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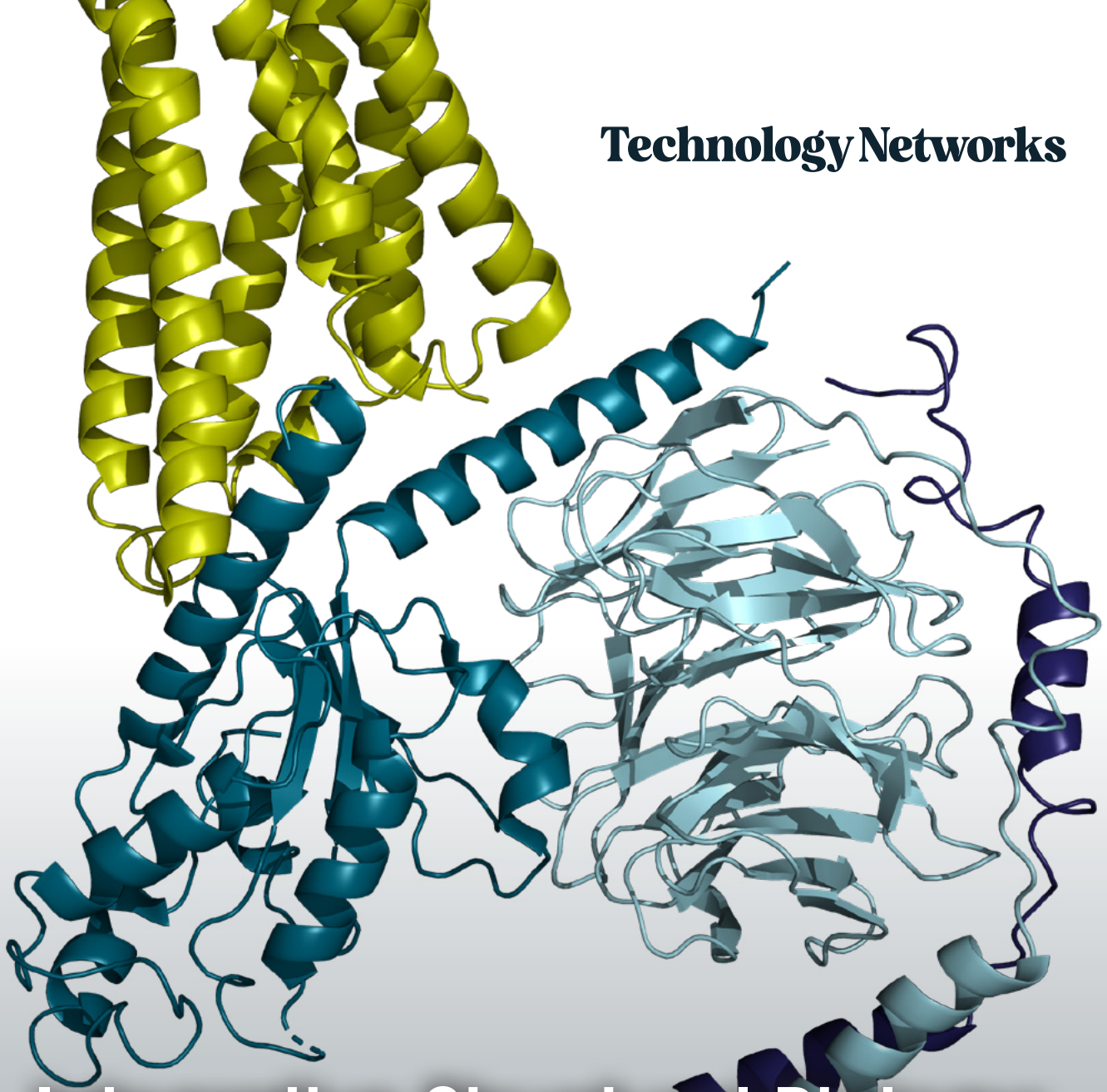

Integrative Structural Biology: High Precision 3D Analysis from Structure to Function

Combining Cryo-ET and Mass Spectrometry

Mass Spectrometry and Its Role in Integrative Structural Biology

Mass Spectrometry for Cryo-EM Sample Screening

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Foreword

In order to truly understand how proteins function in their native environment, researchers must look beyond studying individual proteins alone and examine the assembly and structure of protein complexes within the cell. Advances in mass spectrometry (MS) have had a considerable impact on structural biology research, enabling researchers to characterize biomolecular structures with greater speed, sensitivity and selectivity. Similarly, the emergence of cryo-electron microscopy (cryo-EM) has resulted in the ability to directly visualize complete macromolecular complexes in near-atomic level resolution.

Structural biologists face several challenges when trying to solve the structure of large and dynamic complexes. However, the combination of different techniques, an approach known as integrative structural biology, is revolutionizing the understanding of protein structure, function and dynamics.

Thermo Fisher Scientific offers a range of innovative analytical tools designed to help structural biologists solve complex challenges in the field. This eBook will explore the evolution of MS and cryo-EM and their impact on the field of structural biology, with reference to how researchers are incorporating Thermo Fisher's solutions into their own research.

by Rosa Viner and Albert Konijnenberg

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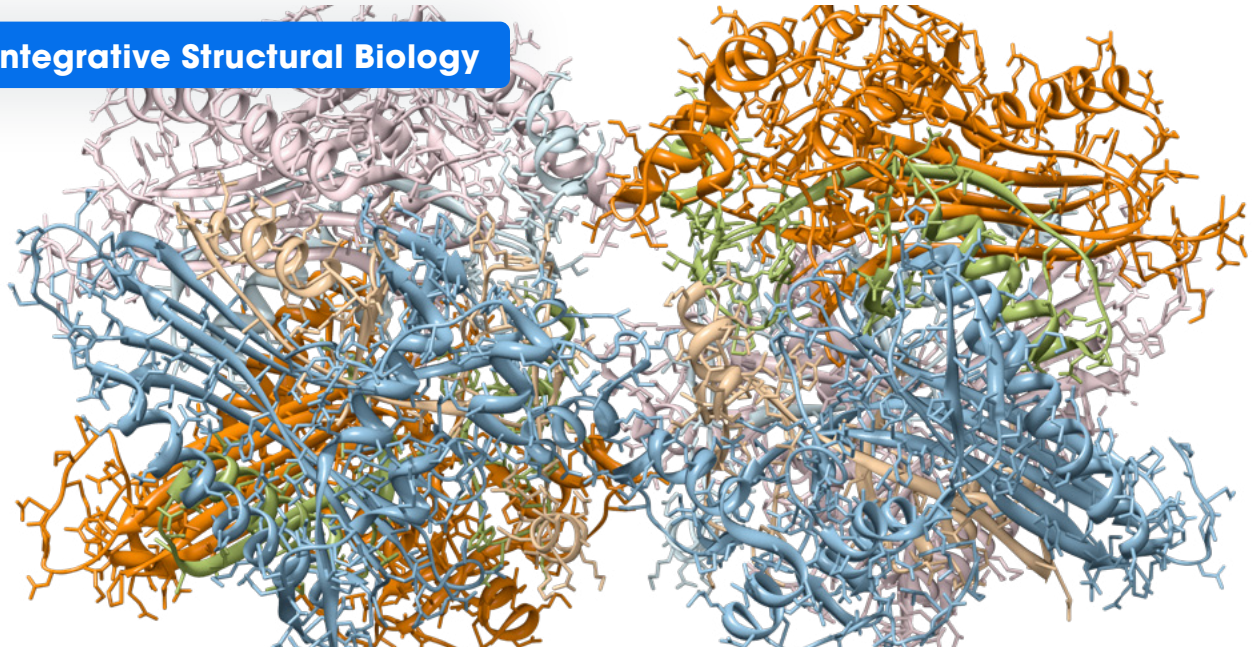


Chapter 1: An Introduction to Integrative Structural Biology

Introduction

Structural biology is a branch of science that has been dominated by high-resolution techniques such as X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR), however these techniques are limited by the size and range of the molecules that they can study. Additionally, the components and configurations of these macromolecules can change, therefore, researchers require strategies that can assess a wider range of heterogenous and complex samples.[1,3,4]

Extracting functional information of proteins – rather than structure alone – is key to tackling key research questions within structural biology, however, generating this information requires input from multiple techniques. Mass spectrometry (MS), cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) offer significant improvements in resolution and have gained attention in the structural biology field. Yet, when used alone they are still limited in their ability to provide unique information about protein structure and function. In recent years, the combination of these approaches – known as integrative structural biology – has enabled researchers to probe structures that were previously inaccessible and in much more detail. This article will discuss the techniques used within integrative structural biology, as well as their advantages and common applications.[1,5–8]

Techniques for structural biology research

Mass spectrometry

MS detects the mass-to-charge ratio (*m/z*) of ionized molecules, allowing scientists to detect and identify these molecules and their variations within a sample.[9] Although its traditional use focuses on protein sequencing, recent developments have enabled MS to become a powerful tool for protein structure analysis.[1] Technological advances enabled MS to analyze macromolecules in their native complexes. MS-based methods are advantageous due to their ability to analyze heterogeneous samples, across a broad range of molecular weights, that are difficult to study using other techniques. Intact molecules are detected using analyzers that detect ever broader *m/z* values, meaning larger molecules can be analyzed without being digested first. Additionally, combining MS with soft ionization techniques, like electrospray ionization (ESI), improves sample integrity during analysis and allows for maintaining even weak molecular interactions.[1,10] The analysis of non-denatured proteins in their native-like complexes, known as native MS (nMS), provides structural information about post-translational modifications (PTMs), binding interactions, and subunit stoichiometry.[11,12]

Apart from nMS, several MS-based techniques exist that are capable of probing macromolecules in their native complexes and environment by covalent or reversable

labeling, including fast photochemical oxidation of proteins (FPOP), chemical crosslinking MS (XL-MS) and hydrogen/deuterium exchange-MS (HDX-MS).[1] The development of automated systems, as well as methods that have better peak capacity and accept higher levels of unique sequences has resulted in increased adoption of HDX-MS. This technique enables researchers to decipher structural information about large macromolecules in their native complexes.[13] By monitoring deuteration levels from the hydrogen/deuterium exchange of backbone protein amides, HDX-MS is particularly useful for obtaining conformational information of protein complexes and binding interactions.[13] XL-MS is a technique whereby native proteins are chemically crosslinked, providing information on how protein complexes are linked together. Since XL-MS requires that structures are close enough to crosslink, it can also be used to gather spatial information.[14]

Together, these techniques provide unique structural information on a wide array of macromolecular systems that can be used by researchers to characterize proteins more comprehensively.[1] These developments have enabled researchers to analyze proteins directly in cells, tissues and organisms, as well as isolated proteins, with applications including PTMs, soluble proteins, membrane proteins, macromolecular machines and viral structures.[1,11] However, MS techniques alone cannot generate high-resolution protein structures and are commonly paired with other complimentary MS techniques or alternative structural biology approaches, such as cryo-EM.[15]

Cryo-EM

Cryo-EM is a revolutionary tool that has been used to determine the 3D structure of proteins. The process involves rapidly freezing samples in a thin layer of vitreous ice to prevent the formation of ice crystals, preserving samples in a more natural state, while protecting them against the harsh imaging conditions (vacuum and electron beam).[16–18] Modern, high-resolution cryo-EM largely relies on single-particle analysis in frozen and fixed samples. 2D projections of randomly orientated molecules are then collected and analyzed to reconstruct a 3D structure.[19,20] This 3D structure can then be used to determine protein function in health and disease and identify potential drug targets.

Furthermore, cryo-EM is commonly used to gain structural and functional insights into macromolecules and their complexes. Proteins are frozen in their native complexes and can be imaged in less stable confirmations, allowing cryo-EM to capture the different functional states of macromolecule complexes. Through averaging multiple particles in different orientations, cryo-EM can achieve highly-resolved structures at (near) atomic level.[21] Consequently, common applications include the analysis of macromolecule/macromolecule complexes involved in gene, protein and immune regulation (such as the spliceosome, ribosome and inflammasome), as well as membrane proteins and viral structures.[22,23] Whilst cryo-EM is good for studying the 3D structure, due to the need for averaging, dynamic aspects are typically lost. Additionally, in order for this technique to be successful, researchers must already know what they are looking for.

Cryo-ET

Cryo-ET is a form of cryo-EM in which several 2D projections of a sample are collected at different tilt angles. These images are then used to reconstruct a 3D structure.[24] As with cryo-EM this technique preserves the sample in a more native state, avoiding macromolecule damage from factors such as dehydration and the microscope vacuum.[18] Cryo-ET can be used in a variety of complex samples *in-situ*, such as studying molecular machines in whole cells or organisms. Consequently, it can provide spatial and functional information for macromolecular machines across a variety of applications, such as the study of mechanisms in healthy cells (e.g., mitosis), infected or diseased cells (e.g., the trypanosome flagellum in African sleeping sickness) and diagnostic applications (e.g., detecting abnormal platelets in ovarian cancer).[25] Cryo-ET was traditionally limited by sample thickness and typically used for thin samples such as bacteria or small tissue segments. However, the emergence of cryo-focused-ion beam milling overcame these limitations in sample thickness, by "carving" thin regions (100–250 nm) out of the frozen cells, exposing internal macromolecules for structural imaging.[26] It can be difficult to obtain images at all orientations in cryo-ET therefore it is best to use this method in conjunction with other approaches to compensate for the limited resolution that is obtained.[27]

Integrative MS and Cryo-EM/ET

Combining structural biology approaches (known as integrative structural biology) offers more comprehensive structural and functional information of macromolecules in their native environment, overcoming limitations of a single technique (such as sample heterogeneity and limited resolution) (Figure 1). Integrative structural biology approaches that combine MS and cryo-EM can determine the structure and function of large, complex protein machinery, such as the nuclear pore complex and intact ribosomes.[28] In addition, advanced integrative techniques combining MS and cryo-ET can identify mutated proteins in diseased cells, such as cancer cells. For example, thousands of proteins and protein complexes can be identified in samples from healthy and cancerous tissues using MS techniques. However, cancer-associated mutational changes that disrupt protein complexes can be detected during MS analysis as variations in relative abundances.Affected complexes may then be targeted for detailed structural analysis by cryo-ET to understand how the mutations impact protein structure and aid therapeutic design.[29] Finally, through integrated modeling, the data from various techniques can be combined to provide the most detailed picture of the protein or complex under investigation.

