175-1
64 Zolg, D.P., Wilhelm, M., Schmidt, T., Medard, G., Zerweck, J., Knaute, T. et al. (2018) Proteometools: systematic characterization of 21 post-translational protein modifications by liquid chromatography tandem mass spectrometry (LC-MS/MS) using synthetic peptides. *Mol. Cell. Proteomics* **17**, 1850–1863 https://doi.org/10.1074/mcp.TIR118.000783
65 Ashwood, C., Pratt, B., MacLean, B.X., Gundry, R.L. and Packer, N.H. (2019) Standardization of PGC-LC-MS-based glycomics for sample specific glycotyping. *Analyst* **144**, 3601–3612 https://doi.org/10.1039/C9AN00486F
66 Kong, A.T., Leprevost, F.V., Avtonomov, D.M., Mellacheruvu, D. and Nesvizhskii, A.I. (2017) MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry–based proteomics. *Nat. Methods* **14**, 513–520 https://doi.org/10.1038/nmeth.4256
67 Solntsev, S.K., Shortreed, M.R., Frey, B.L. and Smith, L.M. (2018) Enhanced global post-translational modification discovery with MetaMorpheus. *J. Proteome Res.* **17**, 1844–1851 https://doi.org/10.1021/acs.jproteome.7b00873
68 Bittremieux, W., Laukens, K. and Noble, W.S. (2019) Extremely fast and accurate open modification spectral library searching of high-resolution mass spectra using feature hashing and graphics processing units. *J. Proteome Res.* **18**, 3792–3799 https://doi.org/10.1021/acs.jproteome.9b00291
69 Chiba, T., Peasley, K.D., Cargill, K.R., Maringer, K.V., Bharathi, S.S., Mukherjee, E. et al. (2019) Sirtuin 5 regulates proximal tubule fatty acid oxidation to protect against AKI. *J. Am. Soc. Nephrol.* **30**, 2384 https://doi.org/10.1681/ASN.2019020163
70 Galvan-Pena, S., Carroll, R.G., Newman, C., Hinchy, E.C., Palsson-McDermott, E., Robinson, E.K. et al. (2019) Malonylation of GAPDH is an inflammatory signal in macrophages. *Nat. Commun.* **10**, 338 https://doi.org/10.1038/s41467-018-08187-6
71 Basisty, N., Meyer, J.G. and Schilling, B. (2018) Protein turnover in aging and longevity. *Proteomics* **18**, e1700108 https://doi.org/10.1002/pmic.201700108
72 Alevra, M., Mandad, S., Ischebeck, T., Urlaub, H., Rizzoli, S.O. and Fornasiero, E.F. (2019) A mass spectrometry workflow for measuring protein turnover rates in vivo. *Nat. Protocols* **14**, 3333–3365 https://doi.org/10.1038/s41596-019-0222-y
73 Fornasiero, E.F., Mandad, S., Wildhagen, H., Alevra, M., Rammner, B., Keihani, S. et al. (2018) Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. *Nat. Commun.* **9**, 4230 https://doi.org/10.1038/s41467-018-06519-0

74 Heo, S., Diering, G.H., Na, C.H., Nirujogi, R.S., Bachman, J.L., Pandey, A. et al. (2018) Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E3827–E3E36 https://doi.org/10.1073/pnas.1720956115
75 Alvarez-Castelao, B., Schanzenbächer, C.T., Langer, J.D. and Schuman, E.M. (2019) Cell-type-specific metabolic labeling, detection and identification of nascent proteomes in vivo. *Nat. Protocols* **14**, 556–575 https://doi.org/10.1038/s41596-018-0106-6
76 Steward, K.F., Eilers, B., Tripet, B., Fuchs, A., Dorle, M., Rawle, R. et al. (2020) Metabolic implications of using bioOrthogonal non-canonical amino acid tagging (BONCAT) for tracking protein synthesis. *Front. Microbiol.* **11**, 197 https://doi.org/10.3389/fmicb.2020.00197
