Instrumentation at the leading edge of proteomics

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INTRODUCTION

The proteome, or collection of proteoforms expressed in a biological system, is dynamic and heterogeneous. As our appreciation for the complexity of the proteome has evolved, so have the technologies we use to interrogate its composition. A rapid expansion in the field of proteomics was driven by the advent of soft ionization techniques more than three decades ago. At first, proteomics focused on capturing protein sequence information using mass spectrometry (MS). This led to tools to automate peptide and protein sequencing with tandem MS (MS/MS). As those tools matured, our field recognized the limits of qualitatively cataloguing gene products without quantitative or other contextual information.[1,2] This realization drove a multi-pronged expansion of MS-centric technologies that seek to capture various aspects of proteins, including their abundance, modification states, conformation and structure, and spatiotemporal relationships.[3–7] Here we review innovations in MS-based instrumentation that continue to expand our ability to survey the proteome with ever increasing sensitivity, speed, and flexibility. The march of progress in MS instrumentation has been steady over the better half of the past century, but our discussion here focuses on developments within the past five years. These advances have ushered in an exciting era in proteomics, where MS is poised to be the dominant platform for exploring biological phenotypes in basic and translational sciences for the foreseeable future.

Understanding why MS instrument development has been central to advances in proteomics requires understanding the analytical needs of proteome characterization. Proteomics emerged in the 1990s and early 2000s as an indispensable tool for measuring effector molecules of biological systems. Delivering on the promise of proteomics, however, has proven challenging. The heterogeneity of protein regulation, e.g., splice isoforms and dynamic post-translational modifications (PTMs), expands the observable human proteome far beyond the ~20,000 protein-coding genes in our genome to several million.[8–10] Further, the expression of these proteoforms is highly variable, spanning a billion-fold dynamic range with some genes giving rise to single copy proteins.[11,12] Capturing the complex milieu of protein machinery and coordinated functions within the cell necessitates instrumentation with speed, sensitivity, and versatility.

These analytical challenges are not insurmountable. In fact, they have inspired several generations of instrumentation advances that push toward increased sampling breadth and depth, lower sample requirements, and structural insight for proteins and their complexes.[13–18] Here we aim to 1) describe emergent MS instrumentation and related technologies, and 2) provide a snapshot of how the field is leveraging these advances for proteome characterization. Because instrument development is a vibrant and active field within proteomics, it is not possible to describe all advances. Instead, we focus on tools used widely in the proteomics community. Additionally, advances in the “before” and “after” steps that bracket MS data acquisition are indispensable features of modern proteomics. Rather than include detailed discussion of them here, we point readers to several excellent reviews on both biochemical techniques to process samples and bioinformatics tools to translate data into biological knowledge .[19–26]

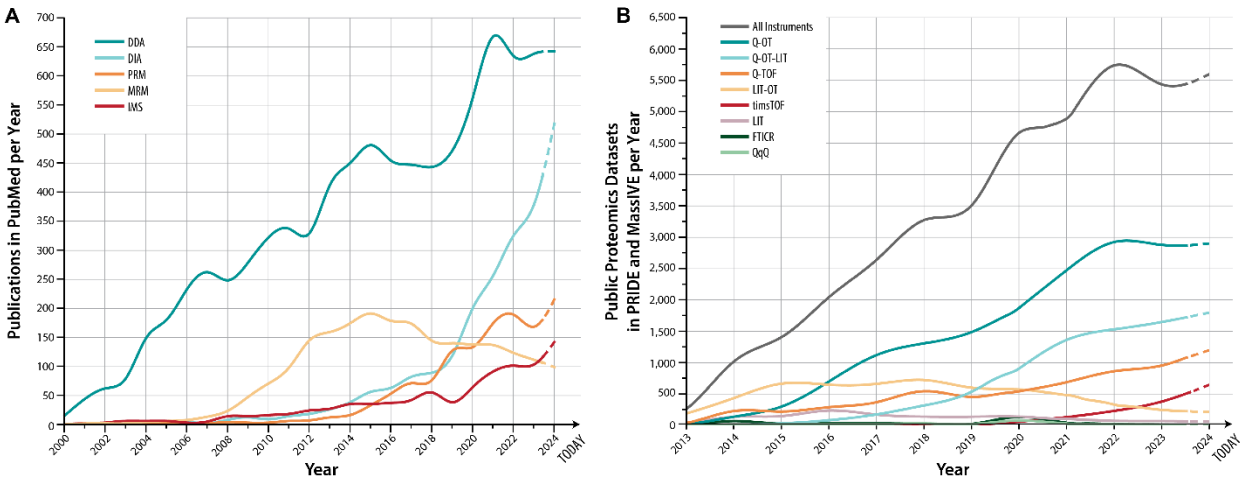


Figure 1. Snapshot of data acquisition strategies employed and instrument usage over time . A) Proteomics data acquisition strategies employed from 2000 to today indicate fading and emerging approaches in targeted, untargeted, (i.e, discovery-based) proteomics. Publications accessible in PubMed which mention in their title or abstract the terms data-dependent acquisition (DDA), data-dependent acquisition (DIA), parallel reaction monitoring (PRM), multiple reaction monitoring (MRM), or ion mobility spectrometry (IMS) in addition to mass spectrometry and proteomics were counted and plotted from 2000 to 2024. B) Instrument usage over the past decade reveals emerging technologies. All public proteomics datasets deposited in PRIDE and MassIVE data repositories were downloaded and annotated and usage for eight commonly used instrument platforms were plotted over time. Q – quadrupole; OT – Orbitrap; LIT – linear ion trap; TOF – time of flight; timsTOF – traveling ion mobility spectrometry TOF mass spectrometer; FTICR – Fourier Transform ion mobility spectrometry; QqQ – triple quadrupole mass spectrometer.

ACQUIRING MS-BASED PROTEOMIC DATA

MS-based proteomics relies on ionization of peptide or protein molecules and manipulation of those ions by electromagnetic fields within a mass spectrometer to either measure their mass-to-charge (*m/z*) ratio or to fragment them into sequence-informative product ions prior to mass analysis. How and when ions are manipulated before mass analysis is an active area of development. The ability to control ion populations continues to evolve as advances in instrumentation enable new ways to design scan sequences.

Scan functions often depend on the instrument platform being used, as the hardware available can dictate how ions can be manipulated. Data acquisition approaches are typically grouped into data-dependent and data-independent methods, named for how the instrument prioritizes which *m/z* regions to analyze at any given moment. **Figure 1a** summarizes trends in various acquisition strategies that are discussed below. Even with the diversity of methods and instrumentation available today, the evolution of proteomic instrumentation can be distilled to a common theme: improvements in proteomic data quality come from more efficient use of ion populations entering the mass spectrometer. Electrospray ionization (ESI), for example, generates billions of ions at any given moment.[27] Modern MS instruments seek to capitalize on advances in hardware and instrument control software to maximize the transformation of these ions into proteomic information.

Data-dependent acquisition (DDA)

DDA takes its name from the prioritization of MS/MS spectra acquisition based on precursors detected in survey MS spectra during an experiment, usually prioritizing ions based on relative abundance. This approach enables discovery-driven experiments with spectrum-centric data

analysis that has been the foundation for proteomics for over two decades. **Figure 1a** reflects this sustained popularity. DDA experiments have given rise to multiple MS-level label-free and isotopic labeling-based quantitation methods[28,29], where area-under-the-curve (AUC) of MS^1-level intact peptide chromatograms are used for relative quantitation. DDA can generate large sets of high-quality MS/MS spectra that are often matched with relative ease to theoretical spectra derived from sequence databases. That said, it also leads to stochastic precursor ion sampling, which can be a disadvantage with respect to run-to-run reproducibility and detection of low abundance analytes that are less likely to be sampled. These challenges can be addressed with other acquisition strategies, like targeted approaches and data-independent strategies discussed below. Even with these disadvantages, DDA remains a powerful method for identifying a large number of proteins in a sample, especially when theoretical sequence databases are the main reference information available or when alternative fragmentation methods (see below) are required.

One DDA-centric approach to improve throughput and data completeness within a defined set of samples is multiplexing via isobaric labels, which enable sample pooling and parallel data acquisition.[30–32] Commercial isobaric tags include tandem mass tags (TMT)[33] and isobaric tags for relative and absolute quantification (iTRAQ).[34] Several non-commercial options with relatively straightforward and/or low-cost synthesis have also been developed.[35–37] Commonly used 10- and 11-plex TMT kits have recently been superseded by a proline-based tag (TMTpro)[38,39], which was originally introduced as a 16-plex platform in 2019 and has since been upgraded to an 18-plex option in 2021.[40] The isobutyl-proline immonium ion reporter structure of the TMTpro tags enables increased fragmentation efficiency and signal compared to the dimethylpiperidine-based reporter of the TMT 10- and 11-plex reagents.[38,39] Further, by including a longer mass normalization group between the reporter and amine reactive group, additional carbon-13 and nitrogen-15 atoms can be variably incorporated to boost the multiplexing capacity of the reagents. Experiments using isobaric labels rely near-exclusively on systems with Orbitrap mass analyzers (see below). This is largely because high resolving power is needed to distinguish mDa differences that arise from mass defects of atoms and their isotopes (i.e., a ~6 mDa difference when using ^{13}C versus ^{15}N).[41] This so-called neutron-encoding has been explored through several strategies[42] and enables the higher plexing in current TMT schemes. Isobaric labeling also brings several challenges that have spurred the development of more nuanced acquisition strategies discussed further below.

Targeted acquisition

While the goal of DDA is to generate identifications in discovery-style experiments, targeted acquisition sits on the opposite end of the experimental design spectrum. Targeted proteomic experiments aim to detect and quantify known peptides of interest. Popular targeted acquisition techniques are multiple reaction monitoring (MRM, also called selected reaction monitoring, SRM) and parallel reaction monitoring (PRM).[43] In both MRM and PRM, predetermined precursor ions are selected for MS/MS regardless of their detection at the survey MS scan level. This is directly opposite to the detection requirement for MS/MS selection in DDA. Furthermore, MRM and PRM MS/MS scans can be optimized for individual precursors via scheduling, collision energy adjustments, and more instrument parameters. MRM experiments are largely performed using triple quadrupole (QqQ) systems, where the third quadrupole selects for a single fragment ion at a time and rasters through predetermined product ions for each precursor in sequential fashion. By contrast, PRM is usually performed on Orbitrap and time-of-flight systems and collects high resolution/accurate mass MS/MS scans containing all product ions, which simplifies method development and provides additional selectivity.[43,44] During PRM, the third quadrupole of a QqQ

is replaced with a high resolution/accurate mass analyzer to enable simultaneous detection of all target product ions in a single high resolution mass analysis. PRM performed with low resolution in the linear ion trap can also have advantages.[45] In both MRM and PRM, identification and quantitation are typically performed by aligning product ion chromatograms (rather than precursor ion chromatograms as in DDA) and using AUC information. The speed and sensitivity of QqQ instruments typically give MRM a slight advantage over PRM, but the balance of simplicity of method design and selectivity of multiple fragment ions per spectrum makes PRM a competitive option.[44,46,47] Introduced in 2012, PRM has steadily gained popularity (**Figure 1a**).[47] MRM is far more prevalent than indicated in **Figure 1a** given its wide use in clinical, biopharmaceutical, and other industrial proteomics, but data from these fields often do not get deposited into public repositories.

Data-independent acquisition (DIA)

Targeted approaches technically collect MS/MS scans in a given *m/z* range "independent of" detection of the peptide of interest, but the term data-independent acquisition (DIA) is typically reserved for discovery-based strategies that do not rely on *a priori* knowledge to create predefined lists of peptide targets. DIA methods aim not only to be broadly informative like DDA, but also to acquire MS/MS information across the LC time domain (i.e., product ion chromatograms) like targeted approaches. Rather than choose narrow isolation windows based on the presence of precursor ions in survey MS scans, DIA strategies isolate successive windows (typically ~10-25 *m/z* bins) iteratively across a defined *m/z* space for a given cycle time, after which the acquisition of successive windows repeats. The resulting highly multiplexed MS/MS spectra contain fragments from multiple precursors, which makes database searching less straightforward in DIA experiments. Instead, large DDA-based spectral libraries are traditionally acquired on a highly fractionated pooled sample, and library spectral matching is used for chromatographically aligned product ions to generate peptide identifications.[48,49] Because of the acquisition speeds required for these approaches, DIA methods almost exclusively use collision-based fragmentation (see below), but can be coupled with DDA-based library building methods that use alternative dissociation methods.[50]

This reliance on experiment-specific, empirically acquired DDA spectral libraries is still common[51–53], but is diminishing as spectral prediction[54–56], pseudo-MS/MS spectra (e.g., directDIA)[57–59], and gas-phase fractionation approaches[60–62] rapidly develop. Indeed, while the term DIA was coined in the early 2000s,[63] the popularity of DIA reached an inflection point in the early 2010s as methods like SWATH-DIA emerged on quadrupole-time-of-flight hybrid instruments.[64,65] DIA datasets can provide both MS- and MS/MS-level quantitation: i.e., precursor and product ion AUC data. The vast majority of DIA experiments rely on label free quantitation using product ion AUC because multiple-ion measurements provide more robust quantitation.[66–71] That said, several groups have explored the utility of stable isotopic labels for DIA methods, including metabolic labeling with SILAC[72–75] and chemical labeling via mTRAQ, dimethyl labeling,[76] and other custom tags.[77] Isotopic labeling can also be used for MS/MS-level quantitation in DIA experiments, although data analysis is not straightforward.[78–82] Muntel et al. showed that DDA with TMT quantitation and label free DIA quantitation both offer high utility, with slightly higher identifications and quantitative precision with DDA-TMT compared to better quantitative accuracy for DIA.[83] Furthermore, convergence of targeted and discovery strategies are blurring the lines between what were originally considered disparate strategies.[84,85] Even as studies race to benchmark identification rates in various experimental regimes, MacCoss and colleagues have poignantly noted that quantifiable peptides are truly the currency of modern proteomics, and that methods should be judged by the number of quantifiable peptides they can reproducibly sample across an experiment.[86]

