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Ion mobility–mass spectrometry for structural proteomics

Yueyang Zhong, Suk-Joon Hyung, and Brandon T Ruotolo*

Department of Chemistry, University of Michigan, 930 N. University Avenue, Ann Arbor, MI, 48109, USA

Abstract

Ion mobility coupled to mass spectrometry has been an important tool in the fields of chemical physics and analytical chemistry for decades, but its potential for interrogating the structure of proteins and multiprotein complexes has only recently begun to be realized. Today, ion mobility–mass spectrometry is often applied to the structural elucidation of protein assemblies that have failed high-throughput crystallization or NMR spectroscopy screens. Here, we highlight the technology, approaches and data that have led to this dramatic shift in use, including emerging trends such as the integration of ion mobility–mass spectrometry data with more classical (e.g., ‘bottom-up’) proteomics approaches for the rapid structural characterization of protein networks.

Keywords

high throughput; macromolecular; non-covalent complexes; protein network; protein topology; structural genomics

Proteins rarely act in isolation, resulting in massive, dynamic multiprotein machines and networks that govern most critical cellular processes [1]. As such, the structural characterization of these large-scale multicomponent protein complexes has been a key goal of structural biology for decades [2]. More recently, attention has turned to high-throughput technologies capable of assessing the structure and topology of many complexes in a serial fashion. The ambitious goals set in such studies include the structural annotation of large protein networks, thus enabling a 3D view of vast webs of interacting proteins [3]. High-throughput versions of x-ray crystallography and NMR spectroscopy have been important and pioneering tools in this process, and seek to directly convert isolated proteins and complexes into atomic-resolution representations of the interacting proteins involved in such networks (**Figure 1**) [4]. Such technologies currently dominate the landscape of structural genomics and proteomics research.

While this direct approach has been highly successful for a large number of protein complexes and networks, the vast majority of multi-protein systems provide significant challenges for x-ray and NMR-based approaches. For example, although the detection limits for both technologies continue to improve, relatively large amounts of protein are still required to acquire usable data. In addition, as the complexity of the protein network under investigation increases, so do many parameters that complicate NMR and x-ray analysis, such as the increased presence of protein flexibility, heterogeneity and poly dispersity [2,4].

These properties are found in abundance within membrane-associated protein complexes [5] – a class of protein assemblies that are among the most sought-after therapeutic targets [6]. Furthermore, since neither technology seeks to separate components during analysis, both require highly purified samples. These and other challenges highlight the need to develop new approaches aimed at high-throughput multiprotein structure determination [2,4,7].

Mass spectrometry (MS) and, more recently, ion mobility–MS (IM–MS) of intact complexes is emerging as one of many such alternative approaches in the field of structural proteomics [2,4,8–14]. In these experiments, an alternate route to a final high-resolution map of the protein complex is sought, where protein connectivity and topology are defined in stages, prior to detailed homology modeling or partial atomic-resolution data being used to complete the process (**Figure 1**) [15,16]. For many years, MS technology, driven by the needs of proteomics and other biology-related application areas, has been honed as a high-throughput methodology. This makes MS a more obvious choice for high-throughput data production than many other tools capable of producing protein structure data. Moreover, MS of intact protein complexes has been highly successful in determining the details of protein–protein interactions within assemblies [13,17–19]. Such 2D contact maps (**Figure 1**) are generated by exhaustive measurements of proteins and subcomplexes produced upon the disruption of intact assemblies [16,17,20]. Such disruption experiments can be carried out both in solution [20,21] and in the gas phase [22,23], and both approaches will be covered in some depth here. In this way, 3D models are built by integrating distance and size constraints from IM–MS measurements, along with other structural biology datasets and molecular modeling [15,24].

In this review, we endeavor to highlight the advances that are driving the development of IM–MS for structural proteomics. We specifically address the evolution of IM–MS technology, data interpretation, methods of generating enhanced IM–MS data for protein complexes and the integration of IM–MS with established proteomics protocols in efforts to define a structural picture of protein interaction networks. We then look to the future, and predict the state of the IM–MS field as applied specifically to the analysis of intact proteins and their complexes.

