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Proteomics has lagged behind other -omics, hampered by technological limitations which only provided static snapshots instead of interactions and dynamic states.

Innovations in mass spectrometry technology are now facilitating high-throughput large-scale protein analyses with increased sensitivity, allowing researchers to unlock the mysteries of the proteome.
Systems-Level: Multiplexing with Tandem Mass Tags

To understand the functions of individual proteins and their place in complex biological systems, it is often necessary to measure changes in protein abundance relative to changes in the state of the system. Modern proteomics has evolved to include a variety of technologies for the routine quantitative analyses of both known and unknown targets. Discovery-based relative quantification is an analytical approach that allows the scientist to determine relative protein abundance changes across a set of samples simultaneously and without the requirement for prior knowledge of the proteins involved.

Tandem mass tags (TMTs) are isotopomer (isomers containing identical isotope identities and numbers but differing in positions) labels designed to overcome reproducibility and dynamic range issues when attempting to quantify proteins via liquid chromatography/mass spectrometry based methods.1 TMTs are typically composed of a mass reporter, a mass normalizer, an amine reactive group, and a cleavable linker,2 and they bind to the N-terminus or lysine residues. Upon fragmentation, cleavage of the linker gives rise to a unique reporter ion for each tag at a specified mass-to-charge (m/z) ratio.3 Protein quantitation is accomplished by comparing the intensities of the reporter ions.3

While TMT-based approaches are similar to other peptide labeling techniques (e.g., using isotopes), a pair of peptides tagged with identical TMTs are not chemically identical, but also have the same overall mass and will co-migrate in chromatographic separations, thus leading to more accurate quantification.1 TMTs are amenable to higher-throughput applications, as up to eleven digested peptide samples, each labeled with a unique TMT, can be analyzed simultaneously.1,2,12

TMTs come in different formats, each optimized for different applications. Amine-reactive TMTs are well suited for total peptide quantitation, while sulfhydryl-reactive iodoTMTs are best for working with cysteine-labeled peptides, and carbonyl-reactive aminoxyTMTs are adept at quantitating glycans, steroids, and oxidized peptides.4 TMT-mediated multiplexed quantitation has suffered from reporter ion ratio distortion originating from the fragmentation of co-isolated interfering species, resulting in decreased accuracy, precision, and dynamic range.3,5 While additional gas-phase manipulations (e.g., MS3) and proton-transfer reactions could negate these interfering signals,1 these methods incurred a significant sensitivity penalty.5 The use of synchronous precursor selection (SPS) with MS3 is capable of overcoming reporter ion ratio distortion while avoiding sensitivity penalties.3,6

The SPS-MS3 process entails selecting multiple (up to 20) MS3 fragment ions using isolation waveforms with multiple frequency notches (MultiNotch). The selected ions are then fragmented again (MultiNotch MS3), producing a dramatically more intense reporter ion population with better ratio accuracy compared to subjecting a single MS3 ion to MS3.5 This process also, by carefully defining the selected isolation notches of the SPS isolation waveform, maintains the selectivity of MS3, altogether resulting in a significant increase in the number of quantified peptides.3,6

SPS-MS3 has been already employed to reproducibly quantify 172,704 protein abundance changes between individual cancer cell line proteomes, taking only three separate experiments to accomplish this feat. Additionally, Erickson et al.7 have used the technique to study protein phosphorylation, identifying 38,247 phosphopeptides corresponding to 11,000 phosphorylation sites in less than 48 h. SPS-MS3 has been integral in facilitating the construction of a draft map of the mouse pluripotent stem cell spatial proteome,8 characterizing the response of the yeast ubiquitylome to cold,9 and cancer-cell induced changes to bystander cell proteomes.10

