NYC Integrative Structural Biology Symposium

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Understanding Cooperativity Effects in Chemical Probing Experiments

Ethan Arnold*, Daniel Cohn*, Emma Bose*, Gregory Wolfe, Alisha N Jones
** co-presenting*

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Small molecule chemical probes, which form covalent bonds with atoms of flexible nucleotides, are commonly used to gain insight into the structure adopted by an RNA. Atomistic molecular dynamic (MD) simulations recently revealed that binding of RNA by chemical probes is affected by cooperative effects, leading to an observed reactivity that is dependent on the concentration of the chemical probe. In this work, we used selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) and dimethyl sulfate (DMS) chemical probing experiments to explore whether the structure of an RNA is modulated by chemical probe binding events. We find that as the concentration of a chemical probe increases, modified nucleotides locally modulate the RNA structure, resulting in the increase or decrease of chemical probe reactivity in surrounding nucleotides. This cooperative effect is dependent on both chemical probe concentration and size. This work highlights the importance of optimizing chemical probing experiments to accurately predict RNA secondary structures.

Helical reconstruction of VP39 reveals principles for baculovirus nucleocapsid assembly

Friederike M. C. Benning, Simon Jenni, Coby Y. Garcia, Tran H. Nguyen, Xuewu Zhang, Luke H. Chao

Massachusetts General Hospital, Boston, MA

Baculoviruses are insect-infecting pathogens with wide applications as biological pesticides, in vitro protein production vehicles and gene therapy tools. Its cylindrical nucleocapsid, which encapsulates and protects the circular double-stranded viral DNA encoding proteins for viral replication and entry, is formed by the highly conserved major capsid protein VP39. The mechanism for VP39 assembly remains unknown. We determined a 3.2 Å electron cryomicroscopy helical reconstruction of an infectious nucleocapsid of Autographa californica multiple nucleopolyhedrovirus, revealing how dimers of VP39 assemble into a 14-stranded helical tube. We show that VP39 comprises a unique protein fold conserved across baculoviruses, which includes a Zinc finger domain and a stabilizing intra-dimer sling. Analysis of sample polymorphism revealed that VP39 assembles in several closely-related helical geometries. This VP39 reconstruction reveals general principles for baculoviral nucleocapsid assembly.

Gatekeeper Mutations Activate FGF Receptor Tyrosine Kinases by Destabilizing the Autoinhibited State

Alida Besch, William M. Marsiglia, Moosa Mohammadi, Yingkai Zhang, Nathaniel J. Traaseth

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Many types of human cancers are being treated with small molecule ATP-competitive inhibitors targeting the kinase domain of receptor tyrosine kinases (RTKs). Despite initial successful remission, long term treatment almost inevitably leads to the emergence of drug resistance mutations at the gatekeeper residue hindering the access of the inhibitor to a hydrophobic pocket at the back of the ATP binding cleft. In addition to reducing drug efficacy, gatekeeper mutations elevate the intrinsic activity of the tyrosine kinase domain leading to more aggressive types of cancer. However, the mechanism of gain-of-function by gatekeeper mutations is poorly understood. Here, we characterized FGF receptor tyrosine kinases harboring two distinct gatekeeper mutations using kinase activity assays, NMR spectroscopy, bioinformatic analyses, and MD simulations. We show that gatekeeper mutations destabilize the

autoinhibited state and lead to conformational changes at the DFG motif that culminate in an equilibrium shift to the active state of the kinase.

Coarse-grained modeling of oligonucleosomes for prediction of short-range spatially correlated cleavage

Ariana Brenner Clerkin, Nicole Pagane, Devany West, Andrew J. Spakowitz, Viviana I. Risca

The Rockefeller University, New York, NY

We use a mesoscale chromatin simulation framework to show a nucleosome repeat length-dependent and nucleosome wrapping-dependent effect on chromatin folding. We perform Monte Carlo simulations with a handful of computational beads per segment of linker DNA. We implement DNA and nucleosome sterics as well as an internucleosome potential to capture the energetically favorable interaction between the basic H4 tails and the acidic patch on H2A/H2B. We validate parameter selection against experimental Cryo-EM and sedimentation assay data. We observe a nucleosome repeat length dependence on fiber compaction and observe a 10 base pair periodicity in compaction capacity using a RICC-seq signal prediction model. Analysis also suggests that compaction and predicted RICC-seq signal is sensitive to the extent of nucleosome breathing.

Square beams for optimal tiling in TEM

Eugene Chua, Michael Alink, Misha Kopylov, Alex de Marco

New York Structural Biology Center, New York, NY

Imaging large fields-of-view at a high magnification requires tiling. Transmission electron microscopes typically have round beam profiles; therefore, tiling across a large field-of-view is either imperfect or results in uneven exposures, which is a problem on dose-sensitive samples. Here we introduce a square electron beam that can be easily retrofitted in existing microscopes and demonstrate its application showing it can tile nearly perfectly and deliver cryo-EM imaging with resolution comparable to conventional setups.