IM–MS technology

Following appropriate sample purification, separation and ionization (typically using nano-electrospray ionization [nESI]) [17], IM separates protein ions based on their ability to traverse a chamber filled with inert neutrals under the influence of an electric field. Larger protein ions undergo a greater number of collisions with the inert neutrals filling the chamber, and therefore have a larger collision cross-section (CCS) than more compact protein ions of similar mass (**Figure 2**) [25]. While this description holds true for most contemporary IM separations described currently in the literature, modern IM technology expands this basic principle into a variety of instrument platforms available for IM–MS experiments. Such instrumentation, as applied to multiprotein complexes, takes three basic forms: drift tube (DT)-type, differential mobility analyzer (DMA)-type and traveling-wave (T-wave)-type instruments. All of these technologies have both strengths and weaknesses for the analysis of multiprotein assemblies [26,27]. Other types of IM technology have yet to be used in the analysis of proteins and their complexes, and as such will not be discussed in detail here. For example, high-field asymmetric waveform IM spectrometry and differential mobility spectrometry, separate ions based on differences in IM generated as a function of separation field strength [28–30]. While both are commercially available, it is currently not possible to use these technologies to reliably measure ion CCS. Consequently, high-field asymmetric waveform IM spectrometry and differential mobility spectrometry are typically not used in experiments aimed at determining multiprotein structure. Other high-resolution

IM technologies, such as overtone IM spectrometry [31,32] and cyclotron-type DT analyzers [33], have yet to be applied to large protein size measurement, but offer exciting opportunities for detailed IM measurements on such systems in the future.

Many of the first IM separations performed on intact proteins and small complexes were conducted using DT-type IM–MS instrumentation [27,34]. DT-IMS measures ion CCS directly and provides high IMS resolving power in research-grade instruments. Such analyzers typically comprise a series of ring electrodes upon which a fixed axial gradient of the electric potential is constructed. A direct proportionality between drift time and CCS is generally used to convert measurements into ion size information. While early versions of these devices suffered from significant ion losses and poor sensitivity when used for the analysis of biomolecules, contemporary implementations of the technology use ion guides either during or after IM separation in order to refocus ions and preserve limits of detection [35–38]. Furthermore, tandem DT analyzers have been used to assess microconformational states within the structural envelope of gas-phase protein ions, and have demonstrated that such structural populations can be stable on the millisecond timescale, thus enabling detailed assessments of gas-phase protein structure [39,40].

DMA-type analyzers have also been utilized to measure the size of a range of protein complexes [41]. These devices function by introducing ions into a region filled with inert gas, often consisting of two plates with offset apertures for ion entrance and exit. A voltage is then scanned and the resulting ion trajectory between the entrance and exit aperture is dependent upon ion CCS. In contrast to DT-type IM separators, which measure a size-proportionate ion drift time, DMA analyzers measure the voltage required for ions to exit the analyzer and be transmitted for MS analysis. Ion mobility is inversely proportional to this voltage, and converting such information into ion CCS is typically performed through a rigorous calibration methodology [42,43]. In an analogous fashion to quadrupole mass filters, DMA analyzers can ‘scan’ over all ion mobilities or ‘select’ and transmit ions with a narrow range of mobilities for more detailed analyses [27,43,44]. In recent experiments, the proteins investigated using this technology have appeared to be more compact in the gas phase than previously thought [42], although a detailed structural analysis of these data and assessment of their broader implications have yet to appear in the scientific literature. Significant databases of protein size, as well as more detailed treatments of multiprotein structure, have appeared based on DMA measurements [45–47], although many of these measurements are performed in the absence of MS measurements in tandem.