SPS-MS3 workflows are subject to methodology and configuration tweaking, depending on experimental parameters and scientist need. Researchers need to balance coverage, quality (in terms of precision and reporter ion yield), and speed to obtain optimized data.11 Nonetheless, the deployment of TMTs in SPS-MS3 protocols has already delivered significant breakthroughs in many diverse scientific fields, and it promises to continue to contribute in the future. As Dr. Wilhelm Haas of Harvard Medical School notes, "what [SPS-MS3] has allowed us to do is really use TMT at a higher throughput with very great accuracy, [...] to look at many samples and really play on the same level as genomics." For references, please see page 11.
**Post-Translation: Glycomics and Glycoproteomics**

Glycans are carbohydrates that are found attached to proteins and lipids. Originally believed to only serve structural purposes, glycans have since been found to affect key cellular functions in development, proliferation, differentiation, and morphogenesis. Altered glycosylation profiles have been linked with congenital and acquired disease states—including cancers, cardiovascular disease, and immune tolerance. As such, glycomic and glycoproteomic profiling has become increasingly important for disease research and therapeutic development purposes. As Dr. Lance Wells of the University of Georgia describes it: “to paraphrase Ajit Varki (UCSD), “despite billions of years of evolution, no living cell has been produced that is not coated with a dense array of glycans” and Gerald Hart (JHMI), “there is not a single human disease or disorder for which glycans do not play a role”.

Glycoproteomic analysis using mass spectrometry (MS) presents unique challenges owing to the macro- (the extent/magnitude of glycosylation at a given site) and microheterogeneity (the range of glycan structures which can be attached at a given site) of the protein-attached oligosaccharides. Thus, protein glycosylation is commonly investigated by analyzing intact glycoproteins, protease-digested glycoproteins (intact glycopeptides), and/or glycans released from glycopeptides. Care must be taken when employing the latter strategy, as not only is structural information not preserved, but false positives can occur.

Glycan microheterogeneity found on glycoproteins means that ionization is required to detect and characterize intact glycoproteins and glycopeptides. However, glycan presence decreases ionization efficiency relative to non-glycosylated proteins/peptides and glycan heterogeneity results in lower abundances for each individual ion—although these issues can be mitigated by separating glycopeptides from normal counterparts. Historically, softer ionization techniques such as ESI and MALDI have been the most commonly used. However, single-stage MS data only provides, through the mass/charge (m/z) ratio, the mass of the glycopeptide, making it difficult to delineate glycoproteins which differ only by conformation, sequence, or glycan linkages unless a high-resolution MS instrument (with mass error below 10 ppm) is used. Glycomics has greatly benefited by the advent and commercialization of hybrid mass spectrometers with high-resolution mass analyzers that have tremendously improved glycosylation detection and analysis via multi-stage MS. Here, various fragmentation methods are employed to generate product ions from precursor ions of a desired m/z ratio for subsequent structural analysis. Dr. Wells highlights that “[multi-stage MS] methods can be developed for specific types of glycans (human O-linked for example) so that intact mass followed by intelligent [multi-stage MS] approaches can clearly define the composition, topology, and linkages for glycans.” Collision-induced dissociation (CID) is commonly used, but provides relatively poor fragmentation, resulting in insufficient peptide information. In response, the development of orbital ion trapping mass spectrometry resulted in the development of higher-energy collisional dissociation (HCD), a specialized CID method with higher sensitivity and ion detection range, albeit still providing relatively poor fragmentation.

Electron-based dissociation methods, which promote deeper cleavages into protein sequences, represent a solution to this issue. The sequential application of HCD and electron transfer dissociation (ETD) using an orbital ion trapping MS instrument yielded amino acid sequence information while preserving modified peptide residues. Finally, a combination of HCD and ETD, termed electron-transfer/higher-energy collision dissociation (EThcD), has resulted in more extensive peptide backbone fragmentation, richer spectra, and better sequence coverage and identification. EThcD can incorporate both glycan and peptide fragments on a single spectrum, revealing significant microheterogeneity details.