77 Ramsey, R.S. and Ramsey, J.M. (1997) Generating electrospray from microchip devices using electroosmotic pumping. *Anal. Chem.* **69**, 1174–1178 https://doi.org/10.1021/ac9610671
78 Xue, Q., Foret, F., Dunayevskiy, Y.M., Zavracky, P.M., McGruer, N.E. and Karger, B.L. (1997) Multichannel microchip electrospray mass spectrometry. *Anal. Chem.* **69**, 426–430 https://doi.org/10.1021/ac9607119
79 Zhu, Y., Piehowski, P.D., Zhao, R., Chen, J., Shen, Y., Moore, R.J. et al. (2018) Nanodroplet processing platform for deep and quantitative proteome profiling of 10–100 mammalian cells. *Nat. Commun.* **9**, 882 https://doi.org/10.1038/s41467-018-03367-w
80 Xu, K., Liang, Y., Piehowski, P.D., Dou, M., Schwarz, K.C., Zhao, R. et al. (2019) Benchtop-compatible sample processing workflow for proteome profiling of <100 mammalian cells. *Anal. Bioanal. Chem.* **411**, 4587–4596 https://doi.org/10.1007/s00216-018-1493-9
81 Budnik, B., Levy, E., Harmange, G. and Slavov, N. (2018) SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biol.* **19**, 161 https://doi.org/10.1186/s13059-018-1547-5
82 Specht, H. and Slavov, N. (2018) Transformative opportunities for single-cell proteomics. *J. Proteome Res.* **17**, 2565–2571 https://doi.org/10.1021/acs.jproteome.8b00257
83 Dou, M., Clair, G., Tsai, C.-F., Xu, K., Chrisler, W.B., Sontag, R.L. et al. (2019) High-throughput single cell proteomics enabled by multiplex isobaric labeling in a nanodroplet sample preparation platform. *Anal. Chem.* **91**, 13119–13127 https://doi.org/10.1021/acs.analchem.9b03349
84 Wilson, R.S. and Nairn, A.C. (2018) Cell-type-specific proteomics: a neuroscience perspective. *Proteomes* **6**, 51 https://doi.org/10.3390/proteomes6040051
85 Zhu, Y., Clair, G., Chrisler, W.B., Shen, Y., Zhao, R., Shukla, A.K. et al. (2018) Proteomic analysis of single mammalian cells enabled by microfluidic nanodroplet sample preparation and ultrasensitive NanoLC-MS. *Angew Chem. Int. Ed. Engl.* **57**, 12370–12374 https://doi.org/10.1002/anie.201802843
86 Myers, S.A., Rhoads, A., Cocco, A.R., Peckner, R., Haber, A.L., Schweitzer, L.D. et al. (2019) Streamlined protocol for deep proteomic profiling of FAC-sorted cells and Its application to freshly isolated murine immune cells. *Mol. Cell. Proteomics* **18**, 995–1009 https://doi.org/10.1074/mcp.RA118.001259
87 Zhu, Y., Podolak, J., Zhao, R., Shukla, A.K., Moore, R.J., Thomas, G.V. et al. (2018) Proteome profiling of 1 to 5 spiked circulating tumor cells isolated from whole blood using immunodensity enrichment, laser capture microdissection, nanodroplet sample processing, and ultrasensitive nanoLC-MS. *Anal. Chem.* **90**, 11756–11759 https://doi.org/10.1021/acs.analchem.8b03268
88 Davis, S., Scott, C., Ansorge, O. and Fischer, R. (2019) Development of a sensitive, scalable method for spatial, cell-type-resolved proteomics of the human brain. *J. Proteome Res.* **18**, 1787–1795 https://doi.org/10.1021/acs.jproteome.8b00981
89 Li, H. and Hummon, A.B. (2011) Imaging mass spectrometry of three-dimensional cell culture systems. *Anal. Chem.* **83**, 8794–8801 https://doi.org/10.1021/ac202356g
90 Liu, X., Weaver, E.M. and Hummon, A.B. (2013) Evaluation of therapeutics in three-dimensional cell culture systems by MALDI imaging mass spectrometry. *Anal. Chem.* **85**, 6295–6302 https://doi.org/10.1021/ac400519c