The majority of IM–MS datasets for multi protein complexes have been generated on IM–MS instruments using T-wave IM analyzers. T-wave IM analyzers are similar in basic construction to DT-type IM devices but differ significantly in their operation. Rather than a linear field gradient, ions are propelled through the analyzer using a series of low-voltage waves [48,49]. Ions are carried by the waves relatively briefly before being subsumed by the wave front in a manner depending on the CCS of the ions being separated, generating a time domain IM separation similar to DT-IM devices [50]. An important feature of this process is that, due to the nature of the separation mechanism employed, T-wave drift times are most often calibrated using standard CCS values for protein complexes rather than being calculated directly from drift time measurements [51]. Apart from being the only IM analyzer currently incorporated into commercially available, high-sensitivity IM–MS instrumentation for ion size measurement in wide distribution, T-wave analyzers offer some modest advantages in terms of separation resolution [52–54].

Often defined in terms of the centroid arrival time of the IM peak normalized to the IM peak width ($t/\Delta t$), drift time resolutions for DT analyzers range from 30 to 150 for research-grade instruments, with those at the high end of the range produced using instruments with very

long flight tubes (>1 m) and high separation voltages (multiple kV) [33,55,56]. Because of the physical principles involved in T-wave IM separation, drift time is correlated to CCS through an exponential relationship [51], which results in a T-wave drift time axis that is effectively 'stretched' relative to those achieved on DT analyzers. This relationship enables T-wave separators to achieve 40–60 CCS resolution ($CCS/\Delta CCS$) using comparatively shorter devices and operating at lower fields and pressures than DT devices of equivalent dimensions [53,54,57].

IM–MS data interpretation

Generating both protein contact and topology information from IM–MS data requires multiple levels of computational analysis. The first stage in this process is typically focused on the interpretation of MS data derived from intact complexes and their constituent subcomplexes, as well as protein subunits generated through careful disruption of intact complexes. Obtaining an accurate component list of proteins within the complex, often from LC-MS protein identification experiments, is a critical, challenging stage in the analysis, as the mass measurement accuracy achieved for intact proteins and complexes is often much lower than those achieved for the peptide ions commonly encountered in 'bottom-up' proteomics experiments. This difference is primarily driven by the relative fragility and size of the multi protein complex ions generated, thus limiting the extent of chemical purification and desolvation that can be used in their MS analysis [58,59]. Often, the mass measurement accuracy achieved is primarily due to the level of desolvation achieved following nESI, with the peak widths and mass shifts recorded for complexes being directly correlated with the amount of non-volatile buffer material adhered to the assembly post-ionization [60,61]. Multiple software approaches have been developed for the automated mass analysis and deconvolution of nESI-MS datasets of protein complexes, enabling the interpretation of MS spectra containing multiple overlapping peaks [59,62–64], and in compiling MS datasets to generate protein complex contact diagrams. Furthermore, recent advances have been made in modeling the relative intensities within MS datasets in a more automated approach to spectral deconvolution [65].

In the interpretation of the IM data for multiprotein complexes, there are two main stages: the conversion of drift times into CCS values, and their comparison with model protein architectures. The first stage in this two-step process is relatively straightforward for DT and DMA-type IM analyzers, which rely upon highly robust calibration and simple algebraic relationships to convert primary data to ion sizes respectively. T-wave analyzers, on the other hand, require more carefully controlled calibration against standards with known CCS values in order to generate accurate results [51,66]. Theoretical treatments of T-wave analyzers have indicated a quadratic relationship between drift time and ion CCS when operated under a relatively narrow set of conditions [50]. Experimental calibration relationships, however, give a range of exponential values for the drift time–CCS relationship, and thus must be determined experimentally during IM–MS data collection in order to achieve optimum accuracy [51,53]. The accuracy of such measurements has increased dramatically in recent reports, primarily owing to the availability of a larger pool of available standards [66], such that modern T-wave CCS measurements are equivalent in accuracy and precision to those produced by other IM analyzers. While some reports have indicated that ions can be significantly heated during T-wave IM separation [67,68], recent results indicate that this effect, if present, does not influence CCS accuracy or precision on second-generation T-wave platforms [53]. Following accurate CCS measurement, model structures are generated *in silico* and their CCS values are estimated computationally for comparison with experimental CCS measures. Recently published IM datasets exhibit a broad correlation between known x-ray structures of complexes and the CCS values recorded for these same assemblies in the gas phase [10,69]. While many approaches exist