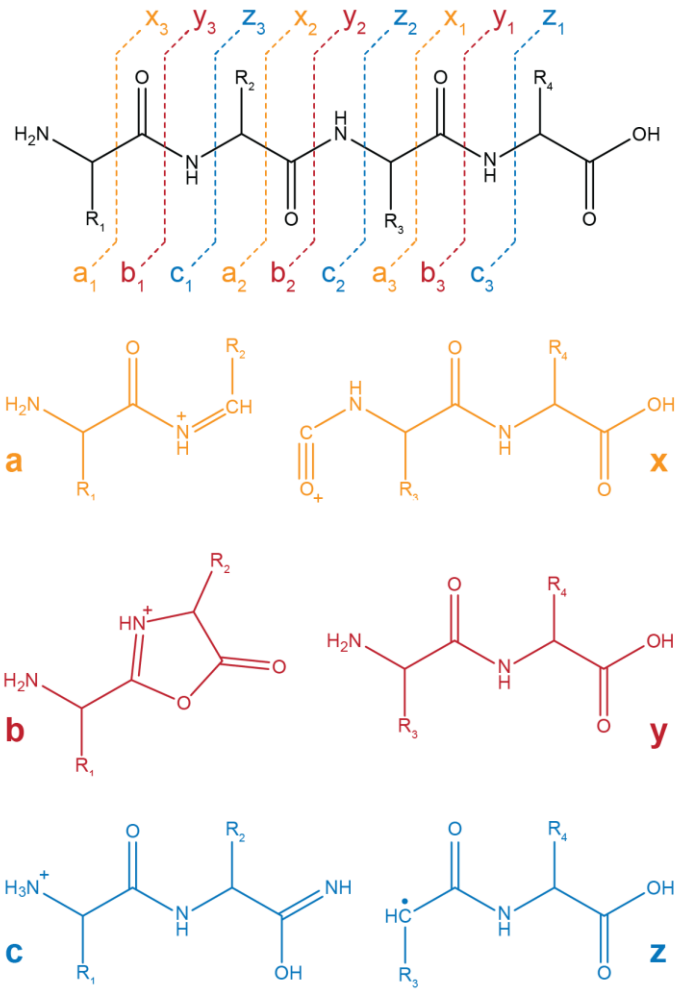


Figure 2. Sequence-informative product ions from peptide backbone dissociation. Ion activation can induce backbone cleavage along several dissociation channels depending on the method of activation performed. Dissociation leads to the generation of complementary a- and x-type ions, b- and y-type ions, or c- and z-type ions.

Ion activation methods

Collisional Activation. Ion activation is a key feature of MS/MS approaches used to characterize the proteome.[87,88] Collisional activation is easily the most ubiquitous form of ion activation across all instrument types, mainly because it is fast, efficient, and easy to implement. The goal of collisional activation is to vibrationally excite peptide ions through collisions with gas atoms or molecules until their internal energy becomes high enough to drive backbone bond fragmentation into sequence-informative product ions. This outcome can be achieved via multiple avenues. Beam-type collision induced dissociation (beam-type CID, bCID, sometimes called higher-energy collisional dissociation, HCD) is a universal approach available on virtually all MS instruments. Here precursor ions are accelerated into a collision cell containing ~5–10 mTorr of gas (usually argon or nitrogen) with enough kinetic energy to break backbone bonds with one ion-molecule collision. Nearly all modern instruments can activate ions using bCID (<1 keV) to form b- and y-type ions that form as N- and C-terminal products of peptide bond dissociation (**Figure 2**). Some

ion trapping devices can also impart collisional activation of selected precursors using resonant excitation to induce collisions of the precursor with a bath gas (resonant CID, rCID). rCID generally relies on multiple collisions with background helium molecules (~3-5 mTorr) to build internal energy, leading to its reference as a "slow heating" method. The slow heating of rCID also leads to b- and y-ion formation, but as soon as dissociation occurs, product ions fall out of resonance with excitation frequencies, meaning that little secondary fragmentation occurs. In contrast, bCID can have secondary dissociation events as multiple collisions between ions and collision gas occur during transfer into the collision cell. This can lead to product-ion dissociation events such as a-type ion formation from b-type products (**Figure 2**), immonium ion generation through specific amino acid side chain losses, and labile modification losses from b- and y-type product ions. Even though dissociation is most often the goal of collisional activation, collisions can also be used to increase vibrational energy of ions to desolvate, unfold, or otherwise structurally alter ions without causing dissociation[89], such as collision-induced unfolding used to probe protein gas phase structure.[90]

Surface-induced dissociation (SID) is an activation method related to collisional activation that has proven especially useful for characterization of native protein complexes.[91,92] Collisions with a surface in SID impart high enough energy to prevent energy redistribution throughout the molecule or multi-molecular complex, which can result in subunit dissociation in protein complexes rather than backbone cleavage or labile PTM loss.[93–95] SID devices have gone through several iterations, and multiple generations have been implemented into research-grade instruments.[96–103] A commercial version of SID is incorporated into the Select Series Cyclic IMS Q-TOF from Waters. Wysocki et al. recently reviewed SID and its use as a structural biology tool.[104]

Electron-based activation. Ion-electron and ion-ion reactions have steadily gained use as an alternative means for fragmentation using electron-driven dissociation, which generates c- and z-type ions through radical-driven cleavage of the N-Cα bond (**Figure 2**).[105–108] Electron-capture dissociation (ECD, ion-electron reactions) and electron-transfer dissociation (ETD, ion-ion reactions) provide complementary information to collision-based methods for peptides and proteins.[109] These and related approaches have been collectively referred to as ExD methods. Ion-electron reactions have historically been confined to Fourier transform ion cyclotron resonance (FTICR) instruments because of the challenges of controlling both cationic peptides/proteins and electrons with alternating radiofrequency (rf) currents, whereas ion-ion reactions for ETD can be conducted in rf-only devices without the need for magnetic fields. Fluoranthene (*m/z* 202.08) is commonly used as the ETD reagent anion generated through electron ionization or chemical ionization in a secondary source. That said, xenon, sulfur hexafluoride, and many other reagents have also been explored.[110–112] One implementation of ETD reagent anion generation called "front-end" ETD generates reagent anions via glow discharge in the high-pressure region at the inlet of the mass spectrometer.[113] This strategy has greatly improved ETD robustness and accessibility on commercial instruments.[113] More recently, ion-electron reactions have gained popularity thanks to new devices, such as 1) the e-MSion ExD cell that uses ring magnets to confine electrons to a region between rf-only devices[114,115], 2) the Omnitrap platform that uses rectangular waveforms and side injection of electrons to enable ion-electron interactions[116], and 3) the branched rf-ion trap on the newest SCIEX ZenoTOF instrument whose architecture allows intersection of an orthogonally injected electron beam with trapped precursor ions.[117,118]

One shared challenge of ExD methods is non-dissociative electron capture or transfer (EXnoD). In EXnoD, electron capture/transfer generates backbone cleavage, but non-covalent gas-phase

interactions keep product ions complexed together, ultimately providing no sequence information.[119] This phenomenon has inspired several groups to explore supplemental activation to improve dissociation efficiencies, including use of rCID[120], bCID[121,122], infrared[123–129] and ultraviolet photoactivation[130,131], and varying electron energies.[132,133] ExD methods also offer access to peptide anion sequencing, which is generally unsuccessful with collision-based approaches.[134–143] Electron-based approaches remain popular for investigations of post-translationally modified peptides and proteins[144–146], non-tryptic peptides such as HLA peptides and those generated with alternative proteases[147–150], and structural proteomics using hydrogen-deuterium exchange or native protein analysis.[151]

Photon-based activation and other methods. Photoactivation via infrared multiphoton dissociation (IRMPD) or ultraviolet photodissociation (UVPD) has been used for peptide and protein activation for over 30 years.[152] IRMPD largely relies on CO_2 lasers that generate 10.6 μm photons. This relatively low photon energy leads to many similar dissociation characteristics of other slow heating methods like rCID, including b- and y-type ion generation[153–155] (**Figure 2**), and IR activation can also be used to heat ions without causing dissociation.[156–160] Although challenges in implementing IRMPD have precluded widespread use, recent work has shown potential promise in considering how to bring IR lasers to modern mass spectrometers.[126,127,161,162]

UVPD describes a more heterogeneous collection of photoactivation modalities, including photons in the 157 nm, 193 nm, 213 nm, 266 nm, and 355 nm regimes, all of which have been explored for proteomics.[163] Higher-energy UVPD methods (e.g., 193 nm and 213 nm) activate peptides through both vibrational and electronic pathways to generate all six backbone product ion types (a-, b-, c-, x-, y-, and z-ions; **Figure 2**) in addition to side chain fragmentation and other dissociation pathways.[164–166] This proclivity for fragment ion generation is a primary benefit of UVPD but also introduces perhaps its greatest challenge: UVPD MS/MS spectra are rich with sequence information, but the numerous dissociation channels cause overlapping ion signals and signal dilution that can ultimately make both manual and automated annotation difficult. The Brodbelt group has been a pioneer in 193 nm UVPD, showing its benefit for peptides, proteins, lipids, and other biomolecules. They have also explored methods to improve its analytical utility through a variety of strategies, e.g., ion parking and fragment protection, proton transfer reactions to simplify spectra, and selective precursor ejection to improve S/N of product ions.[167–169] Even with the benefits shown by work from them and others, the Excimer laser needed for 193 nm photons makes commercialization challenging. Instead, the commercialization of UVPD on Orbitrap Tribrid systems uses a solid-state CryLaS laser to deliver 213 nm (~1.2 μJ per pulse) for photoactivation.[170] Several groups have explored the benefits of UVPD for proteomics and other biomolecules, showing that it can also generate information-rich spectra for intact antibody sequencing[171], modified RNA[172], and bacterial lipids.[173] Non-commercial versions of UVPD on time-of-flight systems have also been explored.[174–180]

MS-CENTRIC TECHNOLOGY USED IN MODERN PROTEOMICS

Perhaps no single decision receives as much attention in a proteomics laboratory as the acquisition of a new mass spectrometer. It is far from a simple decision, especially with numerous instrument features, architectures, and vendor options available. In this section we describe basic operating principles of common mass analyzers and summarize many of the latest and most widely adopted mass spectrometers used in proteomics. This includes performance metrics to highlight the benefits and considerations of each, with metrics largely sourced from vendors themselves. **Table 1** collates this information for several contemporary commercial instrument

platforms. We also made **Figure 1b** using publicly available datasets show trends in instrument use across the field.

Mass analyzers

The principal component of a mass spectrometer is its mass analyzer(s), which manipulate ions based on their *m/z* values. Quadrupoles, ion traps, time-of-flight (TOF), and Fourier transform (FT) devices are the four mass analyzers most widely used in proteomics. Most modern MS instruments are hybrid instruments that combine multiple mass analyzers. Common hybrid instruments include quadrupole-time-of-flight (Q-TOF), quadruple-Orbitrap (Q-Orbitrap), and quadrupole-Orbitrap-linear ion trap (Q-Orbitrap-LIT, or Tribrid) platforms. Note, descriptions of Q-TOFs and Q-Orbitraps throughout this work focus on instruments with dedicated rf-only collision cells, which can also be referred to as Q-q-TOFs and Q-q-Orbitraps. Notably absent from this list are triple quadrupoles (QqQ) instruments, which are ubiquitous and used in innumerable applications via MRM assays.[181,182] That said, they are not often the focus of cutting-edge proteomic developments, where strategies to improve untargeted/discovery methods remain at the forefront. As such, we do not dedicate much time to them here.

Hybrid mass spectrometers enable acquisition schemes that efficiently utilize ion populations entering the instrument, with the goal of maximizing both selectivity and sensitivity. The quadrupole mass filter uses combinations of direct current (DC) and rf electric fields to create stable and unstable trajectories of ions of specific *m/z* values as they traverse the length of the analyzer.[183] This feature allows quadrupoles to serve as an *m/z* filter for mass selection of ions of interest, and rapid scanning functions make them particularly useful as a first stage of mass analysis.[184] Quadrupoles can also be operated in rf-only mode (i.e., without a resolving DC potential) to serve as collision cells for peptide fragmentation.[185] Recent developments in quadrupole hardware have directly impacted hybrid MS platforms. Improvements in transmission efficiencies, scanning functions, and switching speeds have benefited several Q-TOF, Q-Orbitrap, and Q-Orbitrap-LIT systems.[186–189] This is especially true in high-throughput proteomic methods that are trending toward timescales using only several minutes per LC-MS/MS acquisition. In these cases, faster quadrupole rf switching between selected *m/z* isolation ranges enables more MS/MS spectra acquired per unit time.

Linear ion traps (LITs), which often adopt modified quadrupole architectures, can also be used to isolate ion populations. Both quadrupoles and LITs use electron multipliers as detectors, providing relatively high sensitivity. Unlike the scanning function of quadrupoles, however, LITs enable accumulation of large ion populations, making ion traps particularly sensitive mass analyzers.[190] LITs perform mass analysis through sequential ion ejection from the trap using resonant excitation. Scan out of ions (i.e., mass analysis) in LITs is fast, but scan acquisition rates with LITs is often determined by ion accumulation times rather than scan out speeds.[191,192] LITs are notably useful for multiple stages of mass analysis on a population of ions, and they can serve as reaction vessels for ion-ion reactions and other tandem MS modalities.