91 Buchberger, A.R., DeLaney, K., Johnson, J. and Li, L. (2018) Mass spectrometry imaging: a review of emerging advancements and future insights. *Anal. Chem.* **90**, 240–265 https://doi.org/10.1021/acs.analchem.7b04733
92 Aichler, M. and Walch, A. (2015) MALDI imaging mass spectrometry: current frontiers and perspectives in pathology research and practice. *Lab. Invest.* **95**, 422–431 https://doi.org/10.1038/labinvest.2014.156
93 Bemis, K.D., Harry, A., Eberlin, L.S., Ferreira, C., van de Ven, S.M., Mallick, P. et al. (2015) Cardinal: an R package for statistical analysis of mass spectrometry-based imaging experiments. *Bioinformatics* **31**, 2418–2420 https://doi.org/10.1093/bioinformatics/btv146
94 Race, A.M., Palmer, A.D., Dexter, A., Steven, R.T., Styles, I.B. and Bunch, J. (2016) Spectralanalysis: software for the masses. *Anal. Chem.* **88**, 9451–9458 https://doi.org/10.1021/acs.analchem.6b01643
95 Källback, P., Nilsson, A., Shariatgorji, M. and Andrén, P.E. (2016) msIQuant – quantitation software for mass spectrometry imaging enabling fast access, visualization, and analysis of large data sets. *Anal. Chem.* **88**, 4346–4353 https://doi.org/10.1021/acs.analchem.5b04603
96 Kristensen, A.R. and Foster, L.J. (2013) High throughput strategies for probing the different organizational levels of protein interaction networks. *Mol. Biosyst.* **9**, 2201–2212 https://doi.org/10.1039/c3mb70135b
97 Porath, J. and Flodin, P.E.R. (1959) Gel filtration: a method for desalting and group separation. *Nature* **183**, 1657–1659 https://doi.org/10.1038/1831657a0
98 Havugimana, P.C., Hart, G.T., Nepusz, T., Yang, H., Turinsky, A.L., Li, Z. et al. (2012) A census of human soluble protein complexes. *Cell* **150**, 1068–1081 https://doi.org/10.1016/j.cell.2012.08.011
99 Dong, M., Yang, L.L., Williams, K., Fisher, S.J., Hall, S.C., Biggin, M.D. et al. (2008) A "Tagless" strategy for identification of stable protein complexes genome-wide by multidimensional orthogonal chromatographic separation and iTRAQ reagent tracking. *J. Proteome Res.* **7**, 1836–1849 https://doi.org/10.1021/pr700624e
100 Kristensen, A.R., Gsponer, J. and Foster, L.J. (2012) A high-throughput approach for measuring temporal changes in the interactome. *Nat. Methods* **9**, 907–909 https://doi.org/10.1038/nmeth.2131
101 Mulvey, C.M., Breckels, L.M., Geladaki, A., Britovšek, N.K., Nightingale, D.J.H., Christoforou, A. et al. (2017) Using hyperLOPIT to perform high-resolution mapping of the spatial proteome. *Nat. Protocols* **12**, 1110–1135 https://doi.org/10.1038/nprot.2017.026
102 McBride, Z., Chen, D., Lee, Y., Aryal, U.K., Xie, J. and Szymanski, D.B. (2019) A label-free mass spectrometry method to predict endogenous protein complex composition. *Mol. Cell. Proteom.* **18**, 1588–1606 https://doi.org/10.1074/mcp.RA119.001400
103 Wan, C., Borgeson, B., Phanse, S., Tu, F., Drew, K., Clark, G. et al. (2015) Panorama of ancient metazoan macromolecular complexes. *Nature* **525**, 339–344 https://doi.org/10.1038/nature14877

104 Heusel, M., Bludau, I., Rosenberger, G., Hafen, R., Frank, M., Banaei-Esfahani, A. et al. (2019) Complex-centric proteome profiling by SEC-SWATH-MS. *Mol. Syst. Biol.* **15**, e8438 https://doi.org/10.15252/msb.20188438