for estimating the CCS of x-ray and other structural models *in silico* [9], and relating the two datasets can be challenging [15], no computational approaches currently provide greater accuracy for multiprotein systems than simple, properly scaled, projection approximation (PA) calculations [69], and estimated CCS values have been effectively used for a number of years to correlate IM measurements with trial multiprotein topologies [15,22,24]. Such methodologies are computationally inexpensive, and have the benefit of supporting coarse-grained protein representations for rapid modeling and topological prototyping [22,70]. Scaling of such estimates is a key component of relating PA calculations to IM measurements, because without a properly tuned scaling factor, such calculations significantly misestimate the size of potential model structures [9]. Recent computational results support the potential accuracy of scaled PA estimates for the IM analysis of proteins and complexes, as well as introduce a new computational approach, termed the projection superposition approximation, which features a tunable shape factor and retains much of the computational speed of PA calculations [71].

Optimizing the information content of IM–MS for structural proteomics

The IM–MS protocols for structural proteomics described above rely heavily on measurements of intact protein complexes, as well as subcomplexes and subunits, for the determination of multi protein topology. Early experiments relied heavily on MS/MS data, utilizing collision-induced dissociation (CID) to disrupt protein complexes in the gas phase for the determination of complex stoichiometry [20]. Recent CID experiments have revealed that the charge states of precursor ions selected for activation and dissociation can dramatically influence both the structure and identity of the product ions formed. Precursor ions with charges unmodified from those produced from standard nESI conditions can occupy intermediates having undergone both quaternary remodeling and monomer unfolding prior to dissociation [72–74]. By contrast, charge-reduced complexes subjected to CID can produce compact and presumably folded product ions [75]. In rare cases, charge amplification has also been observed to enhance the folded character of product ions produced by multiprotein CID [76]. Either extensive charge reduction or amplification, followed by higher energy CID, can result in the dissociation of covalent bonds within the complex to produce sequence-informative peptide ions from protein ter-mini [23,75]. This last observation suggests the exciting possibility of ‘top-down’-type protein identification experiments performed from multiprotein precursor ions, and has been duplicated using electron-mediated fragmentation approaches [77]. Surface-induced dissociation (SID) technology has recently emerged as an alternative to CID for studying multiprotein complexes, as it can generate subcomplex and presumably compact subunit product ions following multiprotein complex activation [78]. Taken together with the important advances made recently in CID and electron-based fragmentation, gas-phase protein disruption approaches have the ability to access multiple levels of multiprotein organization to greatly inform protein contact map construction.

Despite the proliferation of gas-phase dissociation and disruption methodologies for multiprotein assemblies, such technologies often do not provide sufficient information on their own to deduce a complete protein contact map. As a complementary approach, those studies that have reported high-confidence-level protein contact diagrams have optimized conditions in solution prior to nESI in order to generate subcomplexes with largely orthogonal compositions to those produced by the gas-phase methodologies described above. Such disruption experiments often involve small additions of organic solvent, alterations in ionic strength and solution pH in order to elicit the formation of topologically informative subcomplexes [20,21,24,79,80]. While the phenomenology of this process has been described in multiple reports, and early studies suggested that the evolutionary origins of the protein–protein interface structures within complexes can be used to predict

disruption behavior [81,82], many of the basic principles at work during such multiprotein disruption experiments are still a matter of intense research. Therefore, a relatively exhaustive search of potential solution conditions for optimal disruption, performed in a trial-and-error manner, is usually necessary to develop the information necessary for protein contact map generation (**Figure 3**). Protein disruption data are expected to correlate with the physical and chemical properties of the interacting interfaces between protein subunits [81]. Note that the optimal conditions for subcomplex generation may not overlap with those for other key figures of merit for IM–MS of intact protein complexes. This can result in losses in overall signal intensity, MS resolving power and IM resolution during a search of solution conditions for subcomplex formation. While currently time consuming, such experiments have been critical in establishing the assembly dynamics of viral coat proteins and assigning connectivity within multiple heteroprotein complexes of varying size and structure [24,83,84].