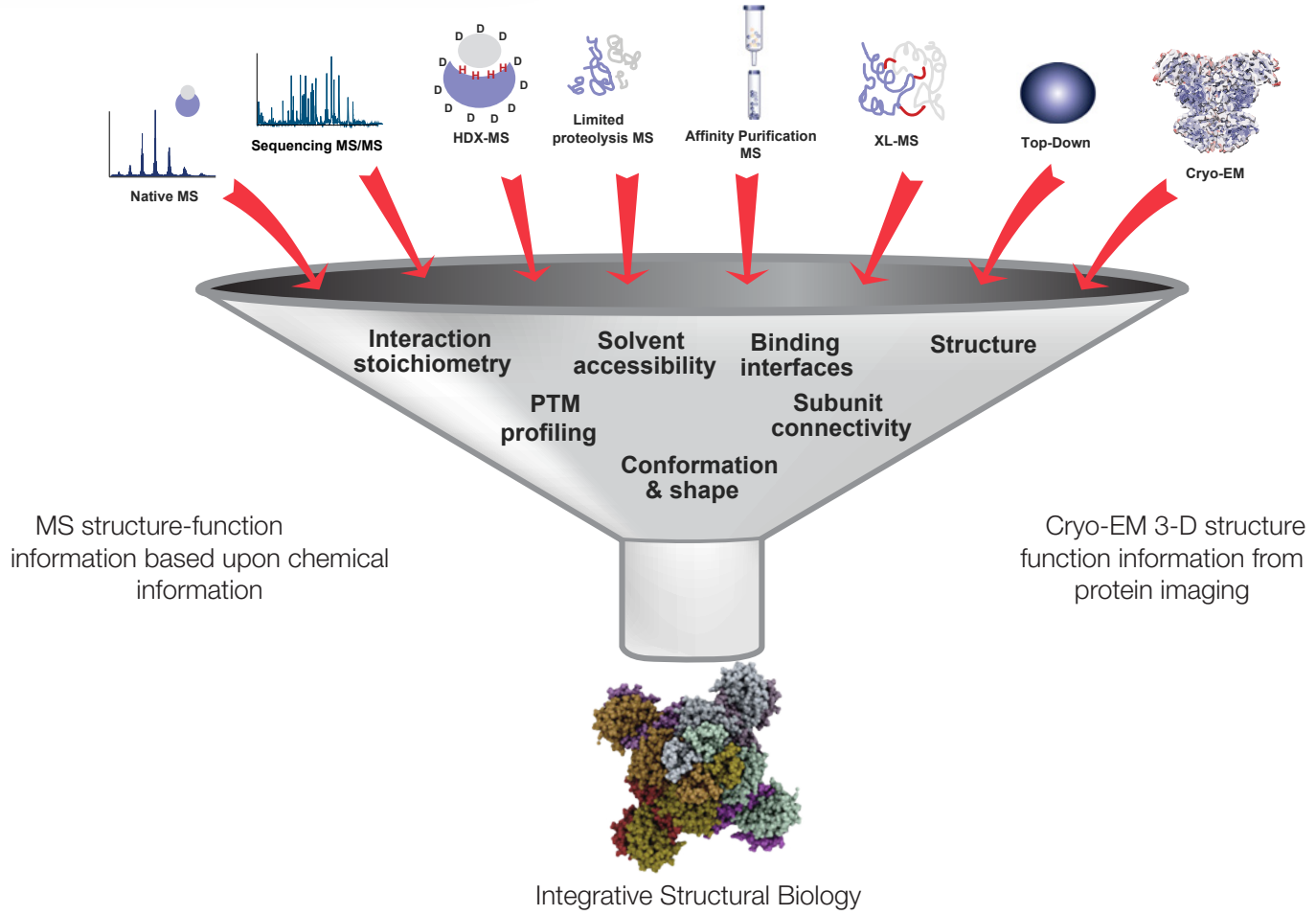


Figure 1. Mass spectrometry–cryo-EM combined workflow.

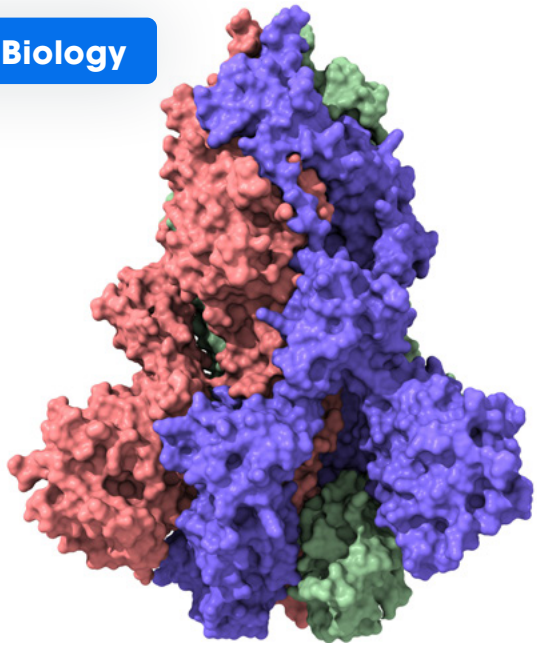
Conclusion

Advances in MS and electron microscopy (cryo-EM and cryo-ET) allow researchers to gain insights into ever larger or more complex macromolecular machines and how their location, configuration and interactions influence their function. In addition, these techniques can be used on samples that cannot be crystalized, under complex conditions that better reflect their native environments. By combining multiple approaches, researchers can build more complete structural models to improve their understanding of the structure and function of complex biological systems.

References

1. Kaur U, Johnson DT, Chea EE, Deredge DJ, Espino JA, Jones LM. Evolution of structural biology through the lens of mass spectrometry. *Anal. Chem.* 2019;91(1). doi:10.1021/acs.analchem.8b05014
2. Campos-Acevedo AA, Díaz-Vilchis A, Sotelo-Mundo RR, Rudiño-Piñera E. First attempts to crystallize a non-homogeneous sample of thioredoxin from Litopenaeus vannamei: What to do when you have diffraction data of a protein that is not the target? *Bioch. Bioph. Rep.* 2016;8. doi:10.1016/j.bbrep.2016.09.011
3. Pastore A. New challenges in structural biology: catching the complexity of dynamic nanomachines. *Front. Mol. Biosc.* 2014;1. doi:10.3389/fmolb.2014.00003
4. Sonn-Segev A, Belacic K, Bodrug T, *et al.* Quantifying the heterogeneity of macromolecular machines by mass photometry. *Nat. Com.* 2020;11(1). doi:10.1038/s41467-020-15642-w
5. Wörner TP, Snijder J, Bennett A, Agbandje-McKenna M, Makarov AA, Heck AJR. Resolving heterogeneous macromolecular assemblies by Orbitrap-based single-particle charge detection mass spectrometry. *Nat. Meth.* 2020;17(4). doi:10.1038/s41592-020-0770-7
6. Fischer N, Neumann P, Konevega AL, *et al.* Structure of the E. coli ribosome–EF-Tu complex at <3 Å resolution by Cs-corrected cryo-EM. *Nature.* 2015;520(7548). doi:10.1038/nature14275
7. Verbeke EJ, Zhou Y, Horton AP, Mallam AL, Taylor DW, Marcotte EM. Separating distinct structures of multiple macromolecular assemblies from cryo-EM projections. *J. Struct. Biol.* 2020;209(1). doi:10.1016/j.jsb.2019.107416
8. White TA, Bartesaghi A, Borgnia MJ, *et al.* Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses: strain-dependent variation in quaternary structure. *PLoS Path.* 2010;6(12). doi:10.1371/journal.ppat.1001249

9. Murayama C, Kimura Y, Setou M. Imaging mass spectrometry: principle and application. *Bioph. Rev.* 2009;1(3). doi:10.1007/s12551-009-0015-6
10. Banerjee S, Mazumdar S. Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. *Int. J. Anal. Chem.* 2012;2012. doi:10.1155/2012/282574
11. Lössl P, Waterbeemd M, Heck AJ. The diverse and expanding role of mass spectrometry in structural and molecular biology. *EMBO J.* 2016;35(24). doi:10.15252/embj.201694818
12. Liko I, Allison TM, Hopper JT, Robinson C. Mass spectrometry guided structural biology. *Curr. Op. Struct. Biol.* 2016;40. doi:10.1016/j.sbi.2016.09.008
13. Engen JR, Komives EA. Complementarity of hydrogen/deuterium exchange mass spectrometry and cryo-electron microscopy. *Trends Bioch. Sci.* 2020;45(10). doi:10.1016/j.tibs.2020.05.005
14. Leitner A, Faini M, Stengel F, Aebersold R. Crosslinking and mass spectrometry: an integrated technology to understand the structure and function of molecular machines. *Trends Bioch. Sci.* 2016;41(1). doi:10.1016/j.tibs.2015.10.008
15. Schmidt C, Urlaub H. Combining cryo-electron microscopy (cryo-EM) and crosslinking mass spectrometry (CX-MS) for structural elucidation of large protein assemblies. *Curr. Op. Struct. Biol.* 2017;46. doi:10.1016/j.sbi.2017.10.005
16. Carroni M, Saibil HR. Cryo electron microscopy to determine the structure of macromolecular complexes. *Methods*. 2016;95. doi:10.1016/j.ymeth.2015.11.023
17. Callaway E. Revolutionary cryo-EM is taking over structural biology. *Nature*. 2020;578(7794). doi:10.1038/d41586-020-00341-9
18. Renaud J-P, Chari A, Ciferri C, *et al.* Cryo-EM in drug discovery: achievements, limitations and prospects. *Nat. Rev. Drug Disc.* 2018;17(7). doi:10.1038/nrd.2018.77
19. Cheng Y. Single-particle cryo-EM—How did it get here and where will it go. *Science*. 2018;361(6405). doi:10.1126/science.aat4346
20. Lyumkis D. Challenges and opportunities in cryo-EM single-particle analysis. *J. Biol. Chem.* 2019;294(13). doi:10.1074/jbc.REV118.005602
21. Ziegler SJ, Mallinson SJB, st. John PC, Bomble YJ. Advances in integrative structural biology: Towards understanding protein complexes in their cellular context. *Comp. Struct. Biotech. J.* 2021;19. doi:10.1016/j.csbj.2020.11.052
22. Fernandez-Leiro R, Scheres SHW. Unravelling biological macromolecules with cryo-electron microscopy. *Nature*. 2016;537(7620). doi:10.1038/nature19948
23. Benjin X, Ling L. Developments, applications, and prospects of cryo-electron microscopy. *Protein Sci.* 2020;29(4). doi:10.1002/pro.3805
24. Murata K, Wolf M. Cryo-electron microscopy for structural analysis of dynamic biological macromolecules. *BBA - General Subjects*. 2018;1862(2). doi:10.1016/j.bbagen.2017.07.020
25. Melia CE, Bharat TAM. Locating macromolecules and determining structures inside bacterial cells using electron cryotomography. *BBA - Proteins Proteom.* 2018;1866(9). doi:10.1016/j.bbapap.2018.06.003
26. Wagner FR, Watanabe R, Schampers R, *et al.* Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography. *Nat. Prot.* 2020;15(6). doi:10.1038/s41596-020-0320-x
27. Strack R. Structures in situ. *Nat. Meth.* 2020;17(1). doi:10.1038/s41592-019-0704-4
28. Faini M, Stengel F, Aebersold R. The evolving contribution of mass spectrometry to integrative structural biology. *J. Am. Soc. Mass Spectr.* 2016;27(6). doi:10.1007/s13361-016-1382-4
29. Powell K. Technology to watch in 2018. *Nature*. 2018;553(7689). doi:10.1038/d41586-018-01021-5



Chapter 2: The Role of Cryo-Electron Microscopy in Integrative Structural Biology

As already mentioned, the resolution of cryo-electron microscopy (cryo-EM) has greatly improved in recent years and it is now rivalling X-ray crystallography. Consequently, cryo-EM – including both single particle analysis (SPA) and cryo-electron tomography (cryo-ET) – is becoming a key technique across a range of applications to determine the structures of heterogeneous and dynamic assemblies that were traditionally difficult to study.[1,2,3] This has resulted in the widespread adoption of cryo-EM; David Julius and Ardem Patapoutian received the 2021 Nobel prize in Physiology or Medicine for discovering the receptors for temperature and touch. Their work was made possible thanks to cryo-EM. Moreover, the application of cryo-EM to study SARS-CoV-2 proteins contributed to the development of effective therapeutics and vaccines during the COVID 19 pandemic.[4] This article will discuss the role of cryo-EM as an essential tool for structural biology research.

Role of Cryo-EM in protein structure research

Dr. Jeff Lengyel used a combination of cryo-EM and biophysical techniques during his doctoral research at the University of Cambridge, UK, and today is the director of product marketing at Thermo Fisher Scientific. He believes that, "cryo-EM is the largest growing research tool for structural biology and is rapidly becoming a routine tool for drug discovery platforms in pharmaceutical and biotech companies." This was made evident during the COVID-19 pandemic, when cryo-EM was used to determine the structure of the flexible SARS-CoV-2 spike protein – a trimer containing three receptor binding domains with variable confirmations. This structure provided the blue-print for the COVID-19 vaccines. He adds, "we also worked with researchers from UC Berkeley to solve a high-resolution structure of a membrane protein from SARS-CoV-2, which is a potential drug target."

In another collaboration, researchers from the University of Cambridge and Thermo Fisher Scientific "generated a high-resolution reconstruction of the GABA (gamma-aminobutyric acid) receptor, which is a critical drug target for several neurological conditions including sleep apnea." Through another collaboration with researchers from the University of Cambridge, they were able to "determine numerous 3D reconstructions of tau filaments". Tau protein can form pathogenic filaments (tau filaments), which lead to a range of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases.[5] Using SPA, the structure of a wide range of tau filaments was determined, allowing them to classify different diseases based on their structure, which led to the discovery of a novel tauopathy. Dr. Lengyel affirms that, "no other structural technique could have been used to do this and it is hoped that these structures could aid the development of treatments for these conditions."

Cryo-ET is capable of handling complex cells or impure samples and can therefore be used to "determine 3D structures of variably shaped viruses (e.g., SARS-CoV-2), bacteria, and eukaryotic cells. When applying cryo-ET to cells, one can generate a 3D image of a section of a cell, allowing visualization of the cellular architecture at an unprecedented resolution, as well as visualization of individual proteins within the cell."

Cryo-EM workflow and challenges

Dr. Alexander Rigort is working in product marketing and development at Thermo Fisher Scientific. He obtained his PhD in cell biology from the University of Bonn before joining the Max Planck Institute of Biochemistry in Munich where he developed and used cryo-focused ion beam instrumentation for applications in electron tomography. He believes that cryo-EM "is currently the best way to study protein or cellular samples in their native state at high resolution". He partly ascribes this to vitrification, which, "makes it possible to rapidly arrest purified proteins or whole intact cells in such a way that hardly any structural changes will occur at the molecular level. Conventional methods for ultra-structural sample preservation cannot do this because they must replace the water content in the cell with a suitable alternative medium, such as resin. At the molecular level, this then leads to changes that make it impossible to determine the exact protein structure. Cryo-ET makes it possible to take high-resolution snapshot images from inside cells. Thus, not only individual proteins can be analyzed, but also their interactions amongst each other as well as their spatial context within the cell."

SPA and cryo-ET differ in their image acquisition; "in cryo-tomography, 2D images of the sample are generated at different tilt angles" from which a tomogram can be reconstructed.[6] He adds; "the way tomography works is conceptually like a computer tomography (CT) scan in radiology but with two important differences. Instead of x-rays, a beam of electrons is used – produced in a transmission electron microscope (TEM) – and instead of rotating the electron source around the cell, the sample is rotated under the electron beam. With cryo-ET, nanometre-resolution 3D images from the interior of cells can be generated. Such detailed 3D images from cells in a near-natural state are only possible due to the very good structural preservation by vitrification, in which the hydrated cell interior is preserved in a glass-like amorphous ice".

Although cryo-tomography offers additional information by investigating "proteins within their functional cellular environment", Dr. Rigort notes that it "currently still lags behind single particle analysis in terms of resolution". Additional challenges for cryo-EM include "the production of electron-transparent samples from cells and, in the future, also from tissue samples". Although fluorescence microscopy can be used to identify target sites, "higher-resolution immersion lenses presently cannot be used for imaging cryo-samples". Moreover, the amount of data generated from cryo-ET can also be a challenge for researchers; the techniques are "very data rich and it can be challenging to process and interpret such data." Looking forward, "there will be a great deal of effort spent on automating the data analysis and interpretation of tomography datasets."