Orbitrap and FTICR mass analyzers are also trapping devices. They rely on motion of trapped ions within the device to generate image currents on detector electrodes, which are then measured as a function of time (i.e., a transient).[193–195] Ion motion is proportional to *m/z*, so that frequencies of ion motion are Fourier-transformed from the time domain to the *m/z* domain to perform mass analysis (i.e., FTMS).[196] Both mass analyzers offer ultra-high resolution (>1 million). Performance metrics to describe them are usually defined in terms of high resolution/accurate mass (HR/AM), which are reported as resolving power at a given *m/z* value or mass accuracies reported in ppm.[197,198] In both FTICR and Orbitrap mass analysis, increasing the transient time

improves mass resolution.[199] For FTICR, increasing the strength of the magnetic field can also improve resolution. Commercial versions offer up 12T and 15T magnets[200], while research-grade instruments boast 21T magnets.[201,202] In Orbitraps, the amplitude of the electric field applied to the central electrode directly affects resolution, but device dimensions must be adjusted for practical application of higher fields. Currently there are two commercially available Orbitraps, with the compact high field trap offering the superior performance.[203] FTICR instruments generally achieve higher levels of mass accuracy, in part because of the stability of superconducting magnets relative to rf voltages. FTICR instruments can also be used as standalone instruments for tandem MS experiments via stored-waveform inverse Fourier transform waveforms for isolation.[204] Even so, they are limited in their sensitivity, acquisition speeds, and practical availability (given high acquisition and maintenance costs of superconducting magnets). Orbitraps have become a dominant mass analyzer in most high-performance proteomics research, where they almost exclusively operate in combination with other mass analyzers in hybrid instrument platforms (**Figure 1b**).[205] Use of external devices to accumulate and store ions prior to their injection into FTMS analyzers boosts sensitivity, and recent work measuring individual ions in Orbitraps highlights their sensitivity for specific applications.[206–211] FTMS is an active area of research with multiple recent reviews that can dedicate more discussion to fundamental principles.[212–216]

TOF mass analyzers are also popular, and recent years have seen a surge in TOF-based instruments (**Figure 1b**). Rather than use ion trajectories and stabilities for mass analysis, TOFs use a defined electric field to accelerate ions toward a detector.[217] Ions with different *m/z* values will have different velocities as they travel along a drift region, resulting in arrival times proportional to *m/z* and charge. This detection regime means that electric field "pushes" can happen in rapid succession, lending very high acquisition rates (>100 MS/MS scans per second, i.e., >100 Hz) to TOF-based systems. High acquisition speeds come at the cost of sensitivity because only so many ions are analyzed per push. Modern TOF instruments seek to address this limitation in various ways discussed below.[218] TOFs also offer moderate-to-high mass resolution (>100,000), which can be improved by increasing the flight path of ions through longer drift tubes and reflectrons that increase the total distance traveled prior to detection.[219] A recently introduced Asymmetric Track Lossles (Astral) analyzer, discussed further below, leverages principles central to TOF mass analysis but uses an electrostatic trap device to stabilize ions in a dielectric field during their 30 meter transversal of the device to significantly improve ion transmission.

Ion mobility spectrometry (IMS)

Many modern mass spectrometers take advantage of ion mobility spectrometry (IMS), which separates ions by size and shape to enable multi-dimensional gas-phase fractionation methods that complement *m/z* measurements made by mass analyzers.[220,221] While mass analyzers often operate in low pressure regimes of 10^{-3}-10^{-5} Torr (ion traps and quadrupoles) or at ultra-low pressures of 10^{-7}-10^{-10} Torr (TOFs and FTMS), IMS uses higher-pressure regions commonly containing helium, nitrogen, or air for separation, storage, and structural analysis of peptide and protein ions.[222] IMS offers increased peak capacity and dynamic range in proteomics experiments, in addition to gas-phase separation of molecular isomers that is not always feasible with liquid chromatography.[223] Recent technological innovations in IMS, especially when paired with state-of-the-art mass analyzers, are empowering breakthroughs in high-throughput proteomics and structural biology via mass spectrometry.[224]

Four common IMS strategies include drift tube IMS (DTIMS), traveling wave IMS (TWIMS), trapped IMS (TIMS), and high-field asymmetric waveform IMS (FAIMS). DTIMS uses a uniform electric field to drive ion movement along a given path length (i.e., a drift tube).[222] As ions traverse

the drift tube, they collide with neutral drift gas molecules that slow their movement toward the detector as a function of their collision cross section (CCS). Separation quality is affected by drift tube length, which is limited by the need for high drift voltages across the drift region. Canonically, the speed of ion mobility separations, in microseconds to milliseconds, has driven the dominance of Q-TOF mass spectrometers as mass analyzers for ion mobility systems, but ongoing work has also coupled DTIMS to FTICR and Orbitrap systems.[225–230] The development of Fourier Transform IMS, modular DTIMS instrumentation, and cheaper alternative power supplies are poised to enable more accessible MS instrumentation and development, especially for IMS.[231–234]

TWIMS uses an electric field applied to a small portion of a low-pressure drift tube. This field then moves along the length of the tube, pushing ions toward the detector. This "wave" relieves needs for high electric fields in DTIMS, but a tradeoff is that TWIMS does not provide directly accessible CCS measurements. Even so, improvement in TWIMS platforms have enabled deeper proteomic characterization in recent years. Increasing the TWIMS path using cyclic devices allows gas-phase separation on the tens-of-meters scale without the instrument footprint demands of a linear system.[235] This strategy was recently commercialized with Waters Corporation's Select Series Cyclic IMS, which is a cyclic IMS-TOF instrument.[235,236] Considerations for such a platform include sensitivity constraints, preventing users from cycling through the ion mobility cell hundreds of times. An exciting variation on TWIMS is structures for lossless ion mobility separations (SLIM) from Richard Smith and coworkers[237–239], which uses printed circuit board designs that improve sensitivity over conventional drift-tube approaches and allow for extended path lengths (>1,000 m in some cases[240]) for enhanced resolution in compact arrangements.

TIMS is rapidly gaining traction as a widely-used gas phase separation technique. TIMS uses gas flow (2 to 3 mbar) to counteract kinetic energy imparted by an analytical DC field, trapping ions in the device in axial positions that correspond to their mobility.[241–243] Higher CCS (usually corresponding to higher *m/z* species) cause higher drag forces from the gas flow, meaning higher field strengths are needed to counterbalance the drag. Thus, lower mobility ions with higher CCS are trapped closer to the exit of the TIMS device. As the analytical DC field strength is progressively lowered to eject ions from the device, higher *m/z* species are released first, followed by ions of increasing mobility and typically decreasing *m/z*. TIMS can resolve congested structural features while also enabling determination of an ion's relative size. Silveira et al. reported high resolution TIMS analysis of several model peptide ions in 2014.[244] The latest generations of timsTOF instruments from Bruker use dual TIMS devices to accumulate ion packets within the first cell and perform ion mobility separations within the second cell, making use of a large majority of ions entering the instrument to boost sensitivity (up to five orders of magnitude on newest instruments). Tandem TIMS (tTIMS), which places an ion gate between two TIMS devices, has also been explored for time-resolved measurements of fragment ions in peptide and protein analyses.[245–247]

FAIMS (also called differential mobility spectrometry, DMS) was originally developed over two decades ago, but has made an impact on proteomics in recent years.[248] In FAIMS, a carrier gas moves ions between two electrodes, where a high voltage asymmetric waveform is applied. Ions exhibit different electric mobilities during the high and low periods of the oscillating waveform, resulting in stable and unstable ion trajectories through the device based on *m/z*, charge, and CCS. A tunable compensation voltage (CV) is used to rescue the transmission of ions of certain mobilities, and CVs can be switched to favor transmission of different ion populations. As FAIMS can be added immediately after ESI prior to the atmospheric pressure inlet of the MS system, separations of ion populations are performed before they enter the MS, adding a complementary gas-phase fractionation step to separate co-eluting peptides and isomers[249–251] or remove

background ions. FAIMS can reduce co-isolation from interfering ions, which can be particularly valuable in quantitative proteomics using isobaric labels [252–255], analysis of post-translationally modified peptides [256–260], single-cell proteomics [261–263], and direct-infusion proteomics that forgo traditional frontend separations and instead rely completely gas-phase separations.[264–266] The benefits of FAIMS for analysis of intact proteins[267,268] and small molecules[269,270] have also been explored.

Common hybrid MS architectures

Q-Orbitraps and Q-TOFs are among the more popular instrument platforms. They build on the QqQ architecture by replacing the third quadrupole with the respective mass analyzer to make them more suitable for both discovery methods and PRM assays. The Q-Orbitrap was first introduced as the Q-Exactive system in 2011.[271,272] A series of instrument platforms followed, including: the Q-Exactive HF with a high field Orbitrap for improved resolution and scan speeds[273,274], the Q- Exactive HF-X with the high field Orbitrap and an electrodynamic ion funnel for improved sensitivity[275], and the Q-Exactive UltraHigh Mass Range (UHMR) with a mass range up to *m/z* 80,000 for intact protein and native MS.[225,276] In 2020, a re-designed Q-Orbitrap platform called the Orbitrap Exploris was introduced with a streamlined architecture for a smaller footprint and easier maintenance.[277,278] This system also interfaces with Thermo's commercial FAIMS Pro source. The Exploris platform offers multiple options, including the Orbitrap Exploris 120 designed primarily for metabolomic analyses and the Orbitrap Exploris 240 and 480 that enable varied scan speeds (up to 22 and 40 Hz, respectively) and superior resolution (up to 240k and 480k FWHM at *m/z* 200, respectively) for high-throughput proteomics applications of complex mixtures. Recent work using ion accumulation in the front optics showed that 100 Hz can be achieved on an Exploris 480, although practical use is likely in the 80 Hz range.[279] The combination of speed, sensitivity, and HR/AM led to quick adoption of the Q-Orbitrap platform across its various generations, as reflected in its leading use in proteomic research (**Figure 1b**). Additionally, several non-commercial modifications have enabled UVPD[280–284], IRMPD[154,164], SID[285,286], and ECD[287,288] on Q-Orbitrap systems, and the newly developed Omnitrap brought multiple ion activation modes to Q-Orbitrap instruments.[289–291]

The speed and simplicity of Q-TOFs have been integral in their appeal for the better part of two decades, but the sensitivity lost through the fast acquisition speeds has been a challenge. Improvements in ion transmission efficiencies to and within TOF analyzers has fostered gains in sensitivity; this is reflected by the recent rise in popularity in studies using Q-TOF instruments (**Figure 1b**), even though Orbitrap systems remain the most popular (at least in terms of deposited datasets). From Bruker, the Impact II introduced in 2015 offers improved resolution (60k) and scan acquisition speeds (60 Hz) over the Compact system,[292] and the MaXis II brings further resolution improvements (80k) with 50 Hz acquisition speeds and the ability to do both bCID and ETD.[293] The Impact II, with both axial and radial ion ejection from its collision cell for improved ion extraction, showed promise for proteomics, but that excitement has been largely transferred to Bruker's newest instrument platform that incorporates trapped ion mobility spectroscopy (TIMS) into the Q-TOF platform, i.e., the timsTOF. In timsTOF instruments, two TIMS cells are oriented upstream from the quadrupole to accumulate ions entering the instrument before ion packets are sequential eluted by CCS and mass selected by the quadrupole. The trade-off between speed, sensitivity, and dynamic range in TIMS has inspired several instrument developments.[294] In the timsTOF SCP, sensitivity down to zeptomolar to attomolar levels is attainable at speeds up to 120 Hz.[295,296] With the newer v.4 TIMS cartridge present in the timsTOF HT and timsTOF Flex, higher scan speeds up to 150 Hz are possible, albeit at a less sensitive attomolar to femtomolar level. Since more ions can be stored and manipulated in the v.4 TIMS cartridge, a wider dynamic range of $1x10^5$ is seen in the HT and Flex relative to $5x10^4$ in the SCP.[297–299] The Flex is compatible with

both ESI and matrix-assisted laser desorption/ionization (MALDI) ion sources[300,301], and real-time data analysis through the parallel search engine in real-time (PaSER) is available on the timsTOF Pro 2 and other newer models.[302,303] The recently released timsTOF Ultra enables scan speeds approaching 300 Hz by integrating sensitivity of the timsTOF SCP with the dynamic range performance of the timsTOF HT, all while creating superior front-end ion transfer efficiency compatible with high scan speeds.[297,304–307] Although not commercialized, UVPD and ECD have been coupled to timsTOF instruments with varying degrees of in-house modifications.[180,308–310]

Several other vendors offer IMS coupled to Q-TOF systems. In the Agilent 6560, a drift tube for DTIMS is placed in front of the quadrupole, similar to the IMS-Q-TOF design of the timsTOF.[311] Other Agilent 6500 series instruments do not have IMS, but a collaboration between Agilent and MOBILion recently introduced the Mobie system that couples SLIM with Agilent's 6500 line of Q-TOFs. ECD capabilities have also been explored for the 6500 family.[312] Waters introduced the Select Series Cyclic IMS system in 2019 that has a 1 m cyclic TWIMS cell oriented after the quadrupole, allowing for isolation of isobaric species, including structural isomers.[235,236] The unique design of the cyclic IMS device enables iterative ion mobility separations before ions are shuttled to and mass analyzed by the TOF cell, and this instrument also offers SID and ECD fragmentation modes.[313] Waters also offers a more traditional Q-TWIMS-TOF system in their Synapt XS model and a non-IMS Q-TOF system called the Xevo G3. The Q-TWIMS-TOF system offers commercial ETD functionalities, in addition to research-grade modified systems that enable UVPD,[314] SID,[285,315–320] and other ion-molecule reactions.[321–328]

The ZenoTOF 7600 from SCIEX is among the newest Q-TOF instruments with an acquisition rate up to 133 Hz, replacing the TripleTOF 6600 model.[329] Unlike the Q-TOFs above, the ZenoTOF 7600 introduced two new devices: 1) a modified branched rf-ion trap (branched ion trap, BIT) for EAD-type activation located after the quadrupole but upstream from a collision cell[330], and 2) a small LIT called a zenotrap located directly after the collision cell but prior to the TOF analyzer. Besides the benefits of new ExD capabilities on this system, the zenotrap accumulates ions immediately before injection into the TOF cell, which can greatly improve sensitivity (attomolar limits of detection[331]) and also extends the upper *m/z* cut-off to 40,000.[330,332,333] The ZenoTOF 7600 does not currently incorporate the SelexION FAIMS source available on other SCIEX instruments, but with the momentum in IMS-Q-TOF development from other vendors, this may change in the future. Additionally, improving resolution to compete with the HRAM capabilities of the Orbitrap is of interest for Q-TOF manufacturers, but large and spatially demanding time-of-flight cells are necessary to confer high resolving power (>50k). The recently released Select Series MRT (Multi Reflecting TOF) from Waters provides extended flight paths up to 50 m using ~50 passes in a 1 m TOF tube to save space while enabling up to ~200,000 resolving power. Ions are focused with each successive pass to aid ion transmission, but sensitivity may be limiting relative to other platforms.[334–337]