In addition to actively destabilizing the structure and connectivity of protein complexes in both the gas phase and in solution, stabilizing protein structures is an equally important goal of IM–MS protocols in structural proteomics. Charge manipulation of protein complex ions produced by nESI, especially charge reduction, can be an effective method of protein stabilization in the gas phase. Recent work has indicated that charge reduction approaches that utilize gas-phase chemistries may be the most effective in the universal structural stabilization of multiprotein complexes [85]. Similarly, small-molecule additives can be used to influence evaporative cooling during nESI, and have been shown to be effective in stabilizing protein–ligand systems [86]. Likewise, small molecules may be added in solution prior to nESI to stabilize the transition between solution and gas phase for protein complexes. Recent studies focused on Hofmeister-type salt systems have discovered dramatic variations between the stabilizing influences of different salts on gas-phase protein structure compared with solution [60,87]. This work also fits into the solution-screening framework described above (**Figure 3**), where multiple salts and small molecules are titrated against IM measurements to gauge protein structural integrity for topology modeling.

Integrating bottom-up proteomics with intact protein IM–MS

Any effort made to structurally assess unknown protein networks by IM–MS incorporates a robust MS-based protein identification workflow in parallel. Most IM–MS workflows for protein complex topology mapping from measurements of intact protein complexes have relied upon a complete list of interacting components, derived from ‘bottom-up’ and denatured protein-based ‘top-down’-type approaches for LC-MS protein identification. IM–MS technology also has a long history in both peptide mass mapping and automated LC-type protein identification experiments, which enables IM to contribute to protein component identification as well as protein structure assignment [88,89]. By incorporating IM separation into standard LC-MS experimental workflows, peptide separation capacity can be increased by one-to-two orders of magnitude [27,70,90–92] and highly parallelized approaches to MS/MS can be implemented to increase sample throughput [93,94]. If these advantages are coupled with the unique structure-based separations [95,96] and chemical tagging methodologies that allow different chemical classes to be distinguished based on the analysis of ‘trend-lines’ within IM–MS datasets [90], a compelling argument can be made for IM–MS workflows that cover both multiprotein interaction topology determination and protein identification.

MS experiments that have aimed to deduce protein contact diagrams for multiprotein complexes have incorporated extensive protein identification schemes into their published workflows (**Figure 4**). Most of these involve partitioning samples into two-to-three tracks, in which one track incorporates intact MS analysis of the complexes and subassemblies

generated via disruption (see above), a second track uses ‘bottom-up’ proteomics to identify the proteins present in the mixture via mass sequence tags and database searching, and a third (optional) track uses intact protein mass measurements on the denatured protein subunits to evaluate the level of post-translational modification present within the constituent proteins. This last track can be critical to successful contact map generation, especially in cases where multiple subunits within the complex have similar sequence masses. IM measurements on protein complexes have recently been incorporated into such workflows (**Figure 4**). For example, IM measurements were used to determine the connectivity and topology of a trimeric protein subcomplex within the eukaryotic initiation factor 3 (eIF3) hetero-complex [24]. In these experiments, protein dimers that compose the trimer in question were observed for all but one of the possible pairs. Such dimer signals could be absent from the MS measurements for many reasons, but IM measurements identified a linear arrangement of subunits, which allowed the model of the entire 13-subunit complex to be restrained with high confidence.

Expert commentary

Over the past few years, IM–MS has been used in a number of cases to determine the structures of aggregating peptide and protein systems [97–99], to perform structural studies on a range of protein homo-oligomers [11,100], to establish the structural details of small heterocomplexes [15] and to refine MS-derived contact maps for large hetero-assemblies [21]. These achievements have been driven equally by the advent of novel research-grade instruments and commercially available IM–MS equipment optimized for the analysis of large protein assemblies. In many cases, advances in instrumentation have provided increased IM resolution and MS resolving power [54]. More importantly, modern instrumentation incorporates IM separation devices with high ion transmission efficiency and low limits of detection, which are capable of highly sensitive analyses [36,38,54]. It is clear that integrating IM separation into any MS workflow aimed at characterizing protein mixtures and interaction networks, in either a ‘bottom-up’ or ‘top-down’ paradigm, will substantially increase the information content of the resulting analysis [101].