Recent innovations

X-ray crystallography was considered the gold standard for structure determination of proteins at the atomic level for a long time. However, it was not always possible to purify and crystallize the proteins under investigation. Dr. Rigort remarks that, "single-particle analysis became increasingly advantageous in that it could be used to investigate proteins or protein complexes that could not be crystallized." Dr. Lengyel agrees that cryo-EM technological advances such as "sample loading, electron sources, automation in data collection and analysis, and, critically, improvements in detector technology" significantly improved the capabilities and applications of cryo-EM. Indeed "the use of the Direct Electron Detector (DED) was one the key enabling technologies that resulted in the cryo-EM "resolution revolution" coined in 2014. In the early days of cryo-EM, the technique was originally only capable of generating 3D low resolution structures (often referred to as "blobology"). However, in 2020, SPA was used to generate an atomic resolution reconstruction (1.22 Å)." At such high resolutions, cryo-EM can generate detailed structural information on proteins, such as hydrogen-bonding networks, solvent molecule interactions, confirmations of amino acids, and glycans.[7]

Although cryo-ET is a lower resolution technique compared to SPA, Dr. Lengyel believes that "if there are structures within a tomographic volume that are the same, they can be aligned to another one to improve the resolution. This is known as sub-tomogram averaging – which is conceptually similar to SPA image processing – and is used to determine structures of proteins." Cryo-tomography allows the determination of supramolecular structures that cannot be purified, the validation of in vitro structures generated by SPA within the cell, or the investigation of conformational changes of proteins. These examples illustrate the potential of cryo-ET and explain why this method has been steadily gaining importance, especially in recent years. The possibilities of cryo-ET could only be fully exploited by accompanying developments in the field of electron detection and sample preparation. "A game-changing technique in this context is the preparation of thin electron-transparent cryo-lamellae from vitrified cellular samples by cryo-focused ion beam scanning microscopes (cryo-FIB/SEM)" supports

Dr. Rigort. For thick samples such as eukaryotic cells or some bacteria, a microscope called a Dual-Beam is used to thin the sample, allowing for tomographic analysis. This is due to the sample being too thick for achieving transmission with the electron beam in the TEM. The Dual-Beam consists of a Scanning Electron Microscope (SEM) combined with a Focused Ion Beam (FIB). The SEM is used to image the surface of the sample and the FIB is used to oblate or mill the sample to TEM transparency, removing bulk material. This specialized Dual-Beam operates at cryogenic temperature.

Dr. Lengyel further outlines the improvements made to cryo-EM through research conducted at Thermo Fisher Scientific; "we are constantly innovating in this space, driving technological development through improvements in both software and hardware. This has allowed us to create an efficient workflow starting from vitrifying the sample, thinning via cryo-FIB/SEM, through automated data collection in the TEM." Ease of use and usability are also constantly being improved. For example, the Tundra electron microscope (dedicated for SPA) has been designed for -all levels of users — by simplifying and automating the workflow, cryo-EM may become more accessible and applicable to a broader research community.

Looking to the future

The future of structural biology will be driven by the integration of different approaches. For example, linking information from mass spectrometry (MS) with data from cryo-EM is expected to become increasingly important. A keyword here is "visual proteomics", where imaging data from tomography is linked to quantitative information about the proteins contained. Together with integrative modeling algorithms, this can be used to decipher the functioning of complex macromolecular machines, as has already been demonstrated for nuclear pore complexes, for example. Dr. Rigort expects that "cryo-tomography will benefit from artificial intelligence research and that this will drive and accelerate new scientific discoveries".

These advances will increase the widespread adoption of the method; as cryo-EM matures, both in terms of sample preparation, and image acquisition and analysis, it is becoming more accessible to a larger community of researchers – who previously had only limited exposure to electron microscopy or structural biology. The technique thus does not stay the sole domain of experts but becomes more widely available. Consequently, when used as a tool to study clinically relevant samples, cryo-EM "will make it possible to analyze differences in physiological or pathological conditions at the ultra-structural level and thus contribute to a better understanding of the underlying mechanisms of disease."

With regards to SPA, Dr. Lengyel predicts that "improvements in sample preparation, data collection, and imaging processing" will make it "easier, faster, and more automated". These improvements "will allow a broader range of biologists to utilize the technique and apply it to more challenging structural biology research questions". Among these challenging questions, "the ultimate goal of cryo-EM is to determine high-resolution structures of proteins and other macromolecules in the cell (in-situ) without purifying them." Acquiring high-resolution protein structures in-situ from cellular samples "will give insights into not only unexpected conformations or binding partners, but also spatial localization and interaction with other proteins. This will allow for completely novel scientific studies such as analysing disease states by visualizing differences in the 3D structures of proteins within their cellular context." With reference to the impact on drug design, he highlights that, "the ultimate dream of the pharmaceutical industry will be to visualize drug binding to its target protein in the cell. This could not only validate the efficacy of the drug but also give insight into any deleterious off target effects."

Studying complex cellular pathways during viral infection, where a virus can undergo large conformational changes, can also be an area of application for tomography in the future. Such intermediate structures may offer viable drug targets, but they can be unstable and change rapidly, making them difficult to purify and study. According to Dr. Lengyel "cryo-ET is already proving to be the only methodology to determine these structures. Many protein complexes also are too transient to be purified from source." With reference to the potential of cryo-EM he adds that "SPA will continue to be the fastest growing structural technique and the ultimate future of structural biology being cryo-ET. The future is very bright for cryo-EM in respect to structural biology."

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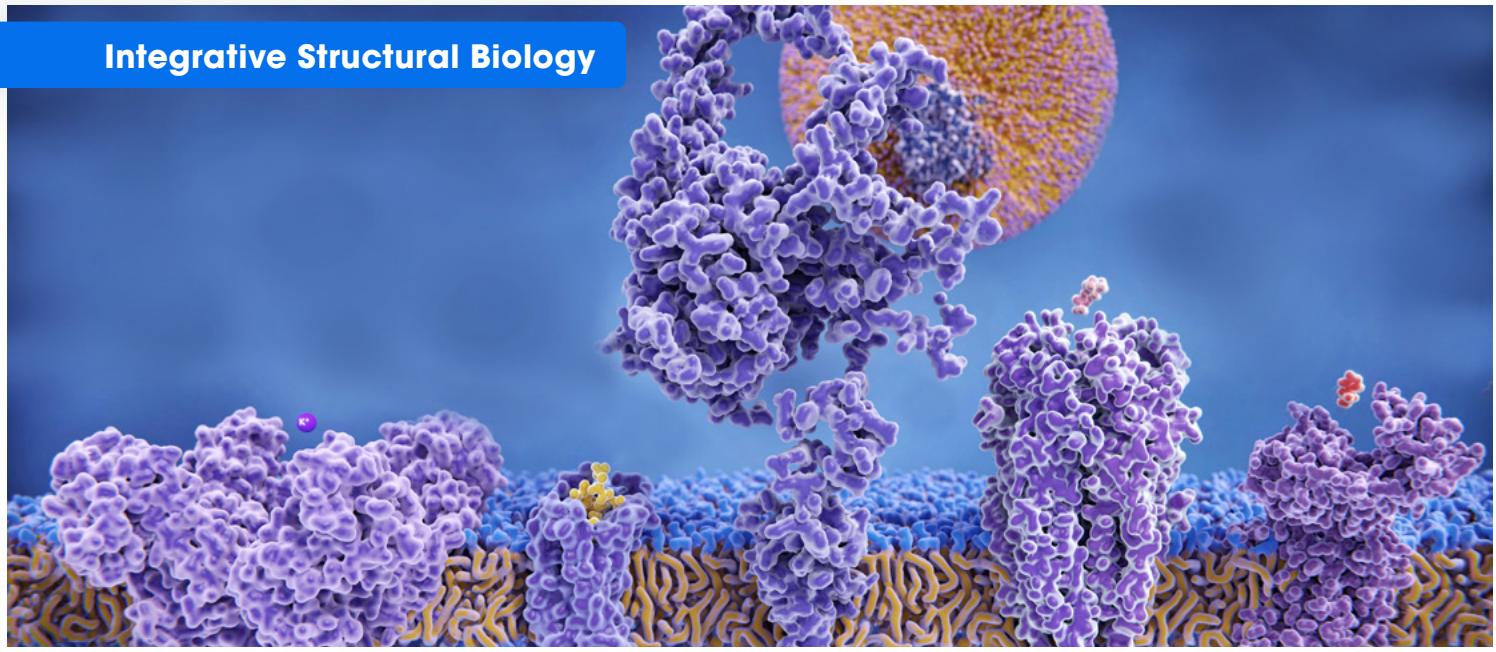
References

1. Lyumkis D. Challenges and opportunities in cryo-EM single-particle analysis. *J. Biol. Chem.* 2019;294(13):5181-5197. doi: 10.1074/jbc.REV118.005602
2. Benjin X, Ling L. Developments, applications, and prospects of cryo-electron microscopy. *Protein Sci.* 2020;29(4):872-882. doi: 10.1002/pro.3805
3. Nogales E. The development of cryo-EM into a mainstream structural biology technique. *Nat. Met.* 2016;13(1):24-27. doi: 10.1038/nmeth.3694
4. Subramaniam S. COVID-19 and cryo-EM. *IUCrJ.* 2020;7:575-576. doi: 10.1107/S2052252520008799
5. Oakley SS, Maina MB, Marshall KE, *et al*. Tau filament self-assembly and structure: Tau as a therapeutic target. *Front. Neurol.* 2020;11. doi: 10.3389/fneur.2020.590754
6. Hagen WJH, Wan W, Briggs JAG. Implementation of a cryo-electron tomography tilt-scheme optimized for high resolution subtomogram averaging. *J. Struct. Biol.* 2017;197(2): 191-198. doi: 10.1016/j.jsb.2016.06.007
7. Nakane T, Kotecha A, Sente A, *et al*. Single-particle cryo-EM at atomic resolution. *Nature*. 2020;587:152-156. doi: 10.1038/s41586-020-2829-0



Chapter 3: Mass Spectrometry and Its Role in Integrative Structural Biology

Traditional methods are limited in their ability to model dynamic molecular interactions, assemblies and communities of molecules at high spatial resolution. Despite the revolutionary impact of cryo-electron microscopy (cryo-EM), it remains difficult to solve many complexes using a single structural method – especially those with a limited sample amount, or compositional or structural heterogeneity. The recent application of various structural mass spectrometry (MS) techniques such as crosslinking MS (XL-MS) and hydrogen/deuterium exchange MS (HDX-MS), has enabled the structural characterization of protein complexes in an environment close to the native state and the study of their dynamics in different cellular states. Integrating this information is critical for developing and refining accurate structural models of proteins and protein systems. The value of the structural information obtained using this integrative approach demonstrates the importance of integrative modeling efforts. This article will explore how the development and incorporation of MS is helping to transform structural biology.[1]

Overcoming challenges with mass spectrometry

Dr. David Schriemer is professor of biochemistry and molecular biology at the University of Calgary. His laboratory predominantly focuses on the development and incorporation of new technologies in proteomics, MS and bioinformatics to enable the structural determination of proteins at cellular level. When speaking on the complementary nature of MS to traditional tools, Dr. Schriemer explains, "in many cases, particularly in cryo-EM, protein complexes can only be partially solved at high resolution due, for example, to domain motions. MS techniques can provide rich constraints to support integrative structural models that deliver greater resolution and accuracy. Adding a set of MS data to traditional techniques can provide the boost needed to generate a complete structural model." One of the techniques adopted by Dr. Schriemer is XL-MS, a technique that involves the chemical installation of hundreds to thousands of "molecular rulers" between two points in a protein system. He elaborates, "we use MS to identify which residues these rulers connect and then build structure from the distance constraints that result. If we get enough crosslinks, we can model protein complexes with a 10 Angstrom (Å) precision, which is pretty good." HDX-MS is another technique used in Dr. Schriemer's lab which is particularly useful for measuring conformational dynamics. HDX-MS can be used to determine how a binding event alters structure and function, and where the most flexible regions of the proteins are. Dr. Schriemer notes that " together with XL-MS, the technique can help us generate more accurate structural models"

Dr. Patrick Griffin is professor of molecular medicine and scientific director of Scripps Research, Scripps Florida. His lab analyzes nuclear receptor structure-function relationships implicated in a wide range of diseases. When trying to map nuclear receptor post-translational

modifications and conformational changes upon ligand activation and/or protein-protein interactions, translocation, etc., he is often limited by the sensitivity of the technique. He adds, "if you're going to do a comprehensive post-translational map of your nuclear receptor of interest from an endogenous system, you have to get enough of the protein out. And you must have high sequence coverage to get a sense of how modified the protein is and how those modifications are changing over time. Additionally, these proteins are often associated with insoluble chromatin fractions, so although you can recover them, there's often significant loss of protein when one does subcellular fractionation."

Being confident that the effects observed are related to direct ligand interaction rather than secondary effects of off-targets is another challenge for the lab. He elaborates, "when studying the influences of synthetic ligands on the receptor, you're also competing against the endogenous ligand. This complexity can prevent you from understanding the selectivity of the synthetic molecules, as most of the endogenous ligands for nuclear receptors hit multiple family members. We often test for selectivity against the most similar receptors, but we don't necessarily know the more broader selectivity profile; those experiments are relatively expensive to have done at contract research organizations and you often only get a binding assay. To see if it's functionally relevant, you then have to do functional assays." The size of the nuclear receptor influences the likelihood of obtaining high-resolution cryo-EM data – which presents another challenge for his research.

Working with larger nuclear receptors (e.g. the steroid receptor family) means that Dr. Griffin's lab can obtain data for rigid regions at reasonable resolution, yet the majority of the protein remains intrinsically disordered. Using XL-MS and HDX-MS, his lab can gain insights into the more flexible regions. The data from those experiments can be used to refine computational models that include density from cryo-EM and or crystallography studies. For example, distinct constraints from crosslinking can be used to further refine the model, providing information on the overall topology of the protein in solution, as well as the reactivity of the amino acids, thereby allowing researchers to build out the structure of the protein. With reference to HDX-MS, Dr. Griffin explains that it provides "a snapshot of the energy landscape of the protein". Using a differential format, allows one to examine "the influences of a ligand on the conformation dynamics, the site of interaction with another protein that it's binding to and the influence of that binding partner on the conformational dynamics of the receptor." Dr. Griffin, adds, "having this additional data provides more information on changes in conformational dynamics so that we can model what the protein complex looks like in solution, more accurately."

Future directions

The emergence of AlphaFold and RoseTTA Fold has revolutionized the field of structural biology; for the first time, protein structures can be predicted with accuracy from primary sequence. While there are still some limitations, the continued improvement of these computational techniques will allow researchers to determine the structure of most individual proteins. Both Dr. Griffin and Dr. Schriemer agree that this, together with the increasing interest in protein complexes, conveys an enormous benefit to structural biology.

Researchers "need to look at the entire cell in structural terms," says Dr. Schriemer, "and this is where MS has a special role to play". Since MS techniques excel at telescoping high resolution structure into larger assemblies (through XL-MS and AI-driven integrative modeling) and at determining structure-function properties (through HDX-MS and ion mobility), the underlying predicted structures can be rapidly assessed for error checking. More importantly, structural investigations of complex systems can be performed without the laborious step of developing the structures of the smaller building blocks. As a result, the structure-function analysis of large systems should proceed much more quickly, allowing scientists to better understand the underlying mechanisms.

With reference to XL-MS, Dr. Schriemer states that "if we can extend techniques like XL-MS to whole cell analysis, we can avoid some of the challenges associated with preparing protein complexes "outside" the cell. Because MS can analyze highly complex samples, we should be able to use most of our structural MS techniques at the cellular level, eventually."[2] Dr. Schriemer adds that, "HDX-MS is really the technique of the future here. New developments will support the conformational analysis of these more complex systems across wider time scales. Proteomics-grade configurations are possible and will make the technique much more sensitive than it currently is. New software solutions that we are building will provide a completely automated data analysis pipeline and remove the current pain of manual data analysis." The future also promises to hold more advanced computational methods. The rise of MS-based techniques and their contribution to integrative structural biology would not have been possible without investment in the development of new algorithms and suites of applications such as the Mass Spec Studio. Future packages will further simplify the analysis to strengthen the synergy between MS and structural biology.