The recently released Astral (Asymmetric Track Lossless) mass analyzer from Thermo Fisher Scientific builds on TOF principles but adds an electrostatic trapping device that combines temporal and spatial focusing across a ~30 m time-of-flight separation. The Astral analyzer was designed to address low ion transmission that limits the sensitivity of TOF instruments[338,339] while also enabling up to 80,000 resolving power, 200 Hz scan speed, and single-ion detection sensitivity.[340] Integrated alongside a HR/AM Orbitrap, the Orbitrap Astral MS introduces a fast-switching quadrupole mass filter congruent with downstream speeds of ion detection, a dual-pressure ion processor cell for ion accumulation[341], fragmentation and subsequent accumulation, ion injection optics for efficient orthogonal extraction into the Astral analyzer, and a high dynamic range conversion dynode detector similar to the linear ion trap (rather than multichannel plates

used in traditional TOFs).[342–344] Five separate populations of ions can be manipulated at once (quadrupole, Orbitrap, ion routing multipole, ion processor, Astral analyzer) to enable MS/MS scan speeds approaching 200 Hz that are matched with HR/AM survey MS scans synchronously acquired in the Orbitrap.[340] Transmission from the ion routing multipole to the detector is estimated to be > 50%, introducing a remarkable gain in sensitivity over conventional TOFs that lose a vast majority (~99%) of ions at the orthogonal extractor or from ion beam divergence. This sensitivity in turn enables ion injection times of 3 ms, driving compatibility with novel methodologies such as narrow-bin DIA and rapid LC-MS/MS experiments, discussed in more depth below.[340,345–348]

Beyond Q-Orbitraps and Q-TOFs, the versatility, sensitivity, speed, and stand-alone capabilities of ion traps drove their adoption into diverse instrument architectures, most notably in combination with high-resolution Orbitrap and FTICR mass analyzers. In the first generation Orbitrap systems, like the LTQ Orbitrap XL,[349] LTQ Orbitrap Velos,[350] and Orbitrap Elite[351] hybrid systems, the LIT functions as both the *m/z* isolation device and the reaction cell for ion activation, although a dedicated collision cell was introduced behind the C-trap in later models[352,353] and multipurpose dissociation cell options were explored.[354,355] In 2013, the LIT-Orbitrap coupling was reimagined as a Tribrid MS system that integrates the Q-Orbitrap-LIT architecture to enable faster scan speeds (~20 Hz) and more versatility through parallelization of various mass analyzer functions.[356] In these instruments, an rf-only storage cell called the ion routing multiple (IRM) exists between the C-trap and the LIT, and the IRM functions as the central routing hub for ions during scan functions in addition to being the bCID collision cell. Tribrid systems have progressed through four generations thus far, including the original Orbitrap Fusion, the Orbitrap Fusion Lumos in 2015[357], the Orbitrap Eclipse in 2019[253], and most recently in 2022, the Orbitrap Ascend.[187] Besides the new architecture, the Orbitrap Fusion introduced commercial versions of synchronous precursor selection (SPS),[358] front-end ETD[113], and EThcD supplemental activation. The Orbitrap Ascend builds on the streamlined design of the Orbitrap Exploris platform and has a second rf-only IRM in front of the C-trap, which affords the ability to manipulate three different ion population simultaneously to boost sensitivity without sacrificing ~50 Hz scan rates.[187,359] Comparatively, scan rates peak at ~40 Hz on Fusion Lumos and Eclipse models. On top of these instrument-specific improvements, the Orbitrap Eclipse and Orbitrap Ascend both can be equipped with proton transfer reaction capabilities, which is an ion-ion reaction that strips charges from ions and thus can be useful to simplify spectra by spreading signal out in *m/z* space.[360–365] The flexibility of the Tribrid platforms has led to their broad use in proteomics, only behind the Q-Orbitrap (**Figure 1b**), and Tribrid platforms have housed several (currently) non-commercial developments, including versions of AI-ETD[126,127,366–368], negative ETD[142], UVPD[369], and IRMPD.[126,127]

Outside of the Orbitrap systems, the other main commercial option for FTMS is Bruker's solariX platform, which offers 7T, 12T, and 15T magnet options that provide >10M resolving power and 0.6 ppm, 0.3 ppm, and 0.25 ppm mass accuracy, respectively. These instruments are Qq-FTICR instruments with the rf-only collision cell (q) having trapping capabilities for ETD fragmentation (including negative ETD), in addition to ion-electron activation (e.g., ECD, EDD, and niECD) in the ICR cell.[370–375] The solariX platform has also been modified for SID as well as UVPD and IRMPD activation, although these are not commercial options.[285,376–381] The performance of these instruments has been enhanced in recent years by introduction of the ParaCell ICR cell on the solariX XR and 2xR platforms, which ultimately improves resolving power for a given magnetic field and thus offers increased performance metrics even when magnetic field strength limitations cannot be overcome.[382,383] Additionally, a collaboration with government labs and several instrument companies (namely, Thermo and Bruker) have resulted in two world-record 21T FTICR instruments that are accessible through collaboration programs.[201,202] Even though FTICR instruments have become increasingly less prevalent in proteomics research, they remain highly

advantageous for intact protein and top-down analyses, in addition to petroleomics, metabolomics, and other analyses that benefit from fine isotope structure determination.[384,385]

Note that many of these instrument platforms have routinely incorporated improvements in ion source developments, which include larger inlets, ion funnels and active beam guides, and improved vacuum systems. In addition to ESI under focus here, MALDI is also a valuable tool for proteomics, especially for in-situ imaging analyses.[386–390] MALDI sources have been explored on several instrument platforms mentioned above, including the timsTOF (commercial), solariX (commercial), LIT-Orbitrap (LTQ model, commercial), and many other TOF-based systems. Work on ambient ionization – e.g., desorption electrospray ionization[391], secondary ion MS (SIMS)[392,393], direct analysis in real-time[394], paper spray ionization[395] – has led to exciting developments like the MasSpec-Pen[396–398] and other techniques discussed thoroughly in recent reviews.[399–401] Still, spatial proteomics through methods like DISCO-MS that combine traditional LC-MS/MS proteomics with advanced imaging/image analysis are also advancing rapidly outside of the spatial proteomic advances of MALDI-centric workflows.[402] Several of these ionization strategies, especially MALDI, have pushed imaging MS toward a fully mature approach for proteomics[403], as well as for metabolomics, lipidomics, and glycomics.[404–407] Note, other instrument architectures (e.g., the GC-Orbitrap[408,409]) have benefits for small molecules and other -omics, but we do not have space to discuss them here.

Exciting instrument developments that couple more exotic ionization sources to mass analyzers have also shown promise for protein expression analysis. Mass cytometry functions as an expanded multiplexed immunolabeling strategy (~45 targets), where antibodies tagged with lanthanide metals are used to label cells.[410] Individual cells can be separated with flow cytometry and ionized via inductively coupled plasma, allowing metal ions to be detected by TOF mass analysis. This strategy provides information about protein expression at a single cell level, which can be highly valuable when target proteins are already known.[411,412] There have been four versions of commercial mass cytometry systems, i.e., the CyTOF family of systems offered by Standard BioTools (formerly Fluidigm), with the CyTOF XT being released in 2021. An iteration on this idea, multiplexed ion beam imaging by TOF (MIBI-TOF) also uses metal-tagged antibodies, but it offers the ability to quantify expression of multiple proteins (~45) in tissues while preserving spatial information, effectively serving as a sensitive and multiplexed immunohistochemistry analysis.[413,414] MIBI-TOF uses SIMS, where a primary ion source shoots atomic ions (i.e., O^{2+}) at the specimen to release secondary ions (*i.e.*, the elemental metal reporters from the antibodies).[415] SIMS is highly sensitive (limit of detection as low as five atoms) and can achieve imaging resolutions as low as 10 nm (~100-fold lower resolution than MALDI), which is below the light diffraction limit to permit super-resolution imaging.[416,417] Ultimately MIBI-TOF results in a high-dimensional image showing expression of multiple proteins. Ionpath offers the MIBIscope System, the only commercial MIBI-TOF system currently available. CyTOF and MIBI-TOF require *a priori* knowledge of protein targets and rely on robust antibody-metal conjugates, which largely prevent them from use as discovery tools.[418] Instead, they are orthogonal tools to standard MS instruments used in proteomics that can provide complementary information.

Overall, commercial instruments dominate the proteomics landscape, but many commercial features are driven by proof-of-principle demonstrations in academic and government labs. Several exciting MS developments reported in recent years that have yet to be commercialized or are on the brink of commercialization include multiplexing of ESI sources into one instrument[419], ion trap arrays and coupling of multiple FTMS/TOF mass analyzers into one instrument[420–423], mass-selective beam deposition and matrix-landing MS for native protein analysis[424–426], electron-

based dissociation of both cations and anions coming to more instrument platforms, and the continued expansion of IMS options to more instrument types. As engineers and researchers continue to innovate in MS hardware design, faster, more sensitive, and more versatile instrumentation will enable new data acquisition strategies and new proteomics discoveries.

Sample handling and liquid chromatography

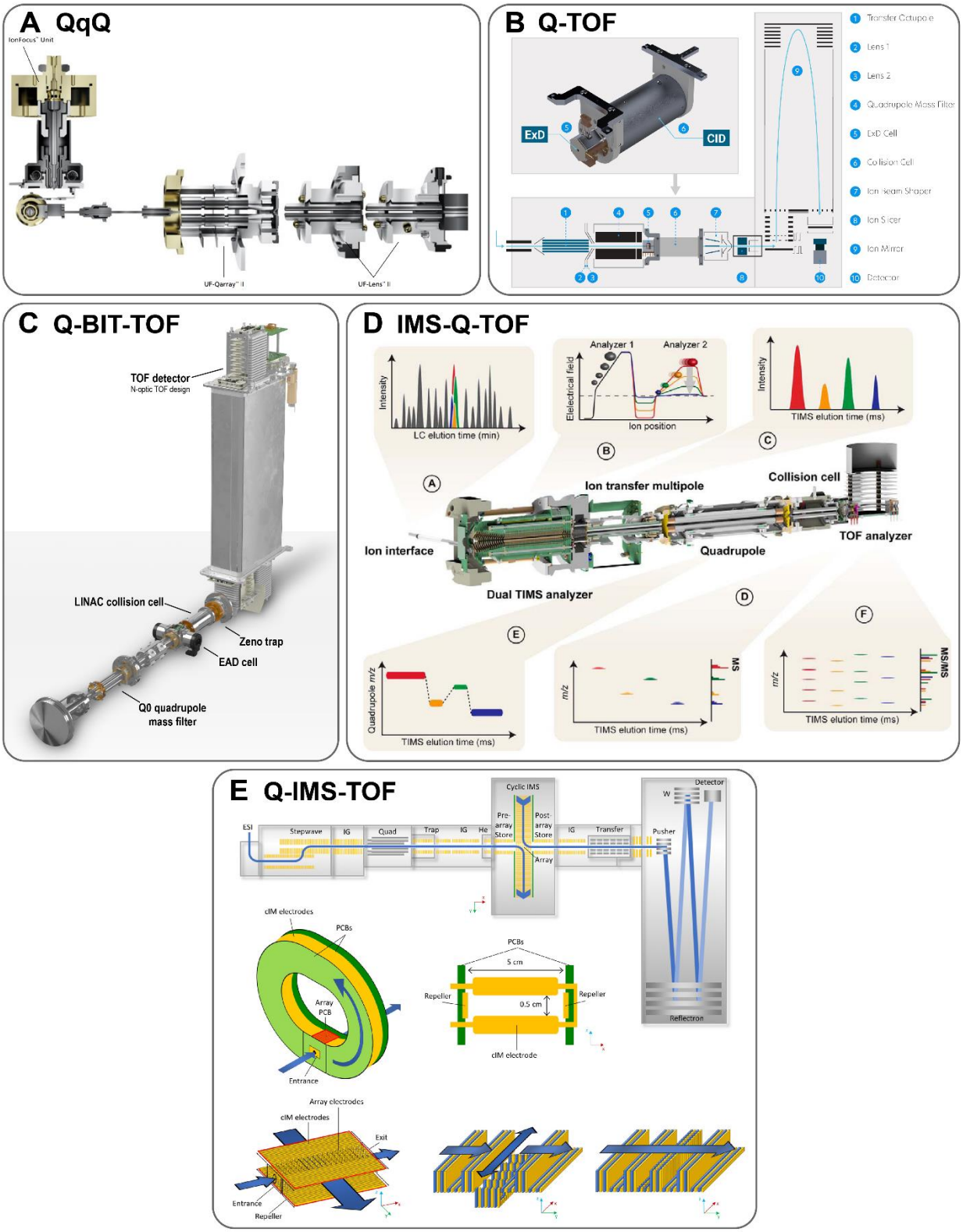
Robust sample preparation and LC are arguably as important as the mass spectrometer itself for quality proteomics data acquisition; as such, new sample handling and LC instrumentation have also been transformative in recent years. Rather than a thorough discussion like those provided elsewhere[427–429], we instead review a few brief examples that promise to have impact in coming years. Perhaps one of the most prominent new additions to the proteomic LC landscape is the EvoSep systems, which use on-line solid-phase extraction and pre-loaded gradients to improve throughput and robustness.[430] Many groups have been adopting EvoSep workflows on a variety of instrument platforms, and pre-defined gradients offered by EvoSep continue to expand in both nano- and micro-flow regimes.[431–433] The Vanquish Neo from Thermo also enables nano- and micro-flow regimes, reflecting an expansion of proteomic methods into both rapid, high-flow methods[434,435] and more sensitive nano-flow methods (which are more common in proteomics).[428,436] Ultra-high pressure chromatography and columns packed at ultra-high pressures have also been explored to improve separation capacity[437–439], while increased reproducibility and separation capacity have also been investigated using micropillar arrays.[440–442] Additionally, prediction of retention times (as well as other peptide features) through machine learning approaches has both improved with more reproducible chromatography, and has also improved interpretation of LC-MS/MS proteomics data.[443–449]

As the field seeks to improve throughput, reproducibility, and sensitivity, sample preparation methods for liquid handling (i.e., manipulating single-cell levels of protein lysates) have also steeply increased. Specialized liquid handlers like those developed for nanoPOTS workflows[450,451] have now been commercialized by CellenOne, and automated sample preparation appears to be key for the high throughput methods needed to analyze single cell proteomes with minimal sample losses.[452–459] Automated sample preparation through equipment like KingFisher, AccelerOme, OpenTrons, TECAN, and many more has become increasingly more prevalent, especially as paramagnetic bead-based digestions and enrichments/depletions prove their utility.[460–470]