It must be noted that glycopeptide analyses benefit strongly from using multiple dissociation methods. “For O-linked [glycans], where the site of attachment on the glycan is hard to predict, electron-based dissociation methods [...] are extremely powerful for assigning the composition of the glycan to a specific hydroxyl-containing amino acid on the polypeptide”, explains Dr. Wells. “[However], Step-HCD approaches can often provide more information than ETD regarding the topology of the glycan attached to the peptide. Approaches using CID in an ion trap are also powerful in that one can capture neutral loss (NL) fragments and fragment the NL fragments again and again until the b and y ions of the peptide are revealed. Thus, for detailed glycoproteomic studies it is often best to scan for glycopeptides using HCD and looking for the generation of oxonium ions. Once identified, a combination of Step-HCD, ETD, and CID NL-triggered [multi-stage MS] can all be used to assist in defining the peptide sequence being modified, the site of attachment, and the topology of the glycan on the glycopeptide. Finally, more recent commercially available fragmentation techniques such as UVPD have not been fully evaluated for either glycomics or glycoproteomics, but may provide unique fragmentation patterns that could further facilitate the assignment of glycans and glycopeptides by mass spectrometry.”

Although the intrinsic complexity and heterogeneity of glycans and glycan-protein interactions has long been a stumbling block for researchers, MS technology is the driving force behind attempts to unravel the glycan code. It has allowed the design and utilization of new workflows, incorporating multiple fragmentation methodologies, to address previously difficult questions. The continued progression of technology and research, including new approaches combining aspects of glycomics, proteomics, and glycoproteomics, will undoubtedly unlock new information regarding the role of glycosylation in human health and disease.

*For references, please see page 11.*
Two proteins of identical mass and sequence can have significantly different biological functions due to differences in conformation. Mass spectrometry-obtained conformation data is important for structural biologists, can shed light on protein dynamism, and can reveal protein-protein interactions.

Two or more proteins physically associate with one another via electrostatic or steric forces, protein-protein interactions fuel essential processes within cells. These interactions may be durable and lasting or ephemeral and transient, indicating the degree to which the response must be tuneable.

Post-translational modifications are chemical signals added to proteins following successful translation which regulate the function of the protein. There are many known PTMs, but the most common ones include:

- **Phosphorylation**: Identified by an 80 Da shift in mass, the addition of a phosphoryl group may be essential to the onset of a biochemical reaction.
- **Acetylation**: Crucial for protein regulation and function affecting stability, localization, synthesis, and metabolism, acetylation can be identified as a 42 Da mass shift.
- **Ubiquitination**: A canonical protein degradation pathway, ubiquitination can be detected following enzymatic digestion by the telltale residues remaining (GG or LRGG on terminal lysines).
- **Glycosylation**: The sweet story of glycosylation involves the addition of sugar residues on proteins, the analysis of which can be complex and require comprehensive fragmentation.
Network Building: Protein-Protein Crosslinking

Proteins often exert their effects by interacting or complexing with other proteins, and the study of protein–protein interactions are therefore fundamental to our understanding of cellular, systemic, and organismal biology. In particular, the detection and characterization of protein–protein interactions is essential for structural biologists.\(^1\) Mass spectrometry (MS) has been used extensively to identify protein–protein interactions, ranging from techniques focused on a single protein of interest such as affinity purification-MS to those aiming to elucidate the greater interactome such as crosslinking (XL)-MS.\(^1\) As Dr. Fan Liu of Utrecht University puts it, "[XL-MS] is very important to biological studies because we can simultaneously look at the architecture of protein complexes and also the detailed interactions - the structural information from those interactions, and this will greatly help us to understand the fundamental functional principles of many protein complexes."