Conclusion

The limitation of almost all structural techniques is the ability to resolve protein dynamics. Although cryo-EM has significantly advanced the analysis of large protein

complexes, it provides an imperfect view of system dynamics. Recent advances in the instrumentation, software and methodology of various MS-based structural methods are enabling scientists to obtain structural information not only from purified proteins but also from direct analysis of proteins in cells, tissues, and organisms.[3] Looking forward, Dr. Schriemer foresees that "perhaps the most exciting use of MS involves bringing high resolution 3D structure to the cell. Techniques like cryo-ET and *in-situ* XL-MS can be combined to generate enough data to model these proteins into their resident complexes without the need of reconstituting protein complexes outside the cell. When this happens, molecular biology will be truly transformed."

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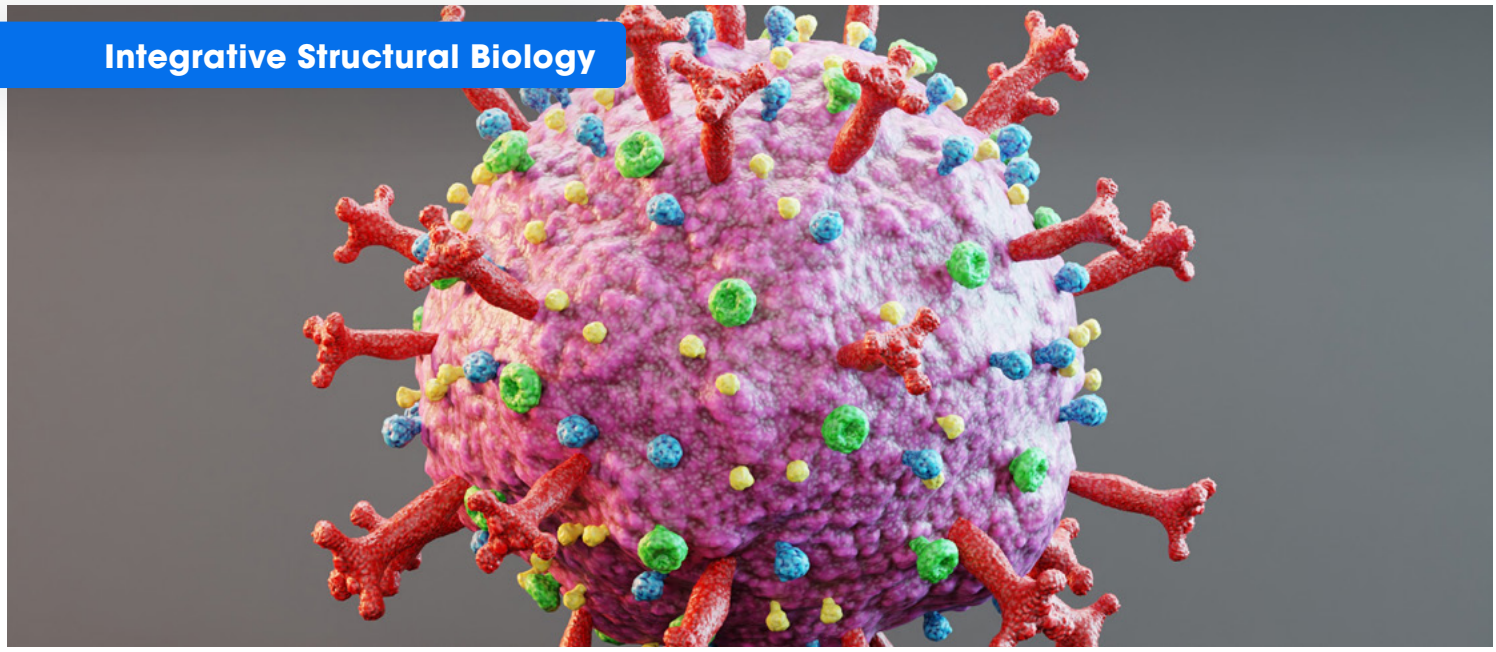
References

1. Faini M, Stengel F, Aebersold R. The evolving contribution of mass spectrometry to integrative structural biology. *J. Am. Soc. Mass Spectrom.* 2016;27(6):966-974. doi:10.1007/s13361-016-1382-4
2. Mahamid J, Pfeffer S, Schaffer M. *et al.* Visualizing the molecular sociology at the HeLa cell nuclear periphery. *Science.* 2016;351(6276):969-972. doi:10.1126/science.aad8857
3. Kaur U, Johnson D, Chea E, Deredge D, Espino J, Jones L. Evolution of structural biology through the lens of mass spectrometry. *Anal chem.* 2018;91(1):142-155. doi:10.1021/acs.analchem.8b05014



Chapter 4: Improving Structural Resolution with Mass Spectrometry

As scientists continue to investigate the structure and function of proteins in their native environment, the need for complementary methods is greater than ever. So far, this eBook has demonstrated that although mass spectrometry (MS)-based techniques and cryo-electron microscopy (cryo-EM) have their individual limitations, their integration allows researchers to probe structure of large molecular assemblies in unprecedented detail.

Dr. Vicki Wysocki is a professor and Ohio eminent scholar at the Ohio State University. Her research group focuses on the development and application of various multi-omics and native mass spectrometry (nMS)-based approaches for the optimization of biomarker discovery, disease diagnosis and prognosis.

Dr. Alexander Leitner is a senior scientist and researcher at the Institute of Molecular Systems Biology, ETH Zürich. His research group develops different crosslinking methods and uses them in combination with mass spectrometric analysis to gain deeper insights into the structural organization of protein complexes at increased resolution.

In this interview, both researchers discuss how they are combining MS-based techniques with cryo-EM and the impact this is having on their work. They also share their insights into the developments they foresee driving the field further in the future.

Q: What impact has mass spectrometry had in structural biology and how can it be used to complement techniques such as cryo-EM?

Alexander (A): An obvious first step for the use of mass spectrometry in structural biology is the characterization of fundamental sample properties. This would include checking for contaminant proteins and confirming the presence of all expected components of the sample under study using conventional bottom-up strategies. In a similar manner, post-translational or artificial modifications (e.g., oxidation) can be identified. Such analysis can ideally be complemented by the top-down characterization of proteoforms, which can confirm processing of protein termini or the coexistence of modifications on the same molecule, although such applications are not as widespread yet.

The main MS methods that focus on structural characterization include native (or non-denaturing) MS (nMS), where the entire protein complex is directly analysed in the mass spectrometer, and bottom-up methods that encode structural information prior to an enzymatic digestion step: hydrogen/deuterium exchange, surface footprinting/covalent labelling methods, and chemical crosslinking (XL-MS). The unique advantage of nMS is that it can inform about the stoichiometry of a protein complex, which is not directly accessible from bottom-up experiments. Hydrogen/deuterium exchange and covalent labelling methods provide information on surface accessibility of residues, their involvement in hydrogen bonding networks, and changes in accessibility if two or more states of a sample are combined. Finally, XL-MS can provide multiple layers of connectivity information, and it is the structural MS method that is most commonly combined with established structural biology techniques.

While applications of XL-MS have initially remained proof-of-concept studies, in the last 10-15 years the method has shown that it can provide highly valuable complementary data. In connection with cryo-EM, its relevance has changed after the introduction of modern direct electron detectors that facilitated the generation of high-resolution maps without relying on auxiliary information. Currently, the main applications of XL-MS in combination with cryo-EM include: confirmation of cryo-EM data by mapping crosslinks on proposed structures; fitting/positioning of subunits with the help of XL-derived restraints; modelling of missing regions invisible to EM due to their flexibility, and, more generally, combination of cryo-EM and XL-MS data with other experimentally or computationally derived information in integrative/hybrid modelling projects.

Vicki (V): Structural biology rarely involves a single technique. Researchers use a variety of approaches as they investigate the structure and function of an unknown protein complex. These approaches vary in their ease of use and in the types of data provided, with readily available low-resolution techniques (circular dichroism, size exclusion chromatography, light scattering, gels) providing less structural information than information-rich tools that are often more difficult to use or larger and more expensive so not available in each lab.

Often, nMS data is easier and quicker to acquire than tools that give high resolution structure and it can be applied in daily work, on intermediates before the ultimate complex has been produced and in parallel to biochemistry studies. nMS, coupled with MS/MS and ion mobility, can provide connectivity information, topology/architecture information, mass of the intact complex and binding partners, heterogeneity information, conformational information, and relative binding affinities. This information complements cryo-EM, especially if cryo-EM is only possible at lower resolution, or in cases where parts of the structure are missing (e.g., because of preferred orientation in the ice). The use of nMS continues to grow as more and more investigators become familiar with its utility. As is common with new technology, fully integrated nMS workflows are not yet commercially available. Research in nMS is a very active, including sample preparation, online separations, spray improvements, mass selection improvements, ion mobility improvements, sensitivity improvements, the ability to do "single molecule"/charge detection MS, and automated computational spectral interpretation and structure refinement. However, there are not yet full solutions available for purchase and easy use by non-experts. Several vendors have made progress though and continue to work with the nMS research community to improve on existing technology. The National Institute of Health (NIH) has also funded individual investigators and centers, e.g., Research Resource for Native Mass Spectrometry Guided Structural Biology.

Q: How have you used cryo-EM and MS within your research? Can you outline some of the common applications of these techniques within the field?

A: My group focuses on crosslinking methods, although in some collaborations we have combined XL-MS with hydrogen/deuterium exchange data generated by other groups. One of the first structural biology projects that I have been involved in showed the power of XL-MS: We used the method to decipher the subunit arrangement of the chaperonin TRiC/CCT[1] and could convincingly demonstrate that the technique was capable of solving questions that were challenging even for established structural biology techniques, at least at that time. Some years later, I was fortunate to contribute to studies on the mammalian mitochondrial ribosome.[2-4] During this project, I witnessed first-hand the "resolution revolution" that changed the way how cryo-EM was perceived by the structural biology community. Both projects initially focused on deciphering the "architecture", i.e. the subunit arrangement of these large, multi-subunit protein complexes. Later, the focus has shifted towards applications that cover more extended biological functions of these complexes. With respect to TRiC/CCT, we have used XL-MS to study interactions with co-chaperones[5] and managed to trap folding intermediates of substrates of this chaperone;[6] in the ribosome field, we have contributed to studies that elucidate the biogenesis of this essential cellular machine.[7]

Apart from these two complexes, noteworthy examples are one of the first applications of XL-MS to membrane complexes (the ryanodine receptor),[8] and epitope mapping and receptor interaction profiling on clinically relevant targets such as complexes from human cytomegalovirus.[9,10] Our most recent collaboration focuses on an E3 ubiquitin ligase complex.[11] I would also like to mention that my group has developed a number of new crosslinking chemistries, protocols and data analysis tools that facilitate biological applications of XL-MS.

V: We have used nMS and cryo-EM as complementary tools in several studies. In one case, complementary MS tools and computations were used to predict structure of the complex toyocamycin nitrile hydratase. We used a combination of techniques, surface-induced dissociation coupled to ion mobility (and, to a more limited extent, solution disruption) to provide connectivity information, covalent labelling to confirm the interfaces, homology modelling and docking, and chemical crosslinking, to provide lowest energy structures for this complex of unknown structure. Later, we used cryo-EM but only low-resolution structures were acquired due to the size of the complex (<100,000 Da) and its motion in the ice. In another case, a manganese oxidizing multicopper oxidase called Mnx, nMS was used to predict connectivity and topology, which matched nicely with the low resolution

cryo-EM structures that became available later. In a third case involving a plant pseudoenzyme-enzyme complex, PDX, nMS clarified enzyme and pseudoenzyme locations in hexameric rings of the dodecamer, information that wasn't available with the 3.2 A cryo-EM structure. This was an interesting case where cell free expression was used to make mixed complexes of enzyme and pseudoenzyme. We could then dissociate these structures by surface-induced dissociation. Lastly, in another case (that is still unpublished), the complex oriented in the ice in such a manner that the full-length protein: DNA structure was not available, however the total composition could still be measured by nMS. Finally, we are currently working on multiple cases, where we are identifying components that are missing in published structures, the presence of contaminants that are complicating cryo-EM attempts, or intermediates in the assembly pathway.

Q: With reference to the methods you are using in your research, why is MS so often used in complementary fashion with cryo-EM?

A: A key advantage of XL-MS is its versatility; being a mass spectrometry-based method, it is not affected by some limitations of conventional structural biology techniques. For example, the sample requirements are typically modest and the method can deal with impurities and sample heterogeneity (although the interpretability of results may be affected for heterogeneous samples).

XL-MS is also increasingly accessible to non-experts. This concerns several steps of the experimental pipeline: the availability of reagents with extended properties (such as gas-phase cleavable reagents), templates for data acquisition methods and integrated data analysis tools. The performance of modern mass spectrometers also helps to increase the depth of coverage, allowing the identification of more crosslinks.

As far as the usability of XL-MS data is concerned, the method is attractive because it yields different layers of contact information.[12] At the lowest level of resolution, if two proteins are found to be connected by a crosslink, this confirms that the two proteins are close in space. By localizing the two residues connected by the crosslink, we can refine that information to the spatial proximity of specific amino acids of the proteins. Finally, the geometry or the chemical structure of the crosslinking reagent helps to define a specific spatial restraint (an upper bound of distance between the crosslinked residues, typically somewhere between 10 and 35 Å). Many computational modelling tools can directly use this measure. Moreover, in contrast to other methods such as surface labelling, hydrogen/deuterium exchange or limited proteolysis, the information is always "binary" in the sense that both interaction partners are identified, and not only changes in accessibility on one partner are observed.

V: Cryo-EM is at an interesting stage of development. The information content is amazing, but the time required from start to completion of a structure can be daunting. Because of the difficulty and expense of the technique, researchers often try to measure structures only at the end of a workflow. In some cases, cryo-EM works well and quickly, but in others, a lot of trials are required to find appropriate conditions for success and success is not guaranteed. Sample preparation is a bottleneck here and instrument access is often limited. While experiments such as titrations can be followed by cryo-EM those types of experiments are not yet routine and often involve experts in the field.

nMS is popular because it is relatively straightforward and can be applied to complexes with a wide range of compositions and sizes. Small amounts of sample are suitable and measurements are relatively fast, allowing experiments such as titrations to be completed in a relatively short amount of time. nMS provides information on whether the sample has a heterogeneous or homogeneous oligomeric state and whether there are contaminants present that are misleading the cryo-EM structure determination. nMS coupled with dissociation and ion mobility can provide mass and sometimes connectivity information on all components in the complex, even for components that are not interpretable in the cryo-EM density maps. Researchers can also obtain information for complexes that may be too small or too heterogeneous for cryo-EM. nMS offers the advantage of coupling directly to separation approaches such as affinity chromatography, online desalting, size exclusion chromatography, ion exchange chromatography, and capillary electrophoresis. With online separations, components of a mixture of species can enter the mass spectrometer separately, e.g., when a monomer or other small x-mer is in excess and would otherwise swamp out the high order oligomer of interest. nMS also works for a wide variety of molecules; proteins and their complexes with small ligands, lipids, RNA, and DNA can all be investigated. This approach is not limited to a single stage in the experimental process and can be used throughout a biological/biomedical study as a researcher begins to define the assembly pathway of a complex. It can also be used to determine whether protein overexpression has produced the protein of interest or whether RNA transcription has produced a clean, single-length RNA or RNA with various lengths. We should mention, though, that nMS is not foolproof either. Sometimes a tricky complex comes along that is not suitable for current nMS approaches (e.g., only stable with glycerol and high Na+ content or isolated or expressed at concentrations too low for manipulation for nMS).