Instrument	m/z Range	Scan Rate (Hz)	Mass Resolution	Mass Accuracy (ppm)	Dynamic Range	Sensitivity	MS^n Compatibility	Ion Source	Ion Mobility	Ideal Applications	Release Year
LIT											
TFS Velos Pro	15 - 4,000	125,000 Da/s	0.075 - 3.0 FWHM	0.1 - 1.5 Da	> 1x10^6	Femtomole	rCID, ETD	ESI, APCI, APPI	FAIMS*	1, 4, 9, 10	2011
QqQ											
Agilent 6495D	5 - 3,000	18,700 Da/s	0.4 - 2.5 FWHM	0.1 Da	> 6x10^6	Atto- to Femtomole	bCID	ESI, APCI	—	1, 4, 9, 10	2023
SCIEX Triple Quad 7500	5 - 2,000	20,000 Da/s	3,100	0.1 Da	> 1x10^6	Atto- to Femtomole	bCID	ESI	—	1, 4, 9, 10	2020
Shimadzu 8060NX	2 - 2,000	30,000 Da/s	0.5 FWHM	0.1 Da	1x10^7	Atto- to Femtomole	bCID	ESI, APCI	—	1, 4, 9, 10	2020
TFS TSQ Altis	5 - 2,000	15,000 Da/s	0.2 - 2.0 FWHM	0.2 - 2.0 Da	> 1x10^6	Atto- to Femtomole	rCID, bCID	ESI, APCI	FAIMS (Pro Duo)*	1, 4, 9, 10	2019
Q-TOF											
Agilent 6545XT	20 - 30,000	50	50,000	< 0.8[i]	1x10^5	Femtomole	bCID, ECD*	ESI, APCI, MALDI*	SLIM*	1, 4, 6, 9, 10	2017
Bruker maXis II	50 - 20,000	50	80,000	< 0.8[i]	1x10^5	Attomole	bCID, ETD	ESI, APCI, APPI	—	1, 4, 6, 10	2014
SCIEX TripleTOF 6600+	5 - 40,000	100	35,000	< 0.5[i]	1x10^5	Atto- to Femtomole	bCID	ESI, APCI	—	1, 4, 6, 10	2019
Waters Select Series MRT	50 - 20,000	10	200,000	< 0.2[i]	1x10^5	Femtomole	bCID	MALDI, ESI	—	1, 4, 6, 9, 10	2021
Waters Xevo G3	20 - 100,000	30	40,000	< 1[i]	1x10^4	Femtomole	bCID	ESI, APCI	—	1, 3, 4, 6, 7, 9, 10	2022
TOF-TOF											
Bruker rapifleX	50 - 8,000	50-100	45,000	< 3[e]; < 1[i]	1x10^4	Femtomole	bCID	MALDI, ESI	—	1, 4, 6	2015
Q-IMS-TOF											
Waters Synapt XS	20 - 64,000	30	75,000	< 1[i]	1x10^4	Femtomole	bCID, ETD	MALDI, ESI	TWIMS (25cm)	4, 6, 7	2019
Waters Select Series Cyclic IMS	20 - 64,000	50	100,000	< 0.5[i]	1x10^5	Attomole	bCID, ECD, SID	ESI, MALDI	TWIMS (>100cm)	1, 3, 4, 5, 6, 7, 9, 10	2019
IMS-Q-TOF											
Agilent 6560	20 - 20,000	50	42,000	< 2[e]; < 1[i]	1x10^5	Femtomole	bCID, in-source CID	ESI, MALDI*	DT-IMS (80 cm)	4, 6, 7	2021
Bruker timsTOF fleX	50 - 20,000	150	60,000	< 2[e]	1x10^5	Atto- to Femtomole	bCID	ESI, MALDI	TIMS (v. 4)	1, 3, 4, 6, 7, 9, 10	2019
Bruker timsTOF SCP	50 - 20,000	120	60,000	< 2[e]	5x10^4	Zepto- to Attomole	bCID	ESI	TIMS (v. 3)	1, 2, 3, 4, 5, 6, 7, 9, 10	2021
Bruker timsTOF HT	50 - 20,000	150	60,000	< 2[e]	1x10^5	Atto- to Femtomole	bCID	ESI	TIMS (v.4)	1, 3, 4, 6, 7, 9, 10	2022
Bruker timsTOF Ultra	50 - 20,000	300	60,000	< 2[e]	5x10^4	Zepto- to Attomole	bCID	ESI	TIMS (v. 4)	1, 2, 3, 4, 5, 6, 7, 9, 10	2023
Q-BIT-TOF											
SCIEX ZenoTOF 7600	40 - 40,000	133	42,000	< 2[e]; < 1[i]	1x10^5	Atto- to Femtomole	bCID, rCID, EAD	ESI, APCI	—	1, 3, 4, 7, 9, 10	2021
Qq-FTICR											
Bruker solariX FTICR	100 - 10,000	1	10,000,000	0.25[i]	2x10^3	Atto- to Femtomole	bCID, ETD, ECD, SORI	ESI, MALDI	—	4, 6	2009
LIT-Orbitrap											
TFS Orbitrap Elite Hybrid	50 - 4,000	12	240,000	< 3[e]; < 1[i]	5x10^3	Atto- to Femtomole	bCID, rCID, ETD	ESI, APCI, APPI	FAIMS*	1, 2, 3, 4, 5, 7, 8, 9, 10	2012
Q-Orbitrap											
TFS Q Exactive HF	50 - 6,000	18	240,000	< 3[e]; < 1[i]	5x10^3	Femtomole	bCID	ESI, APCI, APPI	FAIMS*	1, 4, 6, 7, 8, 9, 10	2014
TFS Q Exactive UHMR	350 - 80,000	22	200,000	< 3[e]; < 1[i]	1x10^5	Femtomole	bCID, in-source CID	ESI, APCI, APPI	FAIMS*	4, 6	2018
TFS Orbitrap Exploris	40 - 8,000*	40	480,000	< 3[e]; < 1[i]	1x10^5	Femtomole	bCID, in-source CID	ESI, APCI, APPI	FAIMS (Pro Duo)*	1, 4, 6, 7, 8, 9, 10	2019
Q-Orbitrap-LIT											
TFS Orbitrap Ascend Tribrid	40 - 16,000*	50	1,000,000*	< 3[e]; < 1[i]	5x10^3	Atto- to Femtomole	bCID,rCID,ETD*, UVPD*, PTCR*	ESI, APCI, APPI	FAIMS (Pro Duo)*	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	2022
Q-Orbitrap-Astral											
TFS Orbitrap Astral	40 - 2,000	200	80,000	< 5[e]	> 1x10^4	Zepto- to Attomole	bCID	ESI, APCI, APPI	FAIMS (Pro Duo)*	1, 2, 3, 4, 5, 7, 8, 9, 10	2023

1: biofluids; 2: immunopeptidomics; 3: deep and unbiased; 4: PTMs; 5: single-cell; 6: native; 7: spatiotemporal; 8: cross-linking; 9: clinical; 10: large-scale

Table 1. Performance specifications for commonly used mass spectrometers for proteomics. Instrument models are grouped by their primary instrumental components including mass analyzers and ion mobility cells. Mass accuracy: [e] with external calibration, [i] with internal calibration. BIT: branched ion trap; bCID: beam-type collision induced dissociation, rCID: resonant collision induced dissociation; ETD: electron transfer dissociation; ECD: electron capture dissociation; EAD: electron activated dissociation; SORI: sustained off-resonance irradiation; UVPD: ultraviolet photodissociation; PTCR: proton transfer charge reduction; APCI: atmospheric pressure chemical ionization; APPI: atmospheric pressure photoionization; FAIMS: high-field asymmetric waveform ion mobility spectrometry; SLIM: structures for lossless ion manipulation; DT-IMS: drift tube-ion mobility spectrometry. Ideal applications: 1) biofluids, 2) immunopeptidomics, 3) deep and unbiased proteomics, 4) post-translational modifications, 5) single-cell proteomics, 6) native proteomics, 7) spatial and temporal proteomics, 8) cross-linking MS, 9) clinical proteomics, 10) large-scale proteomics. * upgrade required.



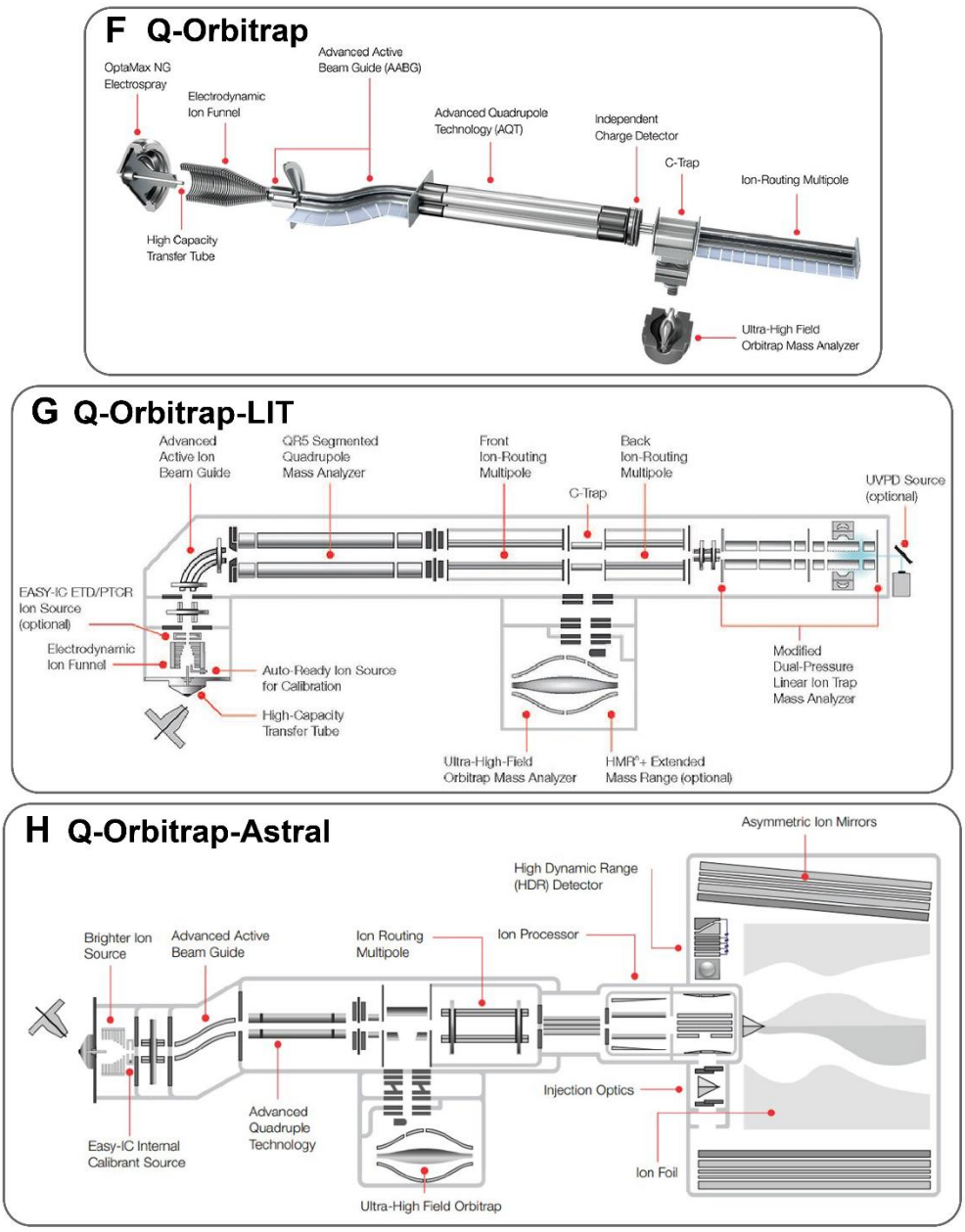


Figure 3. Instrument architectures. Commercially available hybrid MS instrument schematics illustrate similarities and diversity of mass analyzers and how MS components are incorporated into modern instrument platforms. Instrument platforms illustrated are the **A)** Shimadzu LCMS-8060NX Triple Quadrupole, **B)** Agilent 6545XT AdvanceBio LC/Q-TOF with e-MSion AQ-251 Option, **C)** SCIEX ZenoTOF 7600 system, **D)** Bruker timsTOF Pro, **E)** Waters SELECT SERIES Cyclic IMS, **F)** Thermo Fisher Orbitrap Exploris 480, **G)** Thermo Fisher Orbitrap Ascend Tribrid, **H)** Thermo Fisher Orbitrap Ascend. **A)** Adapted/reprinted from Fukui, W., Kudo, T., Toji, Y., Nishiguchi, M., Mukaibatake, K. Robustness Evaluation of the LCMS-8060NX with a Newly-Developed Ion Source, IonFocus Unit (ref. [471]471). Copyright 2020, with permission from Shimadzu Corporation. **B)** Adapted/reprinted from Franklin, R., Hare, M., Meeuwsen, J., Zhu, P., Cooley, R. B., Beckman, J. Top-down Localization of Multiple Phosphorylation Sites using the ExD AQ-251 Option (ref. [472]472). Copyright 2022, with permission from e-MSion, a Part of Agilent. **C)** Adapted/reprinted from https://sciex.com/products/mass-spectrometers/qtof-systems/zenotof-7600-system. ZenoTOF 7600 system (ref. [473]473). Copyright 2024, with permission from SCIEX. **D)** Adapted/reprinted from Mol. Cell. Prot., Vol. 17, Meier, F., Brunner, A., Koch, S., Koch, H., Lubeck, M., Krause, M., Goedecke, N., Decker, J., Kosinski, T., Park, M. A., Bache, N., Hoerning, O., Cox, J., Rather, O., Mann, M. Online Parallel Accumulation–Serial Fragmentation (PASEF) with a Novel Trapped Ion Mobility Mass Spectrometer (ref. [294]294).

Copyright 2018, with permission from Elsevier. **E)** Adapted/reprinted from Anal. Chem., Vol. 91, Giles, K., Ujma, J., Wildgoose, J., Pringle, S., Richardson, K., Langridge, D., Green, M. A Cyclic Ion Mobility-Mass Spectrometry System (ref. [235]). Copyright 2019, with permission from ACS. **F-H)** Adapted reprinted from thermofisher.com (refs. [474–476]). Copyright 2024, with permission from Thermo Fisher Scientific.