While the outlook for IM–MS is generally positive, there are a number of challenges that limit its applicability in certain areas of proteomics. A number of these challenges are in the area of computational IM–MS data interpretation. While existing methodologies allow model structures and experimental constraints to be compared relatively accurately, many sources of potential error still exist in such assessments [9]. Appropriately scaled versions of *in silico* estimates of model structure CCS values, for example, are relatively accurate for currently studied protein complexes; however, it is unknown if such approaches will continue to be accurate for the myriad of structures predicted within the entire proteome. A potentially greater challenge for IM–MS computational data interpretation is the rapid and accurate generation of model structures from relatively limited primary information, or solely from IM–MS constraints [15]. In the absence of x-ray or NMR datasets, or when those datasets only contain partial information, current algorithms for generating coarse-grained approximations of protein complex structure must be substantially improved to assign the structures of large heterocomplexes in a high-throughput fashion.

Experimental challenges for IM–MS measurements revolve around the ability to either stabilize protein structure in the absence of bulk solvent or to disrupt that structure to determine protein connectivity, substructure and topology. Such challenges are amplified in experiments seeking to analyze membrane protein complexes by IM–MS [102]. In these cutting-edge studies, substantial activation is required to remove surfactant and stabilizing molecules in order to make IM–MS measurements, but it is currently unknown if such activation steps dramatically alter protein structures from those that are biologically

relevant. Continued experiments aimed at protein ion charge manipulation [85] and testing the stability of water-soluble protein structures bound to varying populations of stabilizing small molecules [60,61] will undoubtedly further enhance the ability of IM–MS to analyze such membrane protein complex systems.

Five-year view

Currently, there are several major trends in the application of IM–MS. Arguably the most important of these is the push to continuously stretch and apply this technology to the analysis of ever-larger and more complex protein assemblies [58,103,104]. A second key trend is the extensive and growing use of IM–MS to study aggregating protein systems important in amyloid-type diseases [98]. Additionally, the pace of IM–MS technology development is accelerated by extensive use of commercial instrumentation, as well as modified versions of commercial platforms aimed at the enhanced dissociation or charge manipulation of ions for downstream IM–MS analysis [85,105–107]. We expect all of these trends to not only continue, but also to grow and progress significantly over the next 5 years. As a part of this process, we expect the continued and enhanced integration of IM–MS with other structural biology tools, especially electron microscopy [2]. Based on the workflows described here (**Figure 4**), high-throughput versions of the IM–MS technology, capable of rapidly assessing protein topology from relatively small amounts of isolated cellular material, will probably emerge out of the coming 5-year period. Regardless of the details, it is clear that the next 5 years will be an exciting period of growth for IM–MS in the fields of structural biology and structural proteomics.

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Key issues

- Ion mobility–mass spectrometry (IM–MS) is an emergent technology for structural proteomics, capable of assessing multiprotein topologies from complex mixtures using minute amounts of sample.
- There are multiple types of IM technologies frequently coupled to MS, all of which offer advantages and disadvantages for the analysis of large proteins and their complexes.
- Computational analysis is a key aspect of IM–MS data interpretation, and developments in this area are likely to be rapid over the next 5 years.
- There are useful analogies that exist relating the current challenges in IM–MS experiments aimed at determining multiprotein topology and early experiments in high-throughput x-ray structure generation for proteins. Both technologies seek optimal experimental conditions from a vast array of potential solution states.
- Exciting recent developments in the gas-phase dissociation of protein complexes will undoubtedly lead to enhanced capabilities for assessing 3D protein architecture.
- The analysis of multiprotein complexes of unknown composition requires the integration of intact IM–MS measurements with ‘top-down’ and ‘bottom-up’-style proteomics workflows in order to correctly derive protein connectivity and topology.
- Stabilizing highly flexible or deformable protein structures in the absence of bulk solvent, including membrane-bound systems, is a key challenge for the future of IM–MS development, and will undoubtedly require much basic research using model systems.