Q: Could you provide an overview of how these MS techniques are being used more broadly in structural biology studies?

A: I mentioned some of the typical applications already above. In combination with high-resolution structural biology techniques, XL-MS can be used at an early stage - before applying crystallography or electron microscopy - to generate hypotheses. For example, which subunits are in contact with each other in a protein complex, or which domains are involved in an interaction? This information can be used to guide experiments such as the generation of truncated forms of proteins that will crystallize better or reduce the flexibility or conformational heterogeneity in cryo-EM, therefore yielding higher resolution. At a later stage of a structural project, once high-resolution information is available, XL-MS can help to confirm the validity of such models and structures by showing that crosslinks generated in solution reflect what is seen in the crystal or on the grid. Moreover, XL-MS data can be used to "fill in the gaps"; regions that are not resolved due to high flexibility or dynamics can be modelled with the help of distance restraints. In a similar way, additional subunits can be "added" to available high-resolution structures by restraint-based docking. In absence of high-resolution data, the network of crosslinks can still be used to predict the organization of the complex in a more coarse-grained way.

XL-MS has also been successfully applied in conjunction with many other techniques such as nuclear magnetic resonance (NMR) spectroscopy or small-angle X-ray scattering, for example. In more complex systems, it is increasingly used to generate interaction networks or supplement such networks with partial structural information.

V: There are a wide variety of ways that nMS is used in structural biology studies, with a few examples listed below.

1. nMS can be used to simply measure the mass-to-charge ratio (m/z) of a complex to determine its stoichiometry, which is often all that an investigator needs. This can be a single measurement or a repeated measurement for the complex, (for example, during titration) or when different ligands or cofactors are screened for potential binding to the protein complex.

2. nMS can also be used as part of a tandem mass spectrometry system. This approach is typically used if the investigator needs to fragment the complex to learn how the subunits are connected in the complex or to see how many and which proteins, nucleic acids, ligands, metal ions, etc. are present in the complex.

3. When nMS is coupled to ion mobility, investigators can separate and select by m/z and, by shape and charge. Having shape and charge information and drift time measurements that can be converted to rotationally averaged collision cross section measurements can be very useful in deciding between two computational models, for example.

4. nMS can simply be used as a screening tool for designed protein complexes. nMS can quickly determine whether the designed complex matches the design concept or not, allowing rapid screening of multiple designs.

5. nMS can be used to refine structures acquired by other structural biology tools or to guide ongoing research to try to produce a structure that will then be interrogated by an atomic resolution tool, such as cryo-EM. nMS can confirm, for example, which conditions do or do not lead to a homogeneous sample with a dominant oligomeric state of a complex.

6. nMS can be used to determine whether bound lipids are present mainly for stabilization of the complex in a membrane or whether the lipids have a more specific functional role.

Q: Are there any limitations to these techniques?

A: Of course, no experimental method is without limitations and XL-MS in no exception. At a very fundamental level, the method is dependent on the availability of crosslinkable residues in the right location, for example at the binding interface between two proteins. This is particularly challenging for the study of membrane proteins that lack typical targets for crosslinking reactions, especially in transmembrane regions.

Another drawback is that it is usually not possible to direct the crosslinking region to a certain region of your complex or towards a specific protein, at least not with standard methods. Think about the example of ribosome biogenesis mentioned above: It is relatively straightforward to obtain many crosslinks on the ribosome itself, but this is not the information that we are looking for, as the structure is already known. We want to identify crosslinks that connect a specific factor/protein to the ribosome, and at best, only a tiny fraction of the generated crosslinks will give you that information.

XL-MS will never be a technique that will give you high-resolution information on its own, the distances bridged by a crosslink are in the range of tens of Å, therefore, the smaller the target, the less valuable the information. Data from samples with multiple copies of the same protein present are often challenging to interpret on their own because of the intrinsic ambiguity. Some of these limitations can be overcome

by applying specific crosslinking strategies, for example the use of complementary crosslinking chemistries that target different functional groups, or the use of different proteases for digestion. This requires larger sample amounts and more experience to select the most practical approach.

V: Although nMS is a quick and useful technique, there are times when nMS conditions are not suitable for a particular complex. Occasionally, a complex requires a large amount of manipulation to find solution conditions compatible with measurement of the complex (e.g., screening multiple buffer conditions, additions of non-volatiles, multiple pHs, etc.). This is somewhat similar to the need of X-ray crystallography or cryo-EM to screen a variety of conditions before the complex is prepared in suitable conditions for measurement. Other times, nMS works well with simple sample preparation and only a few minutes of measurement. Sometimes, limitations of the technique are really limitations of the mass spectrometers available to the user. If a user does not have a mass spectrometer with an extended m/z range (e.g., by lowering the frequency of the mass selection quadrupole), then the user will have to study protein complexes with a lower m/z range. Alternatively, the user can supercharge the complex to force the ions to show up in the m/z range of the instrument (with care taken to ensure that supercharging does not restructure the complex). Once ions are in the mass spectrometer, the user must tune the instrument appropriately for their application. This can either involve applying harsher conditions to de-adduct the complex, if the goal is to obtain an accurate mass measurement for an accurate stoichiometry prediction or tuning the instrument "softer" to avoid restructuring the ions in order to fragment the native structure. Fragmentation options vary depending on the instrument and lab, so the information gained will depend on what is available. CID/HCD fragmentations tends to give restructured, highly-charged monomer and (n-1) mer, which is great for stoichiometry determination and proteoform identification. SID tends to give substructure products indicative of original connectivity. ExD and UVPD can provide covalent fragmentation that indicates ligand location.

More complex systems, such as nucleoprotein complexes that require metals such as Mg^{2+} will give poorer looking spectra if the complexes are kept more native but these spectra still provide useful structural information. Membrane proteins require spraying from a membrane mimic (detergent micelles, bicelles, liposomes, nanodiscs, SMALPs) and this requires expertise and, sometimes, detergent screening to find the optimum conditions. A given instrument may have limitations in m/z accuracy or in ion mobility resolving power. For the most flexibility, investigators would ideally have access to an instrument that incorporates flexibility in sample introduction and associated chromatography (or related) techniques, flexibility in ion source conditions, a broad m/z selection and measurement range, and highly accurate and precise m/z and ion mobility measurements.

Q: What developments do you see in the future that will drive the use of the technique for the broader integrative structural biology community?

A: XL-MS is rooted in the mass spectrometry community, and therefore follows many of its conventions, for example, related to terminology and how results are reported in publications etc. To be accessible to the broader community that applies integrative structural biology techniques, researchers that generate XL-MS data need to speak the same language like researchers that make use of the data. One way to address this is by enabling structural biologists to generate such data themselves. Another way is to facilitate the uptake of XL-MS data by transparent data analysis and quality control, by uniform reporting standards and by enabling the seamless integration of the data flow from mass spectrometry/proteomics repositories to structural biology resources and tools. Some community initiatives to which I have contributed have laid the foundation for such efforts,[13-15] and in the past few years it has become increasingly common to deposit XL-MS in repositories of the ProteomeXchange consortium. However, such initiatives require sustained funding to generate data standards and support interoperability.

On the technical and methodological side, the recent advances in deep learning-based structure prediction by AlphaFold[16] and RoseTTAFold[17] will without doubt have a huge impact on integrative structural biology. The availability of high-quality models of individual subunits will be very helpful for the structure elucidation of large assemblies. However, flexible regions in such assemblies and dynamic rearrangements that determine their function in vivo will still be more difficult to predict, at least for now. Again, XL-MS can support such endeavours by providing essential information in such cases, as also mentioned in a recent commentary by Cramer.[18] Another direction for which I foresee a bright future for XL-MS will be applications to the modelling of larger assemblies such as fibrillary structures or entire organelles. The combination with cryo-ET looks highly promising to me.

V: There is a need for fully integrated nMS instruments that simplify the sample preparation, sample purification/separation, spraying of the complex, and optimum measurement and dissociation conditions (e.g., surface collisions in addition to collisions with gas). Tools for interpretation of the data and computations to integrate multiple data types to provide the highest resolution structure possible are also required. Charge detection

MS is providing more information on heterogeneity than has been possible in the past. This technique allows measurement of individual ions so that the multiple proteoforms of a single protein in a complex can be identified. Mass photometry is a new tool that builds from interference reflection microscopy and interferometric scattering microscopy. It correlates the light scattered by single molecules with molecular mass and can be used for comparisons with nMS using typical buffers that are not as compatible with native MS.

Integration of nMS data with data from other structural biology tools (circular dichroism, NMR, X-ray crystallography, cryo-EM, size exclusion chromatograpy (SEC) or SEC-MALS (multi-angle light scattering), small angle x-ray scattering) is appealing but requires development, including software development. Multiple low-resolution tools, for example, can provide higher resolution structures than any of the individual tools but computational approaches are needed that integrate the data from the multiple tools into the best structure. The multiple tools can be a wide array of non-MS tools or multiple MS approaches (e.g., chemical crosslinking, covalent labelling). The community also needs to gain more familiarity with nMS and realize how it can complement other approaches. Each structural biology tool has its own niche, and nMS fits into broader structural biology studies in a wide variety of ways. The community should begin to accept that a wide variety of data types should be reported to better characterize protein complexes. While atomic resolution tools are extremely powerful, they sometimes provide a snapshot of one particular structure, captured as a crystal or a particular orientation in vitreous ice but the dynamic behavior of the complex is not captured unless time-resolved experiments are performed or solution measurements are made. In exciting recent developments, research groups are also trying to use nMS as a preparative tool to produce purified complexes that can then be measured with high resolution tools. The bottom line is that there is not a single "one size fits all" approach to structural biology. Many existing tools and developing approaches are needed to fully characterize a system.

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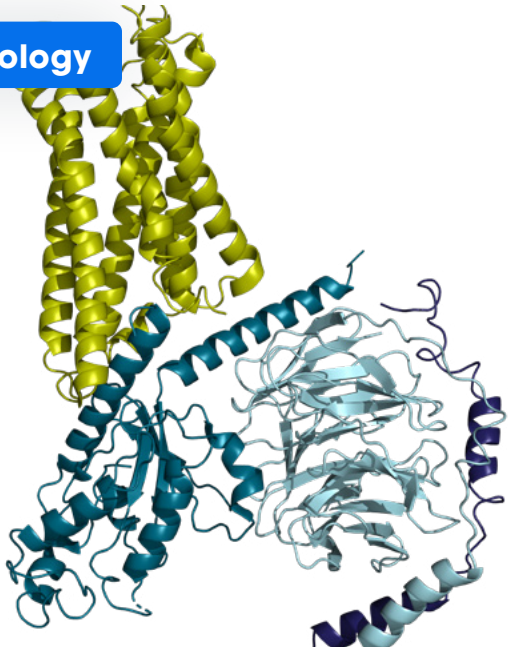
References:

1. Leitner A, Joachimiak L, Bracher A, *et al*. The molecular architecture of the eukaryotic chaperonin TRiC/CCT. *Struct*. 2012;20(5):814-825. doi:10.1016/j.str.2012.03.007
2. Greber B, Boehringer D, Leitner A, *et al*. Architecture of the large subunit of the mammalian mitochondrial ribosome. *Nature*. 2013;505(7484):515-519. doi:10.1038/nature12890
3. Greber B, Boehringer D, Leibundgut M, *et al*. The complete structure of the large subunit of the mammalian mitochondrial ribosome. *Nature*. 2014;515(7526):283-286. doi:10.1038/nature13895
4. Greber B, Bieri P, Leibundgut M, *et al*. The complete structure of the 55 S mammalian mitochondrial ribosome. *Science*. 2015;348(6232):303-308. doi:10.1126/science.aaa3872
5. Gestaut D, Roh S, Ma B, *et al*. The chaperonin TRiC/CCT associates with prefoldin through a conserved electrostatic interface essential for cellular proteostasis. *Cell*. 2019;177(3):751-765.e15. doi:10.1016/j.cell.2019.03.012
6. Knowlton J, Gestaut D, Ma B, *et al*. Structural and functional dissection of reovirus capsid folding and assembly by the prefoldin-TRiC/CCT chaperone network. *Proc. Nat. Acad. Sci*. 2021;118(11):e2018127118. doi:10.1073/pnas.2018127118
7. Greber B, Gerhardy S, Leitner A, *et al*. Insertion of the biogenesis factor Rei1 probes the ribosomal tunnel during 60S maturation. *Cell*. 2016;164(1-2):91-102. doi:10.1016/j.cell.2015.11.027
8. Efremov R, Leitner A, Aebersold R, Raunser S. Architecture and conformational switch mechanism of the ryanodine receptor. *Nature*. 2014;517(7532):39-43. doi:10.1038/nature13916
9. Ciferri C, Chandramouli S, Leitner A, *et al*. Antigenic characterization of the HCMV gH/gL/gO and pentamer cell entry complexes reveals binding sites for potently neutralizing human antibodies. *PLoS Pathog*. 2015;11(10):e1005230. doi:10.1371/journal.ppat.1005230
10. Martinez-Martin N, Marcandalli J, Huang C, *et al*. An unbiased screen for human cytomegalovirus identifies neuropilin-2 as a central viral receptor. *Cell*. 2018;174(5):1158-1171.e19. doi:10.1016/j.cell.2018.06.028

11. 11. Mohamed W, Park S, Rabl J, Leitner A, Boehringer D, Peter M. The human GID complex engages two independent modules for substrate recruitment. *EMBO Rep*. 2021, in press. doi: 10.15252/embr.202152981
12. 12. Leitner A. Crosslinking and other structural proteomics techniques: how chemistry is enabling mass spectrometry applications in structural biology. *Chem. Sci.* 2016;7(8):4792-4803. doi:10.1039/c5sc04196a
13. Iacobucci C, Piotrowski C, Aebersold R, *et al*. First community-wide, comparative crosslinking mass spectrometry study. *Anal. Chem.* 2019;91(11):6953-6961. doi:10.1021/acs.analchem.9b00658
14. Berman H, Adams P, Bonvin A, *et al*. Federating structural models and data: outcomes from a workshop on archiving integrative structures. *Struct*. 2019;27(12):1745-1759. doi:10.1016/j.str.2019.11.002
15. Leitner A, Bonvin A, Borchers C, *et al*. Toward increased reliability, transparency, and accessibility in crosslinking mass spectrometry. *Struct*. 2020;28(11):1259-1268. doi:10.1016/j.str.2020.09.011
16. Jumper J, Evans R, Pritzel A, *et al*. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583-589. doi:10.1038/s41586-021-03819-2
17. Baek M, DiMaio F, Anishchenko I, *et al*. Accurate prediction of protein structures and interactions using a three-track neural network. *Science*. 2021;373(6557):871-876. doi:10.1126/science.abj8754
18. Cramer P. AlphaFold2 and the future of structural biology. *Nat. Struct. Mol. Biol.* 2021;28(9):704-705. doi:10.1038/s41594-021-00650-1



Chapter 5: Combining Cryo-ET and Mass Spectrometry

Although resolving isolated protein structures to near atomic resolution provides a wealth of information on protein function, nothing beats visualizing a protein in its native environment.[1] As discussed in chapter 2, cryo-ET allows researchers to study protein complexes directly within the confinement of the cell. However, as exciting as this is, it also poses challenges as researchers need to know what they are looking for in order to interpret their results. When no prior knowledge is present — for example, when studying perturbations of protein complexes in diseased states — an integrated approach relying heavily on MS can help to identify the unknown elements of the system. By identifying proteins and their dynamic modifications within a large protein complex or an entire cell, MS can help to target analyses and interpret the data obtained using cryo-ET. The outcome of this synergy has broad implications in numerous fields including cell biology,[2] virology[3,4] and drug design.[5]

The role of cryogenic-electron tomography

Gaining insights into protein complexes and molecular machines

The importance of studying proteins directly in their native environment is demonstrated by the significant effect that the number of proteins available in the cytoplasm and macromolecular crowding have on protein conformations and protein-protein interactions.[6] Since many of these interactions are transient, or too weak to maintain in isolation, they are typically lost during the purification process. The ability to accurately resolve structures within their cellular context has therefore been a game changer for structural and cellular biology. For example, the large and intricate nature of the nuclear pore complex (NPC) has made it difficult to determine its structure in the past. However, using cryo-ET researchers can now reconstruct the NPC and resolve a 3D map that correlates its density with the structural signatures of isolated subcomplexes.[7]

Dr. Friedrich Förster is a professor of cryo-EM and head of the In Situ Structural Biology Lab, at the University of Utrecht, the Netherlands. His lab focuses on the development of computational methods and experimental studies (ranging from isolated protein complexes to whole cells) to address structural biology questions from unusual angles at the interface of molecular and cellular structural biology.[8] "In my group, we aim at a structural understanding of processes in the cell, in particular those associated with cellular membranes. Cryo-ET is a great method to gain insights into protein biogenesis at organelle membranes. In combination with other methods, we aim to address questions such as: How are they inserted into membranes? How are they chemically processed? How do they fold and assemble? What goes wrong with these processes in disease?", commented Dr. Förster.