ADVANCED ACQUISITION STRATEGIES ENABLED BY MODERN INSTRUMENTS

As instruments have steadily improved in acquisition speeds, sensitivity, robustness, and ion manipulation capabilities, efforts across the proteomics field have sought to better sample available ions populations. Even as we constructed this review, multiple methods rapidly emerged on several instrument platforms, underscoring the dynamic nature of proteomic methodology. In this section we discuss several strategies developed to leverage modern instrument architectures to improve utilization of ion populations entering the mass spectrometer.

Parallel accumulation serial fragmentation (PASEF)

PASEF, described by Meier and Mann, has seen wide adoption as one of the fastest growing experimental regimes in in shotgun proteomics[477]. PASEF converts one TIMS cycle into multiple MS/MS scans by accumulating all ions in a TIMS device and then synchronizing elution of mobility-separated ions with precursor ion selection via the quadrupole.[188] Precursor ion elution from the TIMS cell occurs in time-separated ion mobility peaks (~2 ms), allowing selection and fragmentation of each packet to occur with little loss in sensitivity. As ions are ejected from the TIMS cell, MS/MS scans can be collected with the TOF at speeds well over 100 Hz. The initial PASEF methods were DDA methods, where a rapidly switching quadrupole (<1 ms) could select many precursors during a TIMS cycle using an isolation window positioned as a function of TIMS ejection time. This step effectively increases efficiency in use of the ion beam without sacrificing the hallmark selectivity of DDA methods. It also allows selection of precursors based on their CCS to bias acquisition toward specific features, e.g., crosslinked peptides versus monolinked peptides.[478] Outside of DDA, Lesur et al demonstrated prm-PASEF, where TIMS separation allowed for more precursors to be targeted per unit time.[479] Meier et al. also demonstrated dia-PASEF, which uses wider, non-overlapping quadrupole isolation windows to collect multiple peptide species as they elute from the TIMS device.[480] Because molecular weight and ion mobility are correlated, peptides of the same charge state and similar *m/z* values elute together so that they can be isolated together in DIA mode without sacrificing the sampling of other ions that have not eluted yet (**Figure 4**). Speedy-PASEF further combines dia-PASEF with analytical flow rate chromatography (~500 µL/min) to increase throughput via minutes-long methods.[481]

Recently, Distler et al. reported midia-PASEF, Skowronek et al. reported synchro-PASEF, and Szyrwiel et al. reported slice-PASEF. Each of these are similar, yet innovative methods which make use of the *m/z* and ion mobility domains of the timsTOF instrument to enable fragment ion extraction and interference removal.[482–484] All are DIA-type methods that change how the quadrupole operates to isolate precursors eluting from the TIMS device, and they are largely analogous to the overlapping bin strategy that has become popular for DIA methods on Q-Orbitrap and Q-Orbitrap-LIT systems[485], except with the additional ion mobility dimension. These overlapping window methods improve precursor selectivity through shifting isolation window positions to overlapping *m/z* ranges on subsequent acquisition sequences. This ultimately provides smaller effective isolation windows despite larger physical isolation windows by de-multiplexing spectra based on shared and unique m/z and mobility dimensions. In midia-PASEF and synchro-PASEF, the quadrupole operates in semi-analogous fashion to Scanning SWATH (see below), stepping 0.9 *m/z* units lower for every TOF spectrum (~100 µs) as precursors elute from the TIMS device.[482,483] While both methods use diagonal scanning of the quadrupole as a function of $1/K_0$ to optimally analyze the peptide ion population, midia-PASEF uniquely employs a 12 Th offset for each of 20 MS/MS scans in a 264 Th parallelogram. Each ion is thus given corresponding RT, m/z, and ion mobility measurements in addition to a defined quadrupole selection range.[482] In slice-PASEF, quadrupole isolation windows are split along the mobility vs. *m/z* diagonal relationship so that windows do not overlap in mobility space but do overlap in *m/z* space.[484] The way these windows are divided in the *m/z* space can be altered in different framing

schemes to tailor to optimal separation of peptides in each mobility bin. Slice-PASEF already shows promise over dia-PASEF for low sample amounts, and synchro-PASEF is also poised to improve specificity over dia-PASEF through defined precursor and fragment ion relationships that arise from selection in both *m/z* and mobility dimensions.

Scanning SWATH and other wide-window strategies

One interesting iteration on DIA with high utility is Scanning SWATH, which was introduced in recent years on SCIEX Q-TOF instruments.[186] Instead of stepping through discrete *m/z* windows, Scanning SWATH uses a sliding quadrupole approach where the precursor isolation window is constantly shifting by a step size of two *m/z* units every two ms. The sliding Q1 isolation window provides a "Q1 profile" to annotate precursor peaks within MS/MS spectra and a time dependency to fragment intensities, which improves precursor identification and shortens MS duty cycles, as there is no need to empty the collision cell between isolation steps.[186] Messner et al. showed that Scanning SWATH enabled by a fast-scanning qTOF can identify and quantify 5,004 proteins from K562 cells in 5 min LC gradient.[186] Ralser and co-workers highlight their 1- and 5-min Scanning SWATH methods on COVID-19 patient serum and plasma at 800 µL/min LC flow rate, enabling robust, low-cost proteomics analysis of up to 180 samples/day, time-scales previously unattainable for MS-based proteomics.[186,486] The so-called "wide window acquisition" that intentionally multiplexes DDA-type scans using ~4 *m/z* windows is also being explored.[487,488] Limited post-processing software options are compatible with such data collection strategies. CHIMERYS enables artificial intelligence-driven detection of multiple peptide spectral matches (PSMs) from a single MS/MS scan.[489] The CHIMERYS software is recently available through Proteome Discoverer and is now compatible with phosphoproteomics and DIA workflows. DIA using LIT mass analysis has also been investigated[490,491], and narrow-DIA bins (~4 m/z windows with overlapping acquisitions for effective 2 m/z bins) on fast scanning instruments, e.g., the Orbitrap Astral MS discussed above, are blurring the lines between DDA and DIA acquisitions schemes.

Boxcar and related approaches

Although not MS/MS based, Boxcar methods on Orbitrap-based systems also use multiplexed narrow *m/z* windows distributed across the full mass range as means to improve intact peptide signal at the MS^1-level.[492] This gas-phase fractionation enriches the proportion of low abundance ions in the total ion population that gets mass analyzed in a given scan. Indeed, some so-called MS^1-only methods have completely forgone MS/MS collection and use rapid LC-MS methods that only measure intact peptides. Similar to DIA, these methods then rely on a DDA-based library that can be used to map precursor *m/z* to peptide sequence using retention time and HR/AM measurements.[493–495]

Methods aimed at isobaric and isotopic labeling

Co-isolation of multiple precursor ions is a major challenge of isobaric labeling strategies. Co-isolation of background species in addition to target peptides results in reporter ion ratio distortion, which leads to compressed fold changes in quantitative comparisons.[496,497] To improve reporter ion tag purity, Q-Orbitrap-LIT systems enable a synchronous precursor selection (SPS)-MS^3 strategy. In the SPS regime, peptides are first fragmented in the LIT by rCID; this generates peptide specific fragments without fragmenting off the isobaric tags. Following the rCID MS^2, scan, multiple product ions (up to ~20) are simultaneously selected by a multi-notch waveform within the linear ion trap for MS^3 analysis using bCID and Orbitrap mass analysis. This additional level of gas-phase purification reduces distortion arising from co-isolation to significantly improve

quantitative results.[498] Conversion of these rCID b- and y-type product ions into TMT reporter ions using bCID fragmentation in the MS^3 analysis is not 100% efficient, so IR photoactivation has also been explored for report ion signal generation and shown to be advantageous, especially for single-cell proteomics.[499,500] Reporter ion parking is performed with an RF ion parking waveform during IR photoactivation to limit over-fragmentation and boost quantitative sensitivity.[499,500] Q-Orbitraps cannot offer this SPS-MS^3 approach without the LIT. Instead, isobaric labeling experiments on these systems are forced to rely on gas-phase fractionation methods (e.g., FAIMS) and offline chromatographic fractionation methods to minimize interfering peaks during precursor ion selection. On Orbitrap instruments, phase-constrained spectrum deconvolution (Φ-SDM), also termed turboTMT, enables higher resolution within the TMT reporter ion region with short acquisition transients.[501] Recently, a full mass range implementation of Φ-SDM was demonstrated for DIA methods, yielding particular advantages in improving the spectral quality and quantitative accuracy of rapid LC gradients.[502] Outside of instrument developments, several groups have also shown the importance of careful sample preparation considerations for isobaric labeling experiments.[503–506] Development and synthesis of novel isobaric labels is ongoing, with DiLeu tags presenting a cost-effective alternative to commercially available tags.[507–510]

Targeted methods typically use isotopically labeled standards for quantitation. Several groups have explored how to increase the number of peptides that can be targeted in a given acquisition.[511–514] Multiplexing PRM methods via isotopic standards has been investigated in multiple formats. TOMAHAQ (triggered by offset, multiplexed, accurate-mass, high-resolution, and absolute quantification) offers a particularly intriguing solution. This strategy combines isobaric labeling of samples with TMT with a spike-in of non-heavy-isotope TMT (TMT_0)-tagged peptide standards for targets of interest. TMT_0-labeled species are then used to trigger acquisition of PRM MS/MS scans of TMT-labeled targets in an SPS-MS^3 format on Q-Orbitrap-LIT systems, which enables simultaneous quantification of >100 peptides from multiple sample conditions.[515] Method development tools to design such sophisticated experiments have helped disseminate approaches like TOMAHAQ[516], but it has yet to become commonplace. Sample multiplexing-based targeted pathway proteomics, where peptide standards from proteins of interest in a given biological pathway are targeted, has also shown great promise.[254,515] Until recently, such an approach required synthesis of many peptides; however, with GoDig and real-time peptide elution calibration, over 200 proteins can be targeted for quantitative proteomics with sample multiplexing within a single LC-MS/MS run with no need for synthetic peptide internal standards.[517] The field of chemoproteomics, with the aim of large scale small-molecule drug discovery, has seen a boom in progress in recent years[518–527], driven in part by the incorporation of TMT labeling to the workflow.[528,529] The recently announced TMT 34-plex isobaric labels are sure to advance the speed and robustness of quantitative proteomics, especially when coupled with faster, more sensitive instrumentation.[530]

Real-time searching strategies

Advanced instrument methods have enabled impressive gains in data quality, but some decisions about acquisition parameters cannot be known *a priori*. Intelligent data acquisition methods developed over the past decade aim to direct autonomous instrument decision making in real-time based on empirical data. Groundwork for modern methods were laid by decision-tree and triggering methods that have evolved in targeted proteomics and in a selection of appropriate dissociation strategies.[531–533] For example, methods that blend targeted and DDA approaches based on real-time elution order calculations[534,535] or methods that dynamically adjust MS/MS windows in DIA have shown promise.[536]

Particularly interesting are strategies to enable real-time identifications that can direct instrument control and decision making. MaxQuant Real-Time[537] and inSeq[532] were among the first to report online identification strategies that required only milliseconds to search spectra. Following those leads, others have continued to pioneer real-time parameter adjustments.[512,534,538] In the last several years, real-time searching (RTS) has featured prominently in pushing the throughput of TMT-based proteomics. The scan acquisition regime of SPS-MS^3 methods is inherently slow relative to MS/MS-only strategies, and Schweppe et al. addressed this slower duty cycle with Orbiter RTS-MS.[539] With Orbiter, a real-time database search is performed on every MS/MS scan and a quantitative MS^3 is only triggered upon confident peptide identification, yielding methods with twice as efficient data acquisition. In 2022, Schoof et al. described the benefits of RTS-MS^3 and RETICLE over MS^2-based quantitation for single-cell proteomics applications.[262] In RETICLE, precursors are sampled for a rapid, data-dependent ion-trap MS^2 scan and a high-resolution Orbitrap MS^2 scan is triggered only upon RTS peptide identification. From there, the low-resolution triggering scan is disregarded and the high resolution-MS^2 is used for peptide sequence analysis and quantitation. RTS-MS^3 enables increased quantitative accuracy at a similar proteome coverage, while RETICLE outperforms MS^2 quantitation through deeper proteome coverage, quantifying over 1,000 proteins per single cell in a two-hour gradient.[262] Erickson and Gygi showed that active instrument engagement coupled to RTS-MS^3 can quantify more proteins in half the time of DDA-MS^3 while yielding excellent quantitative reproducibility.[540] Real-time library searching is also being explored as a complement to RTS,[541] and RTS has already shown benefits for other biomolecules such as lipids, too.[542]

Even complex acquisition schemes like TOMAHAQ have been improved by real-time instrument control approaches like Tomahto, which provides an array of functionalities including MS^1 peak detection, MS^2 real-time peak matching, MS^2 fragmentation pattern match, SPS ion purity filter, MS^3 automatic gain control, MS^3 quant scan insertion, and target peptide closeout.[254] MaxQuant.Live also greatly increased PRM capabilities on a Q-Orbitrap system using the application programming interface (API).[543,544] Most intelligent data acquisition strategies have been implemented on Q-Orbitrap and Q-Orbitrap-LIT systems through API-based access to instrument control. timsTOF systems can also use a closed parallel search engine in real-time (PaSER), which is currently designed to help with rapid identifications and quality control for offline decision making rather than dynamic instrument control, and PRM-LIVE has improved targeted timsTOF data acquisition.[545]