One of the primary obstacles to studying protein–protein interactions was that many techniques used to investigate proteins caused denaturation and complex dissociation. Chemical crosslinking uses various reagents to introduce covalent bonds between proteins which are either within sufficiently close proximity to one another or interact via noncovalent mechanisms.\(^2\) These artificial bonds can withstand denaturation, thus allowing the protein–protein interaction to be identified and analyzed.\(^2\)

Most crosslinker reagents are bifunctional molecules, meaning that they possess two functional groups with a spacer between them. That having been said, crosslinkers can also contain more than two functional groups.\(^3,4\) These functional groups can be uniform (homobifunctional) or different (heterobifunctional), with different functional groups preferentially targeting different amino acids.\(^5\) Crosslinker molecules have a finite range — two proteins can only be linked together if the distance between the linkage sites is shorter than the distance between the two functional groups in the crosslinker — and thereby convey spatial information which can be used to shape structural modeling.\(^1,3\)

Crosslinking and MS have been used together for several decades now, providing protein-level data at the outset and progressing to peptide-level resolution with the advent of superior instrumentation, protocols, and analytical approaches.\(^1,2\) The peptide-level resolution provided by XL-MS has allowed researchers to identify specific linkage sites, protein folding conformations, and establish the topography of multi-protein complexes.\(^2,3\) Since crosslinking information is encoded onto an non-denatured protein and persists through denaturation/fragmentation, XL-MS is able to provide information regarding their native, in vivo, quaternary structures, as well as any changes to the protein which may occur as a result of a change in physiological/pathological conditions.\(^4\) Moreover, XL-MS is able to contend with heterogeneity — capable of differentiating between different subunits and/or conformations.\(^2\)

Successfully utilizing and optimizing XL-MS means contending with several parameters not encountered when performing MS analysis on conventional proteins. For example, the number and distribution of crosslinking sites across a given protein is largely determinant on the choice of crosslinker. Uneven crosslinker distribution can contribute to uneven fragmentation, meaning that some of the peptide fragments generated may fall outside of an instrument's detection threshold.\(^1\) A combination of different crosslinking reagents and/or cleavage enzymes targeting different peptides/cleavage sites may yield more uniform distribution and/or fragmentation.\(^4\) Similarly, if employing tandem MS, different fragmentation methods will result in different fragment profiles and data resolution.\(^4\) "The most difficult part of the XL-MS workflow is data analysis," says Dr. Liu, "because crosslinked peptides [consist of] two linear peptides covalently linked by a crosslinker. Therefore, there are quite a few technical [challenges], and it is very tricky to identify these crosslinks. In order to [overcome these obstacles], we need to develop novel data acquisition approaches and also data analysis software to facilitate identification of crosslinks."

Since a given XL-MS workflow can involve multiple crosslinking reagents, multiple cleavage mechanisms, and multiple proteins, an MS instrument capable of different techniques and multiple fragmentation methods is a powerful asset, capable of adapting to any set of experimental conditions.\(^4\) The most suitable instrumentation to work on crosslink identification will "provide users with great flexibility," concludes Dr. Liu.

For references, please see page 11.
Where to Start? Top-Down and Middle-Down Proteomics

Historically, mass spectrometry-based proteomics approaches have been “bottom-up” – using protein fragments which have been chemically or enzymatically digested prior to MS analysis. However, bottom-up approaches have clear disadvantages: a digested peptide may not be specific to any given protein, the absence of the necessary peptide fragments may leave large regions of the original protein unidentified, and inopportune digestion sites can separate spatially-related modifications or sequence variations, making it so that they appear unrelated. Dr. Neil Kelleher of Northwestern University sums up the situation aptly by saying that “while decades of research and billions of dollars have gone into optimizing chromatography and mass spectrometers for the analysis of smaller molecules and peptides, the game changes when you look at larger molecules.”

“Top-down” proteomics – starting with the intact protein, which is then fragmented via MS – mitigates and eliminates these issues. While technical challenges have caused the development of top-down methods to lag behind their bottom-up counterparts, MS technology has arrived at a point where 100% sequence coverage and full characterization of proteoforms is now possible using top-down proteomics.