Figure 1. The challenge of structural genomics: converting protein interaction networks into protein structures

High-throughput structural genomics efforts currently underway rely heavily on technologies that can convert an isolated multiprotein complex (A) directly into a structural model of atomic resolution (dashed arrow). The stringent sample requirements involved result in relatively high failure rates for such experiments. An alternative approach (solid arrows) uses mass spectrometry data of intact complexes to generate a contact map (B), integrates ion mobility data and other constraints to build a 3D topology model (C), and utilizes homology modeling or other forms of local constraint to generate the final atomic model (D) for multiprotein complexes. This ion mobility–mass spectrometry approach is projected to be a more universal, more sensitive and higher throughput alternative to contemporary structural biology technologies.

Figure 2. Ion mobility–mass spectrometry data acquisition and basic principles

Ions are generated at the ion source (lower left) and are allowed to drift in an ion guide filled with neutral gas molecules under the influence of an electric field. The ions migrate through this region according to their size-to-charge ratio. They are then injected into a ToF mass analyzer under vacuum for m/z analysis. The resulting data are 3D, containing ion intensity, size and mass information. The various dimensions of the data can be shown as a contour plot (middle, bottom), or 2D selections in drift time or m/z (lower right). A key for the diagram is shown (upper right).

IM: Ion mobility; IM–MS: Ion mobility–mass spectrometry; MS: Mass spectrometry; m/z : Mass-to-charge ratio.

Figure 3. A high-throughput screening process to discover the optimal solution conditions for protein complex topology mapping by ion mobility-mass spectrometry
(A) A 2D screen is developed by varying the composition of solutions in a stepwise fashion over several important variables (e.g., organic content). Ions produced from each solution state are then measured against basic figures of merit: MS resolving power and mass accuracy (pink), IM resolution and collision cross-section (blue), total ion intensity (green), and the percentage of current that carries signal for subcomplexes or monomeric proteins (purple). **(B)** Optimal solutions for each of these classes of information are identified and recorded. In many cases, the optimal solution conditions for each figure of merit are mutually exclusive. The three dots at the bottom of part (A) and part (B) indicate that additional 2D solution screens can be implemented as needed.
 CCS: Collision cross-section; IM: Ion mobility; m/z : Mass-to-charge ratio.

Figure 4. Flow diagram for an integrated ion mobility–mass spectrometry structural proteomics workflow

After protein complex isolation via either standard affinity purification strategies, or following overexpression and reconstitution of the complex *in vitro* (purple box, top), unknown protein samples are split into three channels. One portion of the sample is subjected to denaturation and enzymatic digestion for a combination of ‘top-down’ and ‘bottom-up’-type protein identification experiments. These steps are critical for forming an accurate component list for protein contact map generation. A second sample fraction is submitted to MS for intact analysis, where the assembly is dissociated using a combination of solution and gas-phase approaches to deduce protein connectivity. Information from both of the above sample streams (turquoise) is combined to assemble a protein contact map. A third sample fraction, using optimized solution conditions, is submitted for ion-mobility analysis and measurement of protein size. Ideally, this step can be performed in parallel with MS analysis of the intact protein complex and subcomplexes created by dissociation. Size information on monomers is used to refine structures of the subunits within the complex, and various subcomplexes in a stepwise fashion. A cartoon showing how ion mobility data can refine structures for both a multidomain monomer (purple) and a protein dimer (yellow/blue) are shown, and this information is combined with MS-derived contact map information to provide a complete 3D protein topology (red). This information is then further combined with other sources of information or homology modeling to provide a complete atomic model of the complex of interest.

IM-MS: Ion mobility-mass spectrometry; MS: Mass spectrometry; m/z : Mass-to-charge ratio.