105 Jarvik, J.W. and Telmer, C.A. (1998) EPITOPE TAGGING. *Ann. Rev. Genet.* **32**, 601–618 https://doi.org/10.1146/annurev.genet.32.1.601
106 Huttlin, E.L., Bruckner, R.J., Paulo, J.A., Cannon, J.R., Ting, L., Baltier, K. et al. (2017) Architecture of the human interactome defines protein communities and disease networks. *Nature* **545**, 505–509 https://doi.org/10.1038/nature22366
107 Purcell, A.W., Ramarathinam, S.H. and Ternette, N. (2019) Mass spectrometry–based identification of MHC-bound peptides for immunopeptidomics. *Nat. Protocols* **14**, 1687–1707 https://doi.org/10.1038/s41596-019-0133-y
108 Creech, A.L., Ting, Y.S., Goulding, S.P., Sauld, J.F.K., Barthelme, D., Rooney, M.S. et al. (2018) The role of mass spectrometry and proteogenomics in the advancement of HLA epitope prediction. *Proteomics* **18**, e1700259 https://doi.org/10.1002/pmic.201700259
109 Roux, K.J., Kim, D.I., Raida, M. and Burke, B. (2012) A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J. Cell Biol.* **196**, 801–810 https://doi.org/10.1083/jcb.201112098
110 Kim, D.I., Jensen, S.C., Noble, K.A., Kc, B., Roux, K.H., Motamedchaboki, K. et al. (2016) An improved smaller biotin ligase for BioID proximity labeling. *Mol. Biol. Cell* **27**, 1188–1196 https://doi.org/10.1091/mbc.E15-12-0844
111 Rhee, H.-W., Zou, P., Udeshi, N.D., Martell, J.D., Mootha, V.K., Carr, S.A. et al. (2013) Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science* **339**, 1328–1331 https://doi.org/10.1126/science.1230593
112 Varnaitė, R. and MacNeill, S.A. (2016) Meet the neighbors: mapping local protein interactomes by proximity-dependent labeling with BioID. *Proteomics* **16**, 2503–2518 https://doi.org/10.1002/pmic.201600123
113 Dominko, K. and ?ikić, D. (2018) Glutathionylation: a regulatory role of glutathione in physiological processes. *Arh Hig Rada Toksikol* **69**, 1–24 https://doi.org/10.2478/aiht-2018-69-2966
114 Trinkle-Mulcahy, L. (2019) Recent advances in proximity-based labeling methods for interactome mapping. *F1000Res* **8**, F1000 Faculty Rev-135 https://doi.org/10.12688/f1000research.16903.1
115 Branon, T.C., Bosch, J.A., Sanchez, A.D., Udeshi, N.D., Svinkina, T., Carr, S.A. et al. (2018) Efficient proximity labeling in living cells and organisms with TurboID. *Nat. Biotechnol.* **36**, 880–887 https://doi.org/10.1038/nbt.4201
116 Samavarchi-Tehrani, P., Abdouni, H., Samson, R. and Gingras, A.C. (2018) A versatile lentiviral delivery toolkit for proximity-dependent biotinylation in diverse cell types. *Mol. Cell. Proteomics* **17**, 2256–2269 https://doi.org/10.1074/mcp.TIR118.000902
117 Dong, J.-M., Tay, F.P.-L., Swa, H.L.-F., Gunaratne, J., Leung, T., Burke, B. et al. (2016) Proximity biotinylation provides insight into the molecular composition of focal adhesions at the nanometer scale. *Sci. Signal.* **9**, rs4 https://doi.org/10.1126/scisignal.aaf3572
118 Hung, V., Zou, P., Rhee, H.-W., Udeshi, N.D., Cracan, V., Svinkina, T. et al. (2014) Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric APEX tagging. *Mol. Cell* **55**, 332–341 https://doi.org/10.1016/j.molcel.2014.06.003
119 Lobingier, B.T., Hüttenhain, R., Eichel, K., Miller, K.B., Ting, A.Y., von Zastrow, M. et al. (2017) An approach to spatiotemporally resolve protein interaction networks in living cells. *Cell* **169**, 350–360.e12 https://doi.org/10.1016/j.cell.2017.03.022