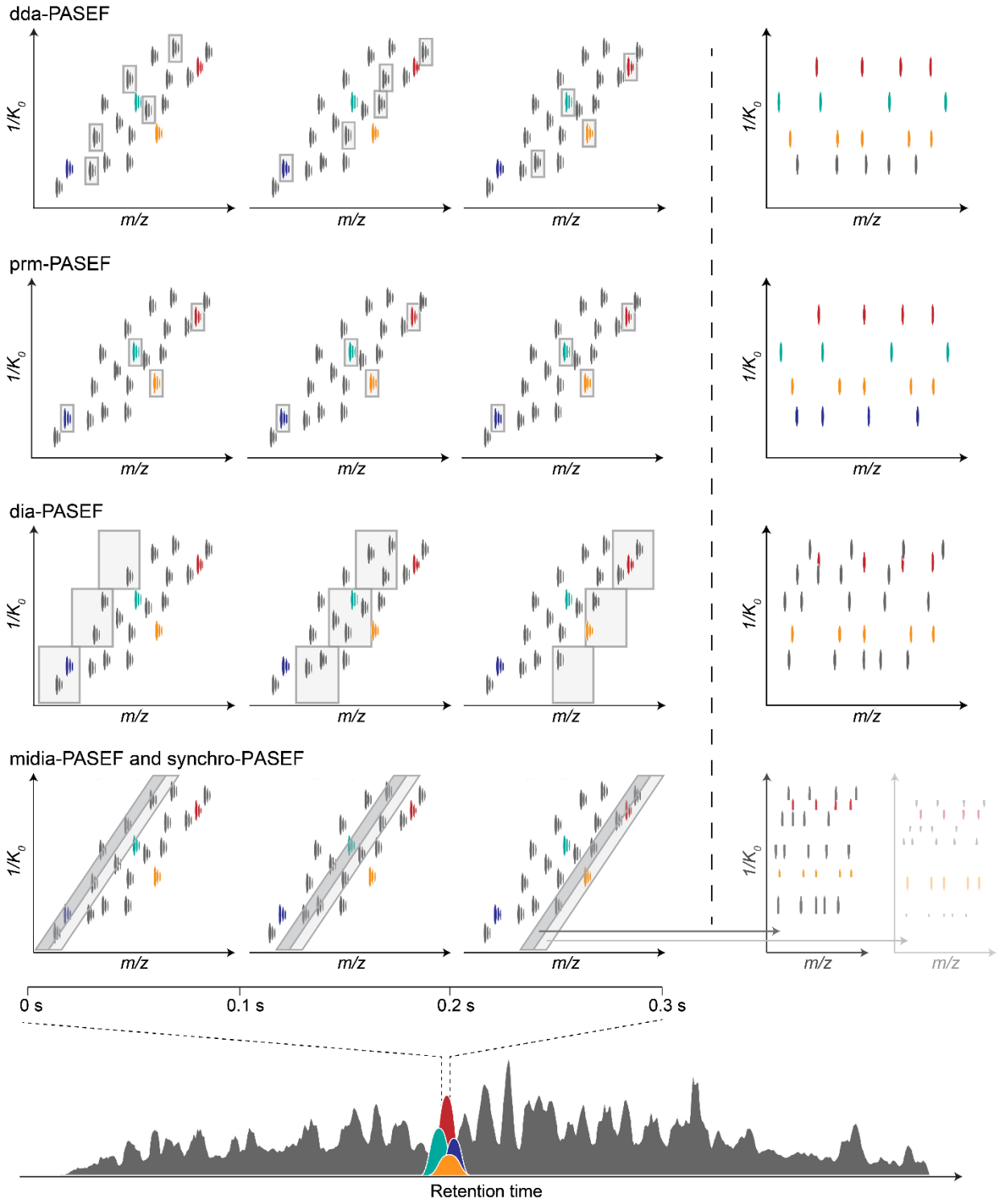


Figure 4. PASEF scan modes. Quadrupole isolation windows (*gray boxes, left panels*) in the two-dimensional $1/K_0$ – m/z plane for dda-, prm-, dia-, and midia-PASEF and synchro-PASEF acquisition schemes with a 100 ms TIMS scan time. The PASEF MS/MS spectra (*right panels*) correspond to the precursor selection in the *third column*. dda, data-dependent acquisition; dia, data-independent acquisition; PASEF, parallel-accumulation-serial fragmentation; prm, parallel reaction monitoring; TIMS, trapped ion mobility spectrometry. Adapted/reprinted from Mol. Cell. Prot., Vol. 20, Meier, F., Park, M. A., Mann, M. Trapped Ion Mobility Spectrometry and Parallel Accumulation-Serial

Fragmentation in Proteomics, (ref [188]). Copyright 2021, with permission from Elsevier, and modified to include midia-PASEF and synchro-PASEF.

EVOLUTION OF EXPERIMENTAL DESIGN IN PROTEOMICS

Given the rapid advances in MS instrumentation and the strategies to acquire data more efficiently, it is no surprise that we have seen shifts in experimental designs favoring higher throughput and/or decreased sample amounts needed for a given experiment. Interest in structural information about the proteome and in classes of biomolecules such as post-translationally modified peptides and intact proteins have also shaped how we use our instruments.

Rapid LC-MS/MS gradients

Modern instruments with advanced data acquisition strategies can detect thousands of proteins in minutes of analysis time. Perhaps one of the most profound pivots in proteomics in the past five years is movement from hour(s)-long LC-MS/MS methods per sample to minutes-long methods to greatly increase throughput. **Figure 5** captures this trend through hand-curated data from numerous recent proteomics experiments.[150,186,262–264,274,275,277,295,347,430,434,436,450,451,480,483,484,486,487,493–495,546–571] Recent efforts (darker colors) emphasize more proteins characterized per hour of LC-MS/MS time (x-axis) over proteomic depth (circle size). The movement to rapid gradients has come as more labs adopt higher flowrate separations. Signal during ESI is concentration dependent, and the field had largely adopted "nanospray" or "nanoflow" regimes, where peptide-containing buffers are driven toward the ESI emitter by LC pumps with flowrates at tens to hundreds of nanoliters per minute. Challenges in LC system robustness and throughput via nanoflow, in addition to desires to iterate through samples faster, have ushered in a recent return to microflow (microliter/min) and other high-flowrate acquisitions. This approach comes with a tradeoff in electrospray sensitivity that can be balanced to some degree by improvement in mass spectrometer sensitivity. Indeed, DIA-based methods now consistently generate datasets of 2,500–5,000 identified proteins in ~5-minute gradients[480,493,494,567,572], although as we note above, quantified identifications, not just mere identification counts, are the currency the field will likely prioritize in coming years. Ion mobility methods (e.g., TIMS, FAIMS) have also dramatically improved data quality in rapid LC-gradients.[277,298,300,482–484,566,572] Still other applications forgo LC altogether and instead use syringe pumps, capillary action, or acoustic ejection to collect data requiring only minutes per sample.[573–579]

Single cell proteomics (SCP) and small sample amounts

With improvements in throughput and sensitivity, the field of proteomics has taken a concerted step toward analysis of nanogram amounts of protein starting material that ultimately enable advances in SCP efforts. At least in practice, SCP requires fast methods that can measure large cohorts of individual cells in reasonable timeframes, so the SCP revolution has been concurrent with the move toward rapid LC gradients. Again, this shift is clearly observed in **Figure 5**, where the y-axis provides unique proteins detected per ng of starting material. The prospect of SCP was once a distant goal prohibited by technical challenges.[580] Today, advances in instrumentation and data acquisition strategies have made SCP a reality[295,487,488,581–587], albeit with ample room for continual improvement.[588,589] Indeed, advances in sample processing (see above) that minimize protein loss have been as important for SCP as MS improvements.[450,451,590] Isobaric labeling has also become a mainstay of SCP through methods like SCoPE-MS and others that use carrier channels to boost MS^1-level detection and reporter ions in MS/MS scans to provide relative quantitation of labeled proteins of individual cells.[560,564] That said, care must be taken in how much

material is used in carrier channels as to not distort reporter ion statistics, which would prevent reliable quantitation of single cell channels.[562,591–595] The sensitivity of PASEF methods has led to focus on SCP efforts for timsTOF work, while Q-Orbitrap and Q-Orbitrap-LIT instruments remain key platforms in SCP thanks to isobaric labeling and RTS strategies like SCoPE-MS and RETICLE. Recently, Schoof and colleagues and Olsen and colleagues reported single-cell proteomics results performed on Orbitrap Astral, doubling the number of proteins quantified related to experiments on the Exploris 480 system.[347,596] Converse to highflow rates for rapid gradients, ultra-sensitive approaches have also been explored with picoflow methods for SCP.[597,598]

Large-scale analyses

Faster, more sensitive methods on current MS instruments also enable expansion of the scale of proteome analysis.[599] Modern proteomic studies have incorporated larger cohorts of samples and better integration of control measurements than ever before. These include MS-based draft maps of the human[150,555,600], mouse[601], Arabidopsis[602], and Medicago proteomes[603]; a landscape of 100 proteomes from diverse organisms across the kingdoms of life[563]; AP-MS interactome experiments on the scale of 10,000+ baits[604–607]; a proteome-wide atlas of small molecule mechanisms of action[529]; multi-omics datasets that combine proteome, transcriptome, metabolome, and/or lipidome analyses for hundreds to thousands of samples[608–614]; encyclopedias of cancer proteomes[5]; and a high-stringency blueprint for the human proteome one decade after the launch of the Human Proteome Project.[600] As such, plasma proteomics and the push toward clinical impacts of proteomics continues to advance[470,615,616], and the progress in proteomics technology has also enabled researchers across the globe to concertedly address questions of infection, disease progression and mechanism, therapeutic treatment, and preventative measures for the COVID-19 pandemic caused by SARS-CoV-2.[617] Strategies to make use of large-scale data available in public repositories has also greatly improv-ed, and several efforts to interactively visualize large-scale datasets have also evolved.[618–623] Even with trends toward high-throughput proteomics that maximize unique proteins per hour of instrument time, highly in-depth, multi-protease proteomics that seek to maximize proteomic sequencing depth and sequence coverage of each protein detected remain valuable.[141,149,150,624–629]

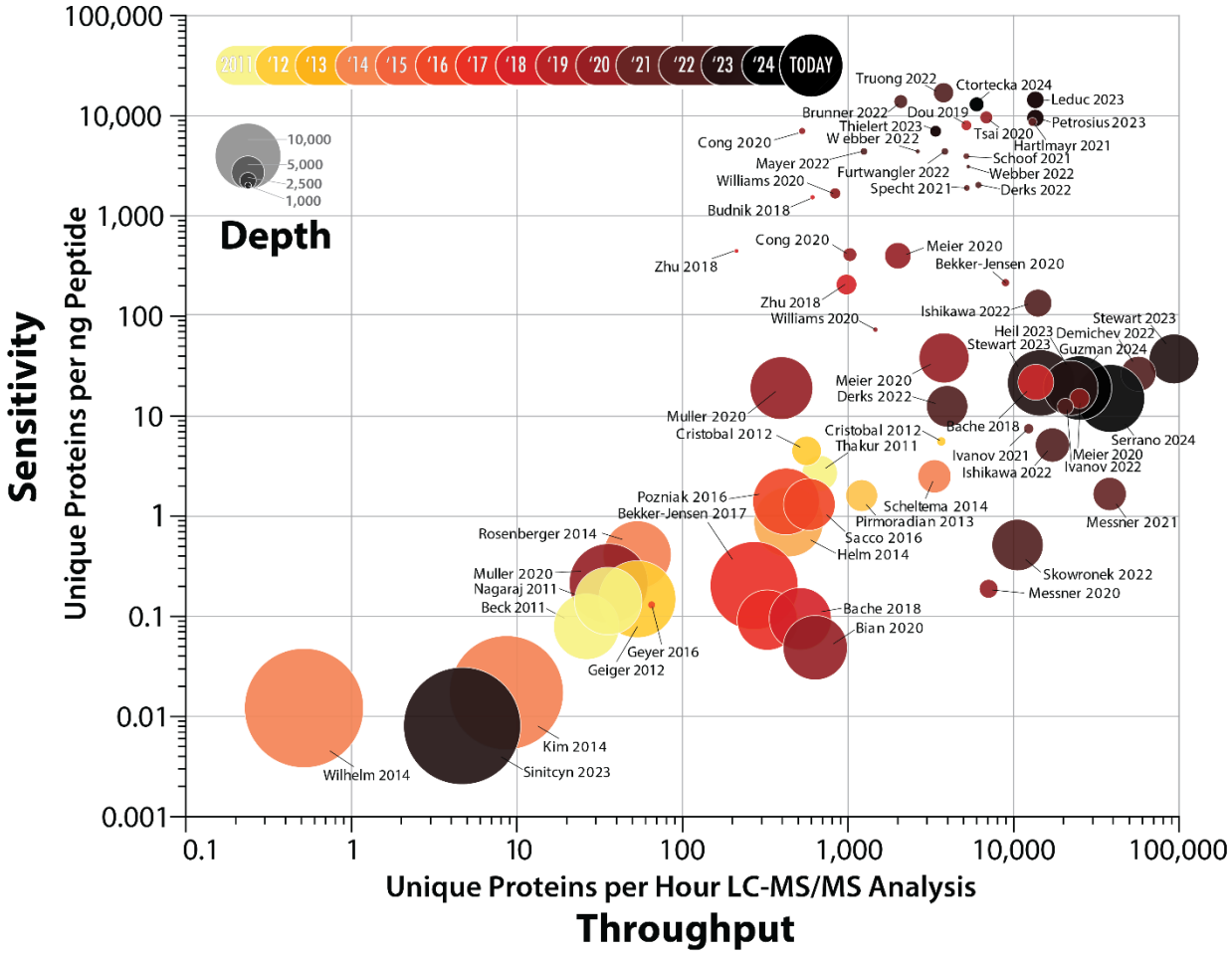


Figure 5. Throughput, sensitivity, and depth are shown for 65 LC-MS/MS-based human proteomics investigations spanning the past fourteen years. The number of unique human proteins identified per hour of LC-MS/MS analysis are plotted against the number of unique proteins identified per nanogram of peptide injected. Dot size corresponds to the depth of human proteome coverage and dot color corresponds to publication year, with darker shades indicating more recent. Note, these data report protein groups identified, not necessarily quantified.