The challenges faced by scientists using cryo-ET alone

A fundamental limitation of cryo-ET is the applicable electron dose. Dr. Förster explains, "while electrons image the sample, they also cause beam damage. Therefore, cryo-ET data are acquired with minimal electron doses that result in extremely weak signals in the raw data. The signal-to-noise ratio ultimately limits the resolution of the 3D reconstructions. Thus, in cryo-ET, we deal with very noisy data for particles whose molecular identity is

unknown – information that is required for molecular interpretation."

It is also difficult to localize and identify complexes with low molecular weight as they produce a weaker signal. Since cryo-ET does not directly readout biochemical information about the sample, several computational methods ranging from template matching to machine learning are required to interpret low-resolution cryo-ET data and parse its individual proteins.[9]

Another fundamental limitation of cryo-ET is that it captures the structure of proteins but not their identity. A high-resolution image obtained by cryo-ET is still a blank reconstruction of the cell; therefore, the proteins constituting every "blob" in the obtained structures must be identified. While a few cellular structures and their proteins are obvious and can be easily identified, such as ribosomes and actin filaments, identification of most blobs is more challenging. For example, cryo-ET reconstructions faithfully depicted the eight-fold symmetry of the pores within the NPC that are essential for passing mRNA from the nucleus to the cytoplasm. While this offered many nuanced details about the NPC structure, it was only after fitting data to existing protein structures that each part of the NPC could be identified. A number of complementary technologies, such as MS, must be used to increase the information obtained from cryo-ET alone.

The benefits of combining MS and cryo-ET

The majority of Dr. Förster's work involves the development and use of "methods that can localize and identify macromolecules in the cell with the highest specificity and sensitivity." Leveraging MS enables researchers to identify a range of proteins within a sample and therefore narrow down the search space, increasing the sensitivity and specificity of the computational methods used to analyze cryo-ET data.[10] He elaborates, "MS provides information on the biochemical content of the sample up front. In other words, peak intensity-based quantification tells us the list of ingredients of the soup we are imaging – and if we should even image at all. In cryo-ET, MS often guides which samples we should invest effort into."

Yet, MS goes beyond identifying proteins; it provides information about post-translational modifications and protein interactions, which are invaluable for downstream modeling. Crosslinking-MS (XL-MS) builds on native MS by using crosslinkers of specific lengths to stabilize interactions within and between proteins further and act as molecular rulers. By providing high-throughput data on side chains involved in protein interactions, XL-MS expands the standard proteomics workflow allowing researchers to see which proteins interact within the "soup" and predict their unknown densities. He is especially excited by the combination of XL-MS and cryo-ET, "we often face the situation that we are able to map large molecules with high specificity and sensitivity in cryo-ET data and discover densities associated with them in subtomogram averages, but do not know exactly what they are. XL-MS plays a key role in identifying these factors and accelerating the biological discovery process." This approach has been instrumental in resolving the NPC, whereby information about protein interactions (obtained by XL-MS) was used to fit protein structures obtained with other methods (e.g., crystallography, NMR, AlphaFold) into cryo-ET reconstructions. In other words, the "noisy data for particles of unknown identity" referred to by Dr. Förster can be interpreted from the data on protein identity, modifications and mutual distances obtained by MS applications.

Another limitation of cryo-ET is that it captures a snapshot of a biological process frozen in time, when in fact biological processes are highly dynamic. This can be solved using MS. For example, for protein complexes that can be studied ex vivo or ex situ, hydrogen-deuterium exchange-MS (HDX-MS) is a powerful technique that can be used to overcome this limitation. HDX-MS measures changes in mass associated with the isotopic exchange of amide hydrogens present at the protein surface. When a protein is incubated in deuterated water (D2O), deuterium (hydrogen containing an extra neutron) exchanges with hydrogen in a time-dependent manner. The rate of exchange for amino acids in exposed regions of the protein is greater than those hidden in the protein's interior. This can be detected during MS analysis, enabling the inference of structural information. However, HDX-MS is limited in its ability to study protein dynamics and conformations in vivo and generally requires purified proteins. Recently a technology called fast photochemical oxidation of proteins (FPOP) has proved able to label proteins directly in cells11 as well as in living nematodes.[12] Instead of incorporating D2O, FPOP uses hydroxyl radicals that are released from the photolysis of hydrogen peroxide to covalently and irreversibly label solvent accessible amino acid side chains.[13] This incorporation followed by a typical proteomics experiment to identify the oxidations allows the study of protein surface structure dynamics and protein-protein interactions on the microsecond-millisecond timescale – making it even more relevant for cryo-ET applications.[14] Therefore, while data from cryo-ET is static and captures protein structures frozen in time, data from HDX-MS and FROP provide information about accessible/hidden and rigid/dynamic regions of the protein, and ultimately, protein conformational dynamics.

The ability to study both individual protein complexes as well as morphological features, makes cryo-ET an interesting technique to study changes between healthy and diseased phenotypes of a cell. Especially for diseases where the full pathways have not yet been explored, there is need for a proteome-wide inventory to interpret the

data obtained by cryo-ET. Although HDX and FPOP have been used at this scale, proteome-wide studies can be challenging due to the nature of the modifications, or the ability to perform a measurement on the right timescale. Recently, an approach called limited proteolysis mass spectrometry (LiP-MS) has been used to study changes in protein-protein interactions and protein conformations on a proteome wide scale.[15] In short, in this approach, proteome extracts are subsequently exposed to a non-specific protease under native conditions, followed by complete digestion with a sequence-specific protease trypsin under denaturing conditions. As the first digestion step occurs under native conditions and is sequence-specific, it generates peptides that are sensitive to the structure of the protein they are derived from. Finally, a bottom-up MS analysis of the same peptides stemming from different conditions reveals proteolytic patterns that will vary for proteins which have changes in conformations or binding state. With the proteome changes mapped, cryo-ET can then be used to selectively study altered complexes at the structural level and visualize the impact of the observed mutations on composition, topology and structure. These technologies will increase our molecular understanding of protein complexes in health and disease and ultimately facilitate the development of drugs that can destroy, inactivate or restore their normal activity.[16]

Dr. Förster is excited about the structural readouts of MS related techniques, he explains that "developments that increase the sensitivity and specificity of XL-MS in complex systems, ultimately in the whole cell, is what I am following most closely. To me, as a distant observer, innovation in crosslinking reagents appears key to further increase sensitivity and specificity of the method in complex settings. There has been considerable innovation in the field, ranging from new chemical groups to MS-cleavable molecules and even crosslinkers that enable affinity purification, and I am looking forward to realizing the full potential of these developments in conjunction with cryo-ET."

Conclusion

The ability to visualize proteins directly in their native environment has the potential to rewrite our understanding of protein function in a cellular context. Especially when studying diseases, the ability to detect protein structure and morphological changes to the cell and correlate this to the genotype is of great value when developing new medicines. This, for example, would allow researchers to trace the genome of a certain cancer cell to perturbation of a specific protein complex, to better understand how to treat the disease.[16] Such an approach would rely heavily on MS to interpret cryo-ET data and facilitating top-down approaches to protein studies – a synergy that will be aided further by instrument automation and accessibility. Looking forward, Dr. Förster notes, "to make MS an integral component of structural biology, the day-to-day usage of MS instruments and specimen preparation protocols must become simpler. Thermo Fisher Scientific has been highly successful in automating instrumentation and pushing to simplify the usage of electron microscopes further, which greatly expanded the user base. Similar efforts in MS together with training events will undoubtedly make MS more accessible among structural biologists."

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References

1. Ziegler SJ, Mallinson SJB, St John PC, Bomble YJ. Advances in integrative structural biology: Towards understanding protein complexes in their cellular context. *Comput. Struct. Biotechnol.* J. 2021;19:214-225.doi: 10.1016/j.csbj.2020.11.052
2. Allegretti M, Zimmerli CE, Rantos V, *et al.* In-cell architecture of the nuclear pore and snapshots of its turnover. *Nature.* 10 2020;586(7831):796-800. doi: 10.1038/s41586-020-2670-5
3. Bárcena M, Barnes CO, Beck M, *et al.* Structural biology in the fight against COVID-19. *Nat. Struct. Mol. Biol.* 01 2021;28(1):2-7. doi: 10.1038/s41594-020-00544-8
4. Lu S, Ye Q, Singh D, *et al.* The SARS-CoV-2 nucleocapsid phosphoprotein forms mutually exclusive condensates with RNA and the membrane-associated M protein. *Nat. Commun.* 01 21 2021;12(1):502. doi: 10.1038/s41467-020-20768-y
5. Renaud JP, Chari A, Ciferri C, *et al.* Cryo-EM in drug discovery: achievements, limitations and prospects. *Nat. Rev. Drug Discov.* 2018;17(7):471-492. doi: 10.1038/nrd.2018.77
6. Luck K, Kim DK, Lambourne L, *et al.* A reference map of the human binary protein interactome. *Nature.* 2020;580:402–408. doi: 10.1038/s41586-020-2188-x
7. von Appen A, Beck M. Structure determination of the nuclear pore complex with three-dimensional cryo electron microscopy. *J. Mol. Biol.* 2016;428:2001-2010. doi: 10.1016/j.jmb.2016.01.004
8. Gemmer M, Förster F. A clearer picture of the ER translocon complex. *J. Cell Sci.* 2020;133(3). doi: 10.1242/jcs.231340
9. Langenfeld F, Peng Y, Lai Y-K, *et al.* SHREC 2020: Multi-domain protein shape retrieval challenge. *Comp. Graph.* 2020;91:189-198. doi: 10.1016/j.cag.2020.07.013
10. Kaur U, Johnson DT, Chea EE, Deredge DJ, Espino JA, Jones LM. Evolution of structural biology through the lens

of mass spectrometry. *Anal. Chem.* 2019;91(1):142-155. doi: 10.1021/acs.analchem.8b05014
11. Espino JA, Mali VS, Jones LM. In cell footprinting coupled with mass spectrometry for the structural analysis of proteins in live cells. *Anal. Chem.* 2015;87(15):7971-7978. doi: 10.1021/acs.analchem.5b01888
12. Espino JA, Jones LM. Illuminating biological interactions with in vivo protein footprinting. *Anal. Chem.* 2019;91(10):6577-6584. doi: 10.1021/acs.analchem.9b00244
13. Cornwell O, Radford SE, Ashcroft AE, Ault JR. Comparing hydrogen deuterium exchange and fast photochemical oxidation of proteins: a structural characterisation of wild-type and ΔN6 β. *J. Am. Soc. Mass Spectrom.* 2018;29(12):2413-2426. doi: 10.1007/s13361-018-2067-y
14. Espino JA, King CD, Jones LM, Robinson RAS. Fast photochemical oxidation of proteins using enhanced multiplexing proteomics. *Anal. Chem.* 2020;92(11):7596-7603. doi: 10.1021/acs.analchem.0c00174
15. Schopper S, Kahraman A, Leuenberger P, *et al.* Measuring protein structural changes on a proteome-wide scale using limited proteolysis-coupled mass spectrometry. *Nat. Protoc.* 2017;12:2391–2410. doi: 10.1038/nprot.2017.100
16. Powell K. Technology to watch in 2018. *Nature.* 2018;553(7689):531-534. doi: 10.1038/d41586-018-01021-5

Chapter 6: Mass Spectrometry for Cryo-EM Sample Screening

Advances in single-particle cryogenic electron microscopy (cryo-EM) are enabling the structural determination of numerous protein assemblies at high resolution and, ultimately, yielding unprecedented insights into their function. Yet, despite its extraordinary capabilities, cryo-EM remains a time-consuming and resource-intensive technique.

Paul Dominic "Dom" Olinares is a research associate in the Laboratory of Mass Spectrometry and Gaseous Ion Chemistry at The Rockefeller University and a member of the National Center for Dynamic Interactome Research (NCDIR). Working with interdisciplinary teams, his research is particularly focused on the use of native MS (nMS) in sample preparation workflows for cryo-EM analysis.

In this interview, Dr. Olinares discusses the limitations of traditional technologies used for sample screening, as well as his role in the development of a streamlined, nMS-based screening workflow and the impact this has had on cryo-EM analysis.

Q: Can you provide an overview of the technologies typically used for sample screening in cryo-EM? Are there any limitations?

Dom (D): Sample integrity, purity and homogeneity are critical determinants in obtaining high-resolution structures. Hence, before one devotes considerable time and resources in performing cryo-EM analysis, rapid and reliable methods for assaying sample quality are needed.

The common methods for checking sample quality include SDS-PAGE, native gel electrophoresis and gel filtration chromatography. These sizing techniques can provide information on sample composition, assembly state and homogeneity but at low mass accuracy and resolution. Negative stain EM is also employed to evaluate particle size and distribution but correlating this information with the presence of a completely assembled target complex can still be ambiguous, especially with potential artifacts. We have worked with structural biologists who have performed all these sample checks and proceeded with cryo-EM analysis only to discover that the particle densities did not correspond to the fully assembled protein complex. With its ability to directly measure the mass of intact protein assemblies and their components at high mass accuracy and resolution, we found that nMS is well-suited for efficient screening and providing feedback on sample stability and homogeneity prior to cryo-EM analysis.

Q: What is native MS-based sample screening and how is it typically used within structural biology research?