120 Konermann, L., Pan, J. and Liu, Y.-H. (2011) Hydrogen exchange mass spectrometry for studying protein structure and dynamics. *Chem. Soc. Rev.* **40**, 1224–1234 https://doi.org/10.1039/C0CS00113A
121 Svejdal, R.R., Dickinson, E.R., Sticker, D., Kutter, J.P. and Rand, K.D. (2019) Thiol-ene microfluidic chip for performing hydrogen/deuterium exchange of proteins at subsecond time scales. *Anal. Chem.* **91**, 1309–1317 https://doi.org/10.1021/acs.analchem.8b03050
122 Jansson, E.T., Lai, Y.-H., Santiago, J.G. and Zare, R.N. (2017) Rapid hydrogen–deuterium exchange in liquid droplets. *J. Am. Chem. Soc.* **139**, 6851–6854 https://doi.org/10.1021/jacs.7b03541
123 Marciano, D.P., Dharmarajan, V. and Griffin, P.R. (2014) HDX-MS guided drug discovery: small molecules and biopharmaceuticals. *Curr. Opin. Struct. Biol.* **28**, 105–111 https://doi.org/10.1016/j.sbi.2014.08.007
124 Masson, G.R., Jenkins, M.L. and Burke, J.E. (2017) An overview of hydrogen deuterium exchange mass spectrometry (HDX-MS) in drug discovery. *Exp. Opin. Drug Discov.* **12**, 981–994 https://doi.org/10.1080/17460441.2017.1363734
125 Puchades, C., Kűkrer, B., Diefenbach, O., Sneekes-Vriese, E., Juraszek, J., Koudstaal, W. et al. (2019) Epitope mapping of diverse influenza Hemagglutinin drug candidates using HDX-MS. *Sci. Rep.* **9**, 4735 https://doi.org/10.1038/s41598-019-41179-0
126 Toby, T.K., Fornelli, L. and Kelleher, N.L. (2016) Progress in top-down proteomics and the analysis of proteoforms. *Annu. Rev. Anal. Chem.* **9**, 499–519 https://doi.org/10.1146/annurev-anchem-071015-041550
127 Smith, L.M. and Kelleher, N.L. (2013) Consortium for Top down P. proteoform: a single term describing protein complexity. *Nat. Methods* **10**, 186–187 https://doi.org/10.1038/nmeth.2369
128 Shen, X., Kou, Q., Guo, R., Yang, Z., Chen, D., Liu, X. et al. (2018) Native proteomics in discovery mode using size-exclusion chromatography–capillary zone electrophoresis–tandem mass spectrometry. *Anal. Chem.* **90**, 10095–10099 https://doi.org/10.1021/acs.analchem.8b02725
129 Skinner, O.S., Do Vale, L.H.F., Catherman, A.D., Havugimana, P.C., Sousa, M.V.D., Compton, P.D. et al. (2015) Native GELFrEE: a new separation technique for biomolecular assemblies. *Anal. Chem.* **87**, 3032–3038 https://doi.org/10.1021/ac504678d
130 Leney, A.C. and Heck, A.J.R. (2017) Native mass spectrometry: what is in the name? *J. Am. Soc. Mass Spectrom.* **28**, 5–13 https://doi.org/10.1007/s13361-016-1545-3
131 Rose, R.J., Damoc, E., Denisov, E., Makarov, A. and Heck, A.J.R. (2012) High-sensitivity orbitrap mass analysis of intact macromolecular assemblies. *Nat. Methods* **9**, 1084–1086 https://doi.org/10.1038/nmeth.2208
132 Kafader, J.O., Melani, R.D., Senko, M.W., Makarov, A.A., Kelleher, N.L. and Compton, P.D. (2019) Measurement of individual ions sharply increases the resolution of orbitrap mass spectra of proteins. *Anal. Chem.* **91**, 2776–2783 https://doi.org/10.1021/acs.analchem.8b04519
133 Skinner, O.S., Havugimana, P.C., Haverland, N.A., Fornelli, L., Early, B.P., Greer, J.B. et al. (2016) An informatic framework for decoding protein complexes by top-down mass spectrometry. *Nat. Methods* **13**, 237–240 https://doi.org/10.1038/nmeth.3731