Post-translational modifications (PTMs)

PTMs are key features of the proteome that contribute to its compositional and temporal heterogeneity, and MS is the gold standard for interrogating PTM status.[144,145] We do not have the space to consider recent developments applied to all PTMs, but offer phosphorylation and glycosylation as two examples of how modern MS instrumentation is making an impact in PTM analysis. Phosphorylation is one PTM with a long history in MS-based proteomics, mainly because it is so fundamentally important to signaling biology and MS is particularly well-suited to characterize phosphosites relative to other technologies.[630–632] The biggest gains in recent years have been the speed and reproducibility with which phosphoproteomes can be quantitatively analyzed. IMS strategies, DIA-based methods, and instruments with rapid acquisition rates have allowed 10,000–30,000 phosphosites to be characterized in 30-minute-or-less analyses using LC-MS/MS.[298,348,633] Automation in sample preparation for large cohorts of samples has streamlined these experiments.[634] The same technology also makes highly in-depth phosphoproteomics possible [635,636], e.g., the functional landscape of the human phosphoproteome from Ochoa et al.[637] and a global atlas of substrate specificities within the human serine/threonine kinome from

Johnson et al.[638] Additionally, reference tools to build both global and targeted phosphoproteomic methods have significantly improved, dovetailing with the gains seen from instrumentation.[639]

Glycosylation is another ubiquitous and fundamental class of PTMs[640], and glycoproteomics has seen rapid advances in recent years thanks to higher quality data on modern instruments and significant improvements in data analysis tools.[641–648] One interesting yet confounding feature of glycoproteomics is differential fragmentation requirements for various glycopeptide classes.[649,650] N-glycopeptides generally can be analyzed with bCID fragmentation (although electron-based fragmentation often gives higher quality, more informative spectra[651]), meaning N-glycoproteomics can be performed on a wide variety of instruments. Even though the majority of glycoproteomic studies to date have been performed on Orbitrap-based systems, advantages for N-glycopeptide characterization have been explored on timsTOF [652–655], Q-TOF[656–659], FTICR[373,660,661], and QqQ[662–666] instruments in addition to Q-Orbitraps and Q-Orbitrap-LIT.[368,667] By contrast, O-glycopeptides often do not retain sugar moieties on fragment ions during bCID, and the lack of an O-glycosylation motif means that localization is not trivial. Thus, ExD fragmentation (most often EThcD) is needed for site-specific O-glycopeptide characterization[668–674], and as such, most recent O-glycopeptide work has been done on Q-Orbitrap-LIT systems. As other ExD implementations mature, they show promise for extending O-glycoproteomic access to other instrument platforms.[132,675,676] As with phosphopeptides, the benefits of various IMS strategies are also being explored for glycopeptides[655,677–684], although the results are still inconclusive on how helpful IMS will be or which IMS method will provide the best boost in analytical power.

Top-Down Proteomics

Top-down proteomics, or MS and MS/MS of intact proteins rather than proteolytically derived peptides, is more widely employed than ever as better instrumentation, more tractable methods, and accessible data analysis software make it more practical.[685–690] Although more technically challenging than shotgun proteomics, top-down approaches offer a more direct view of the functional units of the proteome, i.e., proteoforms; in other words, proteoform information does not need to be pieced back together with peptide-level data, which requires high sequence coverage to do robustly.[691,692] Orbitrap platforms have greatly enhanced top-down proteomic data acquisition,[693] although FTICR systems still offer significant benefits. The extended mass range of TOFs can make Q-TOFs attractive despite lower resolution options, and IMS couplings with Q-TOFs have proven particularly useful for protein characterization.[694–701] The Q-Exactive UHMR platform with extended mass range up to *m/z* 80,000 and the extended mass range of *m/z* 16,000 now available on the Orbitrap Ascend will likely keep Orbitraps prominently featured in future top-down proteomics research. Flexible fragmentation modalities (bCID, rCID, ExD, UVPD) and ion-ion reactions like PTR are also highly advantageous in top-down characterization, and current top-down studies can map thousands of proteoforms across various systems.

One exciting development relevant for intact protein characterization has been implementation of charge-detection MS (CDMS) in Orbitraps. CDMS is a single ion detection strategy used to simultaneously measure charge (via induction of an image current) and *m/z* (traditionally via TOF).[702,703] In this way, CDMS provides mass measurements of large biomolecules (beyond MDa masses) that typically cannot be assigned a charge state in ESI-FTMS because resolution is not sufficient for isotopic resolution.[703] The Kelleher and Heck groups recently demonstrated that individual ion CDMS measurements can be made within commercial Orbitrap systems rather than requiring custom MS hardware often used for CDMS.[207–210,704,705] Orbitrap CDMS is particularly promising for DNA-containing viral capsids, glycoproteins, protein complexes, and amyloid protein aggregates, and it also boosts performance of top-down MS via improvements to

sensitivity and protein sequence coverage.[209,210,705–707] The coming years will be exciting times for CDMS and intact protein analysis, especially as it has (so far) been commercialized as Direct Mass Technology on the newest generation Q-Orbitrap platforms.[207,208,704,708]

Structural proteomics

Deriving protein structure from MS data has long been a goal of the proteomics community, and modern instrumentation has helped make it a reality. From the intact protein angle, native proteomics relies on non-denaturing solvents and ESI to study protein structure in the gas-phase. Native MS measurements can provide information related to protein complexation, protein-lipid interactions, protein-drug binding, and complex three-dimensional structural interrogation (especially when coupled with IMS and MS/MS strategies). Improvements in mass analyzer *m/z* ranges and resolution, transfer optics for large ions, IMS devices, implementations of MS/MS methods, and backend software have all made native MS a rapidly growing and accessible platform in academic and industrial settings. Native MS is also being leveraged to improve other structural biology techniques, such as soft-landing of mass-selected protein complexes for further structural characterization (e.g., cryo-electron microscopy).[424–426,709–714] Hydrogen deuterium exchange and protein footprinting have also seen substantive gains due to steady improvements in instrumentation.[179,326–328,715,716] At the peptide level, methods for crosslinking MS (XL-MS) have enabled large-scale structural proteomics studies, owing in part to the advanced instrumentation discussed herein.[283,717,718] Proximity-dependent labeling has also emerged to help map interactions and corroborate structural studies.[719–724] As some of the more demanding studies within the field, structural proteomics at both the peptide (XL-MS) and protein (native MS) level will likely continue to progress at rapid rates that mirror the exciting instrument developments sure to manifest in coming years.

COMPLEMENTARY TECHNOLOGY TO MS-BASED PROTEOMICS

Although MS is the standard for proteome characterization, it is not the only technology for large-scale protein analysis. Perhaps the most mature alternative is the antibody-based Human Protein Atlas,[725] which was constructed from monumental efforts in antibody-based imaging,[726] where >20,000 antibodies covering ~15,000 human genes are used for immunohistochemical staining of tissue microarray slides.[727–730] These antibody-based profiling data are complemented with MS-based proteomics and transcriptomics to generate a genome-wide analysis of the human proteome.[586,600]

Other notable efforts focus on single-molecule detection using massively parallel technologies. Several companies are developing massively-parallel technologies with single amino-acid resolution (Quantum-SI,[731] Erisyon[732]) or short epitope mapping with multi-affinity reagents (Nautilus Biotechnology).[733,734] Others create functionalized antibodies to give high-throughput proteomics results (Olink, Alamar Biosciences, Quanterix, Nomic, NanoMosaic). With Olink, a proximity-extension assay, in which two antibodies each carry barcoded DNA probes, enables quantitative proteomics by next-generation sequencing (NGS). Encodia also creates DNA information from a peptide sequence for an NGS output. Nanopore sequencing of amino acids is another strategy with growing optimism (DreamPore, Oxford Nanopore). SomaLogic offers a platform with custom nucleic acid aptamers which evolve DNA to selectively bind a protein of interest. They pull-down the bound aptamers onto a DNA microarray, which enables quantification of up to 7,000 proteins spanning twelve orders of magnitude from 55 µL of blood.

MacCoss et al. offer an outstanding perspective examining the challenges of proteome analysis by LC-MS/MS and emergent single-molecule approaches.[735] They point out that a major issue

with admittedly exciting single-molecule methods will be scalability; spatially resolved flow cells can only hold so many molecules, and >1 billion reads are needed to capture the proteome. The cost to use current single-molecule technology for that many reads is prohibitive relative to LC-MS/MS analysis. From our perspective, these alternative approaches are all intriguing developments that will usher in an exciting era in proteomics. That said, we do not see these technologies as competing with or replacing MS as a central player in proteome analysis. Instead, we imagine a landscape where these technologies function in a complementary manner with MS-based proteomics, offering enhanced data quality in specific applications that require increased sensitivity or throughput on defined subsets of the proteome. We predict that MS and alternative proteomic technologies will function in concert, and savvy scientists will quickly learn how to integrate strengths from both rather than pitting them against each other.

LOOKING FORWARD

Proteomics continues to advance rapidly. Many of these exciting developments have been driven by improved MS and MS-centric instrumentation, although sample processing and informatics tools also claim an indispensable role in this progress. Indeed, data analysis tools must progress in-step with improvements in MS instrumentation and data acquisition strategies for the field to capitalize on technological advances. It is then no surprise that proteomic software development is as active as ever, too. A 2019 study pointed to 754 annotated proteomics tools available in bio.tools portal, which itself is not exhaustive.[25] Thus, we can reasonably assume that approximately 1,000 or more software tools are available to proteomic researchers. Many recent informatics tools have been designed specifically to manage data types generated by our newest MS instrumentation, and there are still many gains to be made on how information-rich MS datasets are mined for biological knowledge.

As instrumentation advances and reaches more members of the scientific community, the impact of MS-based proteomics continues to widen its footprint. In this review we highlighted ways in which instrumentation is advancing biotechnology and biological knowledge. Others that we do not have space to dedicate discussion to here include spatiotemporal proteomics[736–738] and immunopeptidomics,[739–750] just to name a few. We created **Figure 6** to summarize the types of methods and applications that modern MS instrumentation has enabled. Looking forward, new instrumentation is surely coming that will continue to combine mass analyzers, IMS devices, and ion activation modalities into exciting hybrid formats. Improvements in how to efficiently use ion populations that enter the mass spectrometer will likely be a common focus, which will continue to bring the impetus for deep proteome characterization and high throughput analyses closer together.

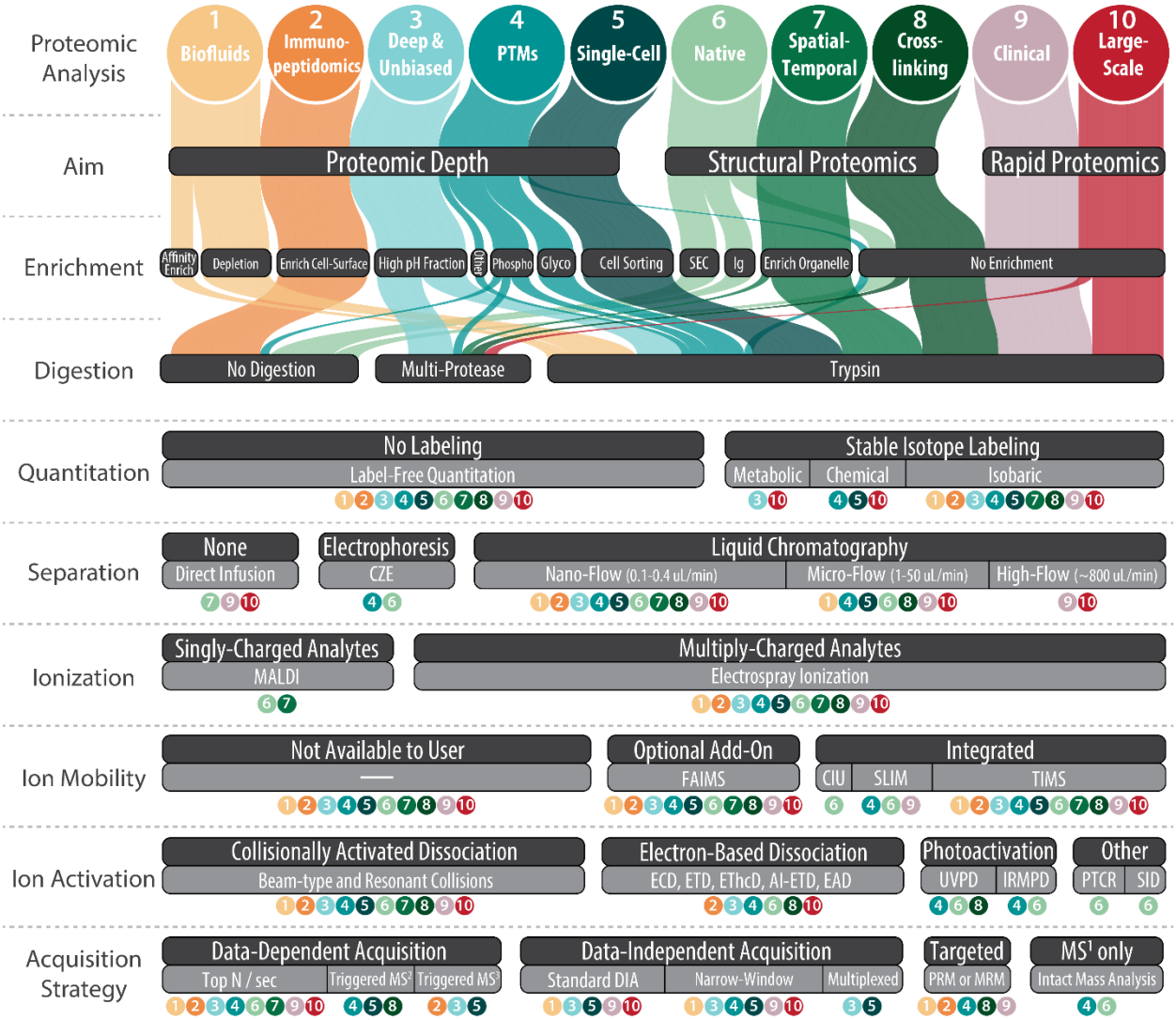


Figure 6. Overview of the breadth of mass spectrometry-based proteomics workflows and their application to ten common proteomics aims. Continued innovation from sample preparation to data acquisition has enabled researchers to capture complex features of the proteome. This figure portrays how various proteomic workflows conducted on modern instruments can address a diversity of analytical challenges inherent to proteome characterization.

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Joshua J. Coon is a Professor of Chemistry and Biomolecular Chemistry at the University of Wisconsin-Madison and the Thomas and Margaret Pyle Chair at the Morgridge Institute for Research. Coon earned his B.S. degree at Central Michigan University and received his Ph.D. at the University of Florida in 2002. At Florida, Coon studied ambient ionization processes under the guidance of Professor Willard Harrison. From 2003 to 2005 he was an NIH postdoctoral fellow with Professor Donald Hunt at the University of Virginia. During his time at Virginia he, with Hunt and John Syka, coinvented electron transfer dissociation (ETD). Coon's research program at Wisconsin is focused on all aspects of biomolecular mass spectrometry.

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