High-performance MS instruments are essential to the detection and characterization of intact proteins, especially for proteomic purposes. In particular, high resolution and sensitivity are critical: the former because proteins which have undergone post-translational modifications can vary in mass by minute amounts relative both to each other and their unmodified counterparts, the latter because the combined signal generated from a single protein will be spread across hundreds of channels. Given these requirements, Fourier transform ion cyclotron resonance (FTICR) and orbital ion trap mass spectrometry, especially when coupled with linear ion traps, have demonstrated their effectiveness for top-down proteomic applications.

The evolution of mass spectrometry fragmentation has also been significantly beneficial for top-down MS proteomics. Collision-based approaches (e.g., collision-induced dissociation [CID] and higher-energy collisional dissociation [HCD]) are useful for peptide identification purposes, but can lack sufficient fragmentation power for the detailed analysis of large intact proteins. Additionally, collision-based approaches tend to result in the loss of post-translational modification groups.

Electron-based methods (e.g., electron-capture dissociation [ECD], electron-transfer dissociation [ETD]) and ultraviolet photodissociation (UVPD) are both capable of retaining the valuable information contained within post-translational modifications. This feature has allowed researchers to use ECD/ETD to extensively study histone methylation/acetylation, as well as protein phosphorylation, glycosylation, and ubiquitination in cells, organisms, and tissues. According to Dr. Jenny Brodbelt of the University of Texas at Austin, “the ability to identify a protein requires that a certain number of amino acids are mapped, thus allowing a match to a sequence in a protein database. Confidence in the identification increases with the number of residues that are matched, and this is one of the attributes of UV-PD that has attracted attention.” Indeed, UVPD has been used to completely characterize individual proteins and study histone post-translational modifications.

Hybrid methods, employing the alternating usage of one of ECD/ETD/UVPD with a collision-based fragmentation technique, have helped mitigate weaknesses found in any given individual technique and improve data acquisition. A combination of ETD and HCD - electron-transfer/higher-energy collision dissociation (EThC) - yielded more extensive proteoform characterization and phosphorylation site localization, while combining ETD and CID also resulted in more comprehensive phosphorylation site identification. Likewise, UVPD and HCD, while not used in tandem on the same sample, did show significant complementarity with regard to their generated data sets. Dr. Kelleher's opinion on this topic is that “if you're using a direct infusion approach, you often have the time to use multiple techniques (HCD, ETD, and UVPD) to fully characterize intact proteoforms - even big ones like antibodies. [...] You often have to choose just one for time considerations, and we typically choose HCD for that task in our "first-pass."

Top-down proteomic approaches have become more feasible due to technological advances with regard to instrument sensitivity and resolution, as well as the development of superior fragmentation techniques. Continued development and advances, particularly with regard to throughput, will help increase the usage of these approaches to solve problems in systems biology: “Whole protein mass spectrometry has already proven valuable for clinical diagnosis of bacterial pathogens in thousands of hospitals worldwide,” notes Dr. Kelleher. "For me, this proves the business and science case for a ~$1/shot assay that is already improving human health. This might even have eclipsed the private-sector activity for clinically deployed use of Bottom-Up based assays using tryptic peptides.”

For references, please see page 11.
### Seeing Deeper: Ultraviolet Photodissociation and Ultra-High Resolution Mass Spectrometry

In tandem mass spectrometry (MS), the process of ion activation entails depositing energy into an ion to generate reproducible bond cleavages, resulting in fragment ions which can be subsequently analyzed to determine structural and sequence information. Several ion activation methodologies exist, including collision-induced dissociation (CID), electron-transfer dissociation (ETD), and photodissociation (PD). CID is widely popular, but does not provide sufficient energy deposition for certain applications or to fragment certain ion types — including large molecules and whole proteins. ETD and other electron-based methods have proven successful in the analysis of some intact proteins, and additionally are able to preserve post-translational peptide modifications. However, electron-based methods are charge-state dependent, and thus give limited sequence coverage for ions in low charge states.