D: nMS enables direct mass measurement of macromolecular assemblies by maintaining their near-native structures and assembly states upon gas-phase transfer from solution. Recent instrumentation developments in nMS have increased its resolving power

and sensitivity and expanded its mass range coverage. We thus use nMS as a diagnostic and a screening platform for rapidly checking whether we had the correct protein and nucleic acid components as well as bound cofactors and ligands, and whether these components are correctly assembled. With high resolving power, nMS can reveal protein heterogeneity arising from post-translational modifications such as glycosylation and phosphorylation as well as contamination and degradation. For example, in one screening experiment, we readily confirmed that the protein subunits had the necessary bound Zinc cofactors (+65 Da each).[1] In another example, we could discriminate a 3'-end nucleotide cleavage (-305 Da) in our bacterial transcription elongation complex containing pre-translocated RNA transcript that ultimately informed us on which RNA constructs to use for successful cryo-EM analysis.[2]

For challenging structural projects wherein the initial sample reconstitution conditions involving multiple components did not yield the desired protein assemblies, we can readily perform iterative native MS-based screening.[3] Using this approach, we systematically varied relevant biochemical parameters (e.g., identity and concentration of protein and nucleic acid components, buffer composition, pH, incubation time, and temperature) to establish optimized sample preparation conditions. Furthermore, the subunit stoichiometries, bound small molecules and protein modifications extracted simultaneously from the same nMS data (acquired during the screening), have been extremely useful in downstream EM density map assignment and structure reconstruction. We found an excellent correlation between observing assembled protein complexes from the nMS screening and subsequently obtaining an analytically useful number of intact particles for cryo-EM analysis. Overall, the molecular weight measurements and sample profiling generated from our time-saving nMS platform have proved so informative that we routinely use it to decide which samples to prioritize and which optimal sample conditions to employ for increasing the success rate in obtaining high-resolution cryo-EM structures.

Q: What research questions are you trying to address within your own work?

D: Our lab develops MS-based methodologies for obtaining key information that facilitate integrative structural studies. Our collaboration with the structural biology lab headed by Elizabeth Campbell and Seth Darst at The Rockefeller University has enabled us to integrate our nMS-based screening platform in their sample preparation and cryo-EM workflows. The main research focus of the Campbell/Darst lab is the structural biology of transcription, an essential and multi-step process wherein genetic information is transcribed into RNA. In bacteria, the DNA-dependent RNA polymerase (DdRp) core complex is the central enzyme that goes through the initiation, elongation, and termination stages of transcription in coordination with many regulatory protein factors. A detailed structural and mechanistic understanding of the bacterial transcriptional cycle is essential not only in gaining insights into gene expression regulation but also in designing new antibiotics. Several structures have been determined that represent the various steps of transcription. However, critical structural intermediates that are transient or unstable remain unexplored, including those formed during promoter melting in transcription initiation and the disassembly of stalled transcription elongation complexes during transcription termination. In these two challenging cases, we initially did not observe a significant amount of the fully assembled complexes (i.e., the DNA or a protein component is not bound or falls off) upon reconstitution. With the help of nMS screening, we were able to efficiently explore a wide range of biochemical conditions and identify critical parameters (e.g., the nucleic acid sequences and incubation times) that facilitated the assembly of the target protein complexes and enabled successful determination of their structures.[3]

Last year, our labs also started working on the RNA-dependent RNA polymerase (RdRp) from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogenic agent behind the current global COVID-19 pandemic. The virus has a relatively large RNA genome that is replicated and transcribed by the RdRp holoenzyme in a replication-transcription complex (RTC). The viral RTC is the target of several polymerase inhibitors, including the clinically approved antiviral drug remdesivir. Elucidating the molecular architectures of RTC bound to relevant accessory proteins will yield insights into the coordination of the multi-step replication-transcription process in SARS-CoV-2 as well as in other RNA viruses and enable rational design of better antiviral drugs that are urgently needed during this current pandemic. Initially, the samples we prepared did not contain a fully assembled RTC and we quickly discovered from the screening that one of the components was truncated enabling us to catch the problem early on and troubleshoot accordingly.[3] Upon addressing this issue and optimizing the RNA constructs used, we were able to determine conditions that yielded a fully assembled RTC coupled with the essential nsp13 helicase and obtain high-resolution cryo-EM structures of this viral assembly.[1]

Q: What techniques or protocols have you developed to enable you to improve throughput and save resource time?

D: We recently published our streamlined nMS-based screening workflow. We have optimized each step in the workflow including rapid buffer exchange into MS-compatible solution with minimal sample losses and

efficient data processing using freely available MS spectra deconvolution software. For native MS analysis, we use the Exactive Plus EMR (Thermo Fisher Scientific), an Orbitrap-based mass analyzer with high resolving power and exceptional sensitivity. These capabilities enable us to work with sample concentrations (0.5-10 µM) that match the concentration ranges employed for single particle cryo-EM analysis.

Overall, each sample or test condition takes about 20-30 min from the buffer exchange step to a list of measured masses and relative peak intensities for all species detected in the sample. This throughput allows us to screen 15-20 different sample conditions (including replicates) per day, which has proved sufficient for our iterative screening pipeline. The nMS timeline enables optimization and iterative screening on a scale of hours or days compared with iterations performed through cryo-EM analyses, which can take several weeks.

We are further developing nMS-based strategies and extending the utility of our screening workflow to aid in structural studies of challenging and dynamic systems. These include coupling nMS with continuous, real-time monitoring of reactions that involve multimeric enzyme assemblies and with limited proteolysis to probe domain topology and map flexible regions in assembled protein complexes.

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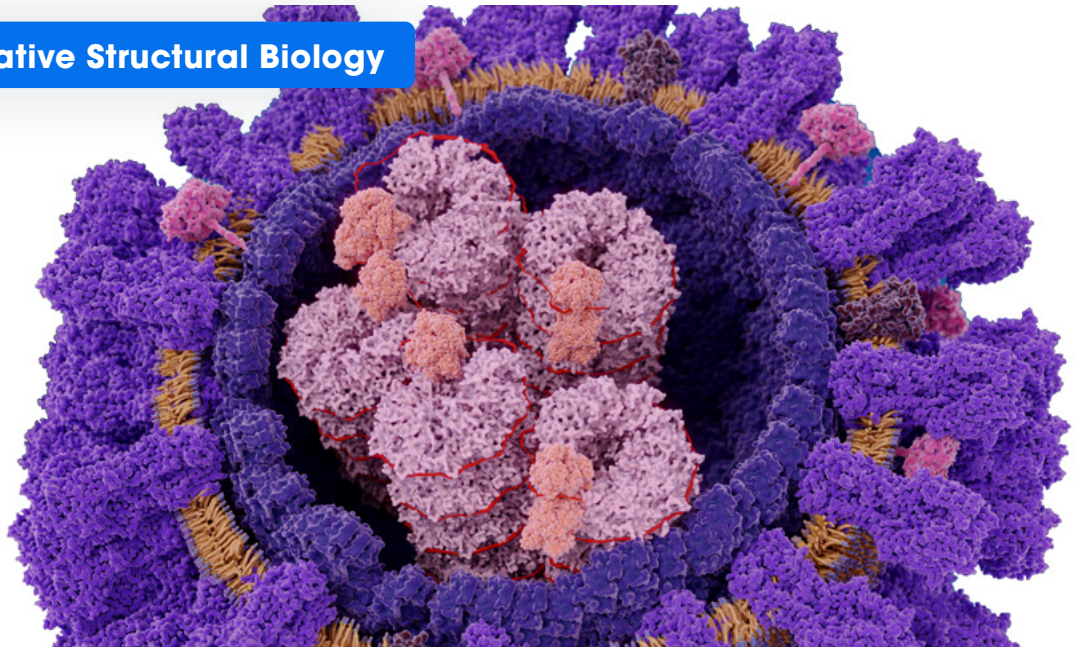
References

1. Chen J, Malone B, Llewellyn E, *et al*. Structural basis for helicase-polymerase coupling in the SARS-CoV-2 replication-transcription complex. *Cell*. 2020;182(6):1560-1573.e13. doi: 10.1016/j.cell.2020.07.033
2. Kang JY, Olinares PD, Chen J, *et al*. Structural basis of transcription arrest by coliphage HK022 Nun in an *Escherichia coli* RNA polymerase elongation complex. *eLife*. 2017;6:e25478. doi: 10.7554/eLife.25478
3. Olinares PDB, Kang JY, Llewellyn E, *et al*. Native mass spectrometry-based screening for optimal sample preparation in single-particle cryo-EM. *Structure*. 2021;29(2):186-195.e6. doi: 10.1016/j.str.2020.11.001



Chapter 7: Integrated Modeling

So far, this eBook has demonstrated the benefits of combining different structure-determination techniques to reconstruct large, complex biomolecular assemblies. Integrative modeling is a crucial step that enables scientists to reconcile and integrate data from different techniques. Hybrid models integrate data from multiple techniques (i.e., mass spectrometry (MS)-based techniques, cryogenic electron-microscopy (cryo-EM), nuclear magnetic resonance (NMR), scattering methods, etc.) into computational molecular modelling methods to obtain comprehensive structural models of biomolecules that cannot not be revealed by any single approach alone.[1,2,3,4,5] The application of integrative modeling has increased significantly in the last few years and today, the data generated by these models are becoming available to a larger community of users.[6] This article will explore the advances in hybrid methods for structural biology and the role of computation in their development and application.

The role of computation in hybrid methods

New algorithms and computational methods that reconcile data from different experimental studies over a wide range of resolution and time scales have been integral to the successful implementation of hybrid methods. One of the scientists at the forefront of this research is Dr. Alexandre Bonvin, professor of Computational Structural Biology at Utrecht University. His group develops bioinformatic and computational approaches to predict, model and dissect biomolecular interactions at the atomic level. "One of our key activities is the development of an integrative modeling software that integrates as much knowledge as possible to guide the modeling of biomolecular assemblies. To this end, we have been developing the HADDOCK software for almost 20 years", he explains. HADDOCK is a pioneer software in integrative modeling of protein interactions; "this software relies on complex machinery and is accessible to users worldwide through a web portal, which enables us to provide computing services to a large community of users – there are currently more than 25,000 registered users (which include researchers in academia and industry)". Dr. Bonvin also explains the importance of these tools; "getting access to the 3D structures of the biomolecular assemblies in the interactome (i.e., the network of all molecular interactions in a particular cell) provides insights into how these molecules interact with each other and exchange information."

Applying hybrid methods

During the last 15 years, hybrid methods have been used to resolve the structure of a wide variety of systems, ranging from complexes of two subunits to large assemblies comprising hundreds of subunits. Table 1 shows some examples of large macromolecular assemblies whose structure was determined by combining data from multiple experiments using computational modeling.

As Dr. Bonvin explains, "Today, we see increasingly that information from a variety of experimental techniques such as crosslinking MS (XL-MS), small angle X-ray scattering (SAXS), cryo-EM, and any other method that gives information about important residues, distances between specific pairs of residues or shapes are being combined to generate 3D models of biomolecular assemblies." With respect to the cryo-EM revolution recently, Dr. Bonvin states that, "it has resulted in a wealth of atomistic resolution new structures, however, as it is not always possible to reach atomic resolution in all parts

Table 1: Selection of structures that have been determined using integrative methods (adapted from [4]).

Structure	Input data
Nuclear Pore Complex[7]	XL-MS, cryo-ET, SAXS
Chromatin remodeler complex INO80[8]	cryo-EM, XL-MS
Polycomb Repressive Complex 2 (PRC2)[9]	EM, XL-MS
Large subunit of the mammalian mitochondrial ribosome (39S)[10]	cryo-EM, XL-MS
RNA polymerase II transcription pre-initiation complex[11]	cryo-EM, XL-MS
Type III secretion system needle[12]	cryo-EM, NMR
Proteosomal lid[13]	nMS, XL-MS
RNA ribosome-binding element from the turnip crinkle virus genome[14]	NMR, SAXS, EM
Translation initiation complex 40S-eIF1-eIF3[15]	X-ray crystallography, EM, XL-MS
Retroviral RNA Packaging Element $[\Psi CD]_2$ [16]	cryo-ET, NMR
Cyanobacterial circadian timing KaiB-KaiC complex[17]	HDX-MS, IM-MS
Aerolysin pore[18]	X-ray crystallography, cryo-EM
ATP synthase membrane motor[19]	cryo-EM, XL-MS,
Human 26S proteasome[20]	XL-MS, EM
Nucleosome remodeler ISWI[21]	XL-MS, SAXS

cryo-ET: cryogenic electronic tomography. SAXS: Small angle X-ray scattering. EM: electron microscopy. cryo-EM: cryogenic electron microscopy. XL-MS: crosslinking mass spectrometry. NMR: nuclear magnetic resonance. HDX-MS: Hydrogen/deuterium exchange mass spectrometry. IM-MS: ion mobility mass spectrometry. nMS: native mass spectrometry.

of a large system, integrative methods are therefore useful and still required to model the missing parts." He thinks that the same is true for data generated using cryo-ET, "likewise, cryo-ET cannot always reach high enough resolution for *de novo* constructions of the 3D structure and, in this case, hybrid methods again can be used to resolve the whole structure". The HADDOCK software has been very successful in resolving several protein-protein complexes. For example, it was used in combination with NMR data to study the complex formed between plectasin and the bacterial wall precursor lipid II, providing valuable information for the design of novel antibiotics.[22] In addition to developing new computational methodologies, Dr. Bonvin uses his expertise to solve biological problems in collaboration with researchers in different parts of the world; "during the pandemic, we experienced a significant increase in the use of our tools and software to model the interactions of the SARS-CoV-2 spike proteins with receptors in human cells, and for drug and peptide screening to block this interaction. We also conducted an *in silico* drug repurposing screening campaign against different SARS-CoV-2 targets in the context of the IMI-CARE project."

The challenges of integrative modeling

As outlined in figure 1, integrative modeling comprises four iterative stages.[3,4] Comprehensive software packages exist that cover all stages of the workflow. Alternatively, different programs or computer codes can be used at each stage.[3]

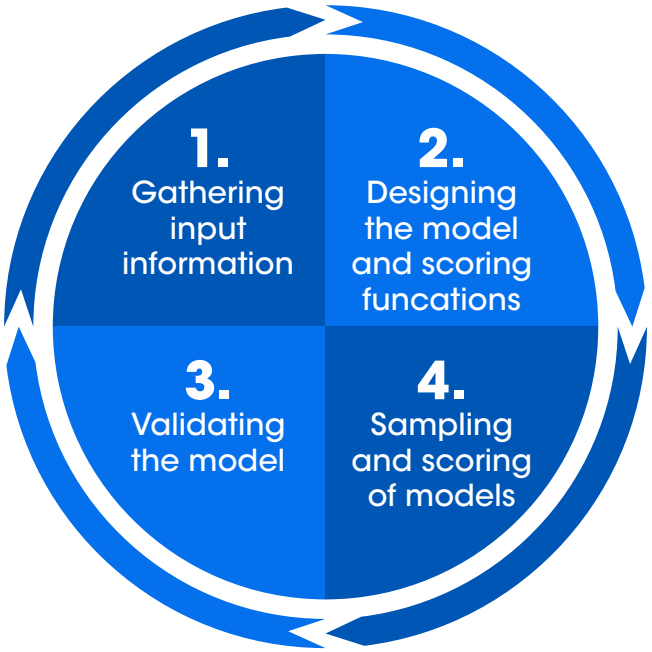


Figure 1: The four stages of integrative modeling

Access to validated data is important for software developers that need to test and benchmark new methods. However, new structure-determination methods are only useful for integrative modeling if the data are accessible. Dr. Bonvin explains that, "without data, we cannot really benchmark our tools; we can generate simulated data, but it's not the same as getting access to real experimental data with real errors and noise. We need more standardization in the way of reporting various types of experimental data, and a better definition of the associated metadata so that the field can collect, process and harvest the correct information". He adds, "hybrid methods need to access all the panoply of experimental data. However, the data are not always available in findable, accessible, interoperable, and reusable (FAIR) repositories. The computational field will benefit greatly from improved organization and sharing of data." In addition, the heterogeneity of data formats is another problem that must be tackled to facilitate data integration; "we spend a lot of time preparing and converting the data from one format to another, before we can use them for a specific application or specific piece of software. That is why, standardization of the data and metadata is so important. Ideally, we'll have a spider web type of model where the different data are connected to the center of the web. You then only need to make a connection to the center of the web to get everything you need. Instead, now we are making connections between the various software and all pieces of individual data. We need a simple interface that allows us to produce data in a format compatible with hybrid methods".

The design of the model is influenced by the quantity and resolution of the available information; different parts of a model may be represented at different resolutions, and one part may be represented at several different resolutions simultaneously.[3] It is during this stage that data from different structure-determination techniques are combined; "researchers are using MS crosslinking data together to stabilize complexes and obtain higher quality cryo-EM data by limiting the conformational heterogeneity". He also comments on some limitations during this stage, "cryo-EM, generates a large volume of data for processing. Therefore, the computational and data infrastructures are often limiting factors. You need to have sufficient computational resources or be able to access and process everything, either locally, in the cloud or on high performance or high throughput computing (HPC/HTC) resources. For example our HADDOCK portal is making use of the HTC resources provided by the EGI and European Open Science Cloud (EOSC) having access to >100'000 CPU cores."[23] A variety of optimization algorithms (e.g., Monte Carlo, gradient decent, etc.) can be used to generate and optimize models.[3]

In order to validate models, they must be analyzed to ascertain ensemble precision and accuracy and to check for inconsistent information.[3] Dr. Bonvin highlights the importance of this final step, "it is important to validate the generated integrative models and that's something that the community is working on. For example, a taskforce has been established by the Protein Data Bank (PDB) to define how to validate such hybrid models." He also emphasizes the importance of training, "we need to teach people how to use the data and software properly, make them aware of limitations, and teach them how to interpret and criticize the results. While in modeling computers may always give you an answer, it doesn't mean that it's correct." When asked about further challenges within the field, Dr. Bonvin highlighted the importance of ensuring reproducibility; "when building workflows for data processing, conversion and computing, it is crucial to properly document the entire pipeline from data collection to the final hybrid model, as this will increase the reproducibility and uptake of these methods."

The future of integrative modelling

Overcoming the challenges outlined above will further expand the applications of hybrid methods. Dr. Bonvin acknowledges that in the future "there will be new types of experiments and associated data coming out that we'll have to learn to integrate." He predicts that Bayesian approaches, bioinformatics and artificial intelligence will become increasingly important in this context. However, since these methods often rely on large amounts of data, he insists that "it will be crucial to overcome the challenge of having proper data and associated metadata documented and accessible."

Finally, Dr. Bonvin foresees that hybrid models will start playing an increasing role to resolve large membrane complexes; "most methods (both experimental and computational) have been mainly targeted at soluble systems. Still a large fraction of the proteome (and interactome) does involve membrane proteins. These are notoriously more difficult to study experimentally. I foresee that this is an area of research where hybrid methods will start playing a significant role". Likewise, he thinks that capturing and modeling dynamic changes is a challenge that will have to be addressed in the future. Hybrid models will be increasingly used to understand the dynamics of biomolecular systems as they assemble, disassemble, function, and undergo regulation via interactions with other systems. "Biomolecules by definition are flexible", he explains, "their dynamics are often part of their function. It is therefore important to not only map one state, but also the landscape that these structures can take, and how the system evolves in this landscape." Additionally, "the landscape itself might change as function of, for example, the state of the cell and/or all kinds of post-translational modifications", he further explains, "capturing and modelling those changes

is a challenge the field will have to address in the future." Indeed, the inclusion of dynamic and state-dependent information into integrative models holds the promise of breathing life and movement into currently mostly static representations and so visualize the processes that actually drive living cellular systems.[4]

Conclusion

Characterizing the structure and dynamic of biomolecules enables scientists to understand the molecular mechanisms regulating living systems and formulate hypotheses about their function and evolution. Yet, many large, complex biomolecular assemblies cannot be reconstructed using single structure-determination techniques in isolation. It is in this context where integrative or hybrid models reveal their potential and become indispensable. The implementation of hybrid methods might appear intimidating, however, as Dr. Bonvin explains: "we are trying to make our tools accessible to the wider community by providing user-friendly web portals and services that simplify the difficult aspects of setting up computations. Simple forms can be used to submit the data and then we take care of the computations. This facilitates the use of hybrid methods by a large community of users, both experienced researchers as well as students as we see our tools being used as well in education".

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References

1. Van Noort CW, Honorato RV, Bonvin AMJJ. Information-driven modeling of biomolecular complexes. *Curr. Op. Struct. Biol.* 2021;70:70-77. doi: 10.1016/j.sbi.2021.05.003
2. Koukos PI, Bonvin AMJJ. Integrative modelling of biomolecular complexes. *J. Mol. Biol.* 2020;432(9): 2861-2881. doi: 10.1016/j.jmb.2019.11.009
3. Braitbard M, Schneidman-Duhovny D, Kalisman N. Integrative structure modeling: overview and assessment. *Ann. Rev. Bioch.* 2019;88(1):113-135. doi: 10.1146/annurev-biochem-013118-111429
4. Rout MP, Sali A. Principles for integrative structural biology studies. *Cell.* 2019;177(6):1384-1403. doi: 10.1016/j.cell.2019.05.016
5. Srivastava A, Tiwari SP, Miyashita O, Tama F. Integrative/hybrid modeling approaches for studying biomolecules. *J. Mol. Biol.* 2020;432(9):2846-2860. doi: 10.1016/j.jmb.2020.01.039
6. Vallat B, Webb B, Fayazi M, *et al.* New system for archiving integrative structures. *Acta Cryst.* 2021;77:1486-1496. doi: 10.1107/S2059798321010871
7. Kim SJ, Fernandez-Martinez J, Nudelman I, *et al.* Integrative structure and functional anatomy of a nuclear pore complex. *Nature.* 2018;555(7697):475-482. doi:10.1038/nature26003
8. Tosi A, Haas C, Herzog F, *et al.* Structure and subunit topology of the INO80 chromatin remodeler and its nucleosome complex. *Cell.* 2013;154(6):1207-1219. doi: 10.1016/j.cell.2013.08.016
9. Ciferri C, Lander GC, Maiolica A, Herzog F, Aebersold R, Nogales E. Molecular architecture of human polycomb repressive complex 2. *eLife.* 2012;1:e00005. doi: 10.7554/eLife.00005
10. Greber B, Boehringer D, Leitner A, *et al.* Architecture of the large subunit of the mammalian mitochondrial ribosome. *Nature.* 2014;505:515-519. doi: 10.1038/nature12890
11. Murakami K, Elmlund H, Kalisman N, *et al.* Architecture of an RNA polymerase II transcription pre-initiation complex. *Science.* 2013;342(6159):1238724. doi: 10.1126/science.1238724
12. Loquet A, Sgourakis NG, Gupta R, *et al.* Atomic model of the type III secretion system needle. *Nature.* 2012;486(7402):276-279. doi: 10.1038/nature11079
13. Politis A, Stengel F, Hall Z, *et al.* A mass spectrometry-based hybrid method for structural modeling of protein complexes. *Nat. Methods.* 2014;11(4):403-406. doi: 10.1038/nmeth.2841
14. Gong Z, Schwieters CD, Tang C. Conjoined use of EM and NMR in RNA structure refinement. *PLoS One.* 2015;10(3):e0120445. doi: 10.1371/journal.pone.0120445
15. Erzberger JP, Stengel F, Pellarin R, *et al.* Molecular architecture of the 40S eIF1 eIF3 translation initiation complex. *Cell.* 2014;158(5):1123-1135. doi: 10.1016/j.cell.2014.07.044
16. Miyazaki Y, Irobalieva RN, Tolbert BS, *et al.* Structure of a conserved retroviral RNA packaging element by NMR spectroscopy and cryo-electron tomography. *J. Mol. Biol.* 2010;404(5):751-772. doi: 10.1016/j.jmb.2010.09.009
17. Snijder J, Burnley RJ, Wiegard A, *et al.* Insight into cyanobacterial circadian timing from structural details of the KaiB-KaiC interaction. *PNAS.* 2014;111(4):1379-1384. doi: 10.1073/pnas.1314326111
18. Degiacomi M, Iacovache I, Pernot L, *et al.* Molecular assembly of the aerolysin pore reveals a swirling membrane-insertion mechanism. *Nat. Chem. Biol.* 2013;9:623–629. doi: 10.1038/nchembio.1312
19. Leone V, Faraldo-Gómez JD. Structure and mechanism of the ATP synthase membrane motor inferred from quantitative integrative modeling. *J. Gen. Physiol.*

2016;148(6):441-457. doi: 10.1085/jgp.201611679
20. Wang X, Cimermancic P, Yu C, *et al*. Molecular Details Underlying Dynamic Structures and Regulation of the Human 26S Proteasome. *Mol. Cell Proteom.* 2017;16(5):840-854. doi: 10.1074/mcp.M116.065326
21. Harrer N, Schindler CEM, Bruetzel LK, *et al*. Structural architecture of the nucleosome remodeler ISWI determined from crosslinking, mass spectrometry, SAXS, and modeling. *Structure*. 2018;26(2):282-294.e6. doi: 10.1016/j.str.2017.12.015
22. Schneider T, Kruse T, Wimmer R, *et al*. Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. *Science*. 2010;328(5982):1168-1172. doi: 10.1126/science.1185723
23. Honorato RV, Koukos PI, Jiménez-García B, *et al*. Structural biology in the clouds: The WeNMR-EOSC ecosystem. *Front. Mol. Biosc.* 20218:729513. doi: 10.3389/fmolb.2021.729513

Summary

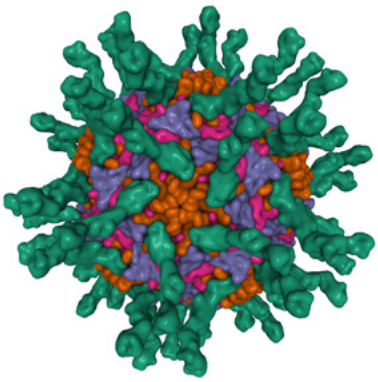
This eBook has explored the various ways that structural biologists are integrating mass spectrometry (MS) techniques with cryo-electron microscopy (cryo-EM) and other structural tools, and the impact this has on their understanding of complex biological systems.

The dynamic and flexible nature of biomolecules poses a major challenge for structural biologists. It is therefore important to develop tools that allow them to map the landscape that these structures can take and the evolution of these systems within the landscape. Advances in MS and cryo-EM provide structural information not only from purified proteins, but also from proteins in their near-native state. This has yielded insights into the impact that location, configuration and interactions have on the function of complex macromolecules.

Yet, the full characterization of a macromolecular system requires the combination of many structure-determination approaches. For example, while the cryo-EM revolution significantly enhanced the analysis of complex protein complexes in their native environment, it is unable to provide information on protein dynamics. In this context, MS can help to interpret the data obtained using cryo-ET. Thus, combining these two techniques is a powerful approach that has broad implications in several fields including cell biology, virology and drug design.

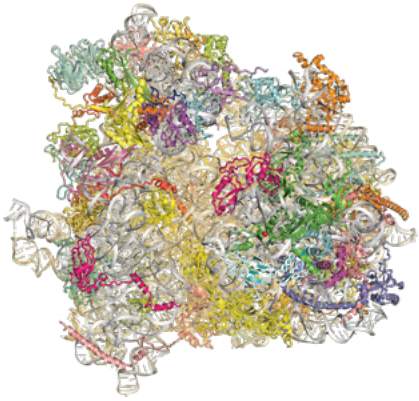
Integrative or hybrid models enable the integration of data from different structure-determination techniques. Thus, the development of new computational methods become indispensable to create comprehensive structural models of biomolecules that cannot be revealed by any single approach alone. Looking forward, bioinformatics and artificial intelligence will play an increasingly important role to ensure that these tools are accessible to the wider community and that they can continue to evolve.

Resources



Higher precision 3D analysis from structure to function

This integrative structural biology flyer discusses how various mass spectrometry techniques can be combined with cryo-EM to provide unique insights beyond protein structures. Read here.

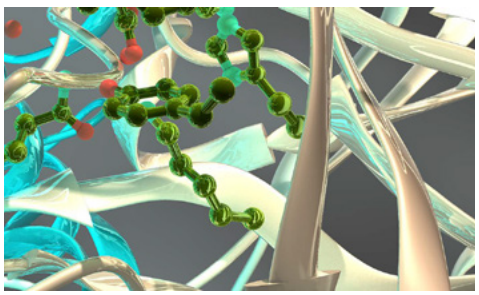


Accelerating the path from structure to function through integrative structural biology solutions

This eBook includes articles that explore the advances in MS and cryo-EM and the applications of these synergistic approaches. Read here.

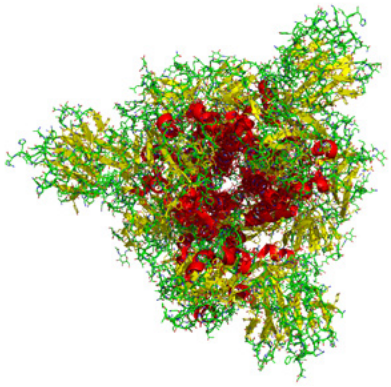
Rapid online buffer exchange for protein screening

This flyer introduces a workflow for rapid sample screening using novel online buffer exchange columns coupled to native mass spectrometry and its applications. Read here.



Crosslinking mass spectrometry (XL-MS) goes mainstream

This white paper focuses on the challenges of performing crosslinking analyses, and how development of the latest tools from sample preparation to data analysis have enhanced the workflow. Read here.



Hydrogen deuterium exchange mass spectrometry for the masses

This white paper focuses on the information provided by HDX-MS experiments, and how new tools developed for processes from sample preparation to data analysis have made this workflow more informative and streamlined. Read here.

Native MS for structural biology research

This white paper focuses on the information provided by native mass spectrometry, and the benefits of incorporating advances in this analytical tool for the workflow. Read here.

Getting Started with cryo-electron microscopy (cryo-EM)

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Webinars

Studying small viral glycoproteins using an integrative structural biology approach

Dr. Joost Snijder, Assistant Professor, Utrecht University, and Dr. Ieva Drulyte, Cryo-EM Application Specialist at Thermo Fisher Scientific, present the cryo-EM structure of the ~80 kDa heavily glycosylated human coronavirus HKU1 hemagglutinin esterase (HE) at a global resolution of 3.4 Å. Register now to watch on demand.

Native mass spectrometry-based screening for optimal sample preparation in single particle cryo-EM

Dr. Dom Olinares discusses recent advances in single particle cryogenic electron microscopy (cryo-EM) have enabled the structural determination of numerous flexible and conformationally heterogeneous protein assemblies at high resolution, yielding unprecedented insights into their function. Register now to watch on demand.

Integrative structural biology going viral

In this 10-minute video, Dr. Albert Konijnenberg, Product Manager, cryo-EM Sample Preparation & Integrative Structural Biology at Thermo Fisher Scientific, discusses the role of cryo-EM in vaccine development and as an assay for mutants. Register now to watch.

Automating native mass spectrometry through the use of online buffer exchange

In this webinar, Dr. Vicki Wysocki discusses the development of online buffer exchange for nMS applications. She describes how the experiment is implemented and how it can be extended by coupling to affinity separation (e.g., IMAC-OBE) to optimize protein overexpression. Register now to watch on demand.

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In this webinar, Dr. Patrick Griffin, Chair of Molecular Medicine, will highlight a new platform for structure-function analysis to dissect activation mechanisms of nuclear receptors (NRs). Register now to watch on demand.

Mass spectrometry for 3D structural determination with cryo-electron microscopy

In this *Ask the Experts*, Dr. Florian Stengel, Dr Henning Urlaub and Dr Christian Dieneman discuss how the combination of cryo-EM and crosslinking helps them to gain in depth understanding of complex biological systems. Register now to watch on demand.