PD offers superior sequence coverage versus collision- and electron-based methods for several reasons, including the fact that both precursor and product ions can undergo photoactivation and dissociation, and that dead-end fragment ions can be converted into useful, data-providing, products. In particular, ultraviolet photodissociation (UVPD) deposits substantially more energy per photon compared to other PD light sources such as infrared, thus providing access to higher energy fragmentation pathways and new dissociation mechanisms, resulting in significantly richer tandem MS spectra and allowing for greater sequence coverage than any other method. 1, 2  

"For intact proteins, UVPD typically causes fragmentation deeper into the mid-section of the sequence, whereas collisional activation tends to favor fragmentation near the N-terminal and C-terminal ends of the protein," explains Dr. Jenny Brodbelt, a leading expert in the field from the University of Texas at Austin. "This factor leads to greater sequence coverage and is particularly beneficial for localizing modifications." Indeed, UVPD has been demonstrated to achieve 100% sequence coverage for intact proteins, and with a photodissociation efficiency of up to 98%, is well-suited for high-throughput top-down proteomics applications when coupled with faster fragmentation techniques such as higher-energy collisional dissociation (HCD). 1, 3 UVPD outperformed CID, HCD, and ETD in terms of characterizing the sequences of proteins in protein-protein and protein-ligand complexes, identifying binding/interaction sites, and providing insight regarding tertiary and quaternary structures. 6

The utility of UVPD is aided by the fact that many organic molecules possess some capacity to absorb UV frequency light. As such, UVPD has demonstrated its efficacy in studies analyzing not only peptides and proteins, but also post-translational modification elements including oligosaccharides, glycosylation, and sialylation, the latter being particularly difficult to analyze. 1, 5, 8 "In the context of profiling protein modifications, UVPD offers two beneficial attributes," explains Dr. Brodbelt. "First, the fact that post-translational modifications are not preferentially cleaved during UVPD increases the ability to determine their locations on the resulting product ions. Second, the large array of fragment ions enhances the opportunity to discern the specific locations of modifications."

"These features extend even to those peptides or proteins with multiple modifications, and the ability to map combinatorial modifications has proven to be one of the more vexing challenges of proteomics," continues Dr. Brodbelt, who cautions that "the production of numerous types and great numbers of different fragment ions disperses the ion current into many channels, thus reducing the overall signal-to-noise ratio of a mass spectrum. This creates a tradeoff between information content and sensitivity."

The superior ion array generated by UVPD has also established the value of the technique in lipidomics. For example, while CID typically produced fragment ions from C-O cleavages (thus limiting detection of phosphate groups [R-O-P] and fatty acid chains [C-C]), UVPD produces ions from amide, C-C, glucosamine, phosphorylethanolamine, and hydroxyl modification cleavages. 10, 11 UVPD fragmentation has thus been used to characterize gangliosides, glycosphingolipids, and glycerophospholipids. An MS instrument equipped with an orbital ion trap which is able to perform tandem MS combining higher-energy collisional dissociation (HCD) and UVPD is well equipped for the analysis of complex phospholipid mixtures, capable of localizing double bonds and detecting changes in isomer composition. 1, 11

The versatility and depth of data provided by UVPD has proven valuable for a broad range of –omics fields, making the fragmentation technique a promising tool. The coupling of UVPD with ultra-high resolution tandem MS (with resolutions now up to 1,000,000 FHMW) further enhances structural characterization and compound quantitation capabilities, and represents another step towards the automated elucidation of proteins and lipids in high-throughput workflows. 1 In the context of protein interactions, 

[... ] UVPD affords another promising approach for identifying proteins within complexes, and recent evidence suggests that variations in fragmentation patterns of different protein complexes correlate with conformational changes of the proteins," notes Dr. Brodbelt, who concludes that "this is one of many new frontiers of UVPD."

For references, please see page 12.
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### Article 5 - Seeing Deeper: Ultraviolet Photodissociation and Ultra-High Resolution Mass Spectrometry

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