Structural Studies of Small Molecule ARNT PAS-B Binders as Novel Modulators of Transcriptional Response

Joseph Closson, Xingjian Xu, Leandro Pimentel Marcelino, Marion Lucia Silvestrini, Riccardo Solazzo, Lillian Chong, Eta Isiorho, Denize Favaro, Kevin H. Gardner

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The hypoxia-inducible factors (HIFs) are a class of heterodimeric transcription factors that regulate metabolic and angiogenic pathways in an oxygen dependent fashion. Overactivation of these pathways such as in the case of Von Hippel Lindau disease can result in tumorigenesis and certain types of cancers. One method of inhibiting these HIFs lies in the recruitment of coiled-coil coactivators (CCCs) that are essential to the function of the complexes. Two small molecule fragments, KG-548 and KG-655,

have previously been found to inhibit CCC binding to the β-subunit of the HIF complexes, aryl hydrocarbon receptor nuclear translocator (ARNT). Our work details the binding locations of KG-548 and KG655 to the isolated ARNT PAS-B domain through NMR, X-ray crystallographic and computational studies, revealing a highly occupied surface binding interface for KG-548, while internal binding of KG-655 appears to induce a conformational change to accommodate the occupation of the cavity. Additionally, we have performed binding assays within the larger HIF2a-ARNT complex to determine the ability of these compounds to bind to the more functional complex, suggesting that KG-548 binding remains of comparable affinity in the HIF2a-ARNT complex, but internal binding ligands KG-655 and KG-279 have lost the ability to bind, suggesting some sort of occlusion of the internal binding mode in the functional transcription factor complex. We aim to utilize this work to develop small molecule tools capable of probing HIF interaction as well as to perform preliminary work into designing cancer therapeutics.

Automated native mass spectrometry screening of membrane proteins for structural biology applications

Natalia de Val, Scott Kronewitter, Paul Gazis, Mick Greer, Weijing Liu, Rosa Viner, Olufemi Adeyemi, Albert Konijnenberg and Edward Pryor

Thermo Fisher Scientific

In the last five years, a sharp increase in membrane protein structures has been determined, driven largely by the adoption of cryo-EM, which is well-positioned to determine previously unobtainable integral membrane protein structures. As the automation and achievable resolution of cryo-electron microscopes is rapidly improving, the critical step is now in preparing good grids, which require a stable and homogeneous sample. Typical characterization methods like SEC can be hard to interpret due to the heterogeneity introduced by the membrane mimetics required for the stabilization of the membrane protein. Here we present a fully automated native mass spectrometry-based workflow, designed for non-experts, to screen membrane proteins for stability, homogeneity, and composition.

This workflow was tested on several membrane protein classes to validate the performance. The workflow can be used in an automated fashion for up to 96 samples, ranging from applications like drug screening to buffer optimization. By switching or omitting detergents, the same workflow can be used to either provide intact analysis of membrane proteins without additional sample preparation or harsh denaturing conditions and even analysis of host cell proteins that are co-expressed or purified with the target protein.

Developments in Advanced Handling, Storage, Transport, and Tracking of Cryo-EM Samples

Joyce Frank, Ben Apker, Dave Closs, Robert Thorne

MiTeGen

Interest in cryoelectron microscopy (cryoEM) is growing rapidly as technical advances in electron detectors and optics dramatically improve imaging capabilities and sample throughput. The federal government, via the NIH, has recently established multiple national centers that will provide user access to this technology. Efficient use of these centers requires improved tools and methods for sample management. Building on experience gained in the high-throughput revolution in cryocrystallography, systems for the advanced storage, transport, and tracking of cryo-EM samples are being developed. We report on our current and planned developments in this area.

An RNA excited conformational state at atomic resolution

Ainan Geng, Laura Ganser, Rohit Roy, Honglue Shi, Supriya Pratihar, David A. Case, and Hashim M. Al-Hashimi

Columbia University, New York, NY

Sparse and short-lived excited RNA conformational states are essential players in cell physiology, disease, and therapeutic development, yet determining their 3D structures remains challenging. Combining mutagenesis, NMR spectroscopy, and computational modeling, we determined the 3D structural ensemble formed by a short-lived (lifetime ~2.1 ms) lowly-populated (~0.4%) conformational state in HIV-1 TAR RNA. Through a strand register shift, the excited conformational state completely remodels the 3D structure of the ground state (RMSD from the ground state = 7.2 ± 0.9 Å), forming a surprisingly more ordered conformational ensemble rich in non-canonical mismatches. The structure impedes the formation of the motifs recognized by Tat and the super elongation complex, explaining why this alternative TAR conformation cannot activate HIV-1 transcription. The ability to determine the 3D structures of fleeting RNA states using the presented methodology holds great promise for our understanding of RNA biology, disease mechanisms, and the development of RNA-targeting therapeutics.

Advances in Vibration Control Strategies for Cryo TEM, E-beam Metrology and other Nanotech tools

Mike Georgalis

TMC / Ametek

Cryo-Transmission Electron Microscopes, Focused Ion Beam and Electron Microscopes, and other ultra-precision instruments installed in nanotech, materials engineering, and life science research facilities are required to achieve extremely high resolution and precise measurements. Today's instruments commonly can achieve sub-nanometer, and even sub-angstrom, resolution. Floor vibration often prevents such tools from meeting their design specifications.

To achieve floor vibration specifications published by the tool manufacturers, architects work with consultants to design extremely quiet buildings at great cost. Is this efficient and effective? Is there an alternative? Architects can design buildings to meet moderate floor vibration levels without too much difficulty. Designing buildings to meet the extremely low vibration levels required for the nanoscale and other advanced science and technology facilities, however, requires significantly more cost with diminishing returns. Even to the extent that the building floors are quiet, once the building is populated with people and machinery, vibration sources are introduced negating much of the benefit of the quiet building design.

Sources of vibration are discussed and different strategies for vibration mitigation are presented including passive vibration isolation, massive isolated plinths and point-of-use inertial active vibration control pedestals.

Exploring Experimental PDB Structures and Computed Structure Models from Artificial Intelligence/Machine Learning at RCSB Protein Data Bank (RCSB.org)

Sutapa Ghosh and RCSB PDB Team

RCSB Protein Data Bank, Rutgers University, Piscataway, NJ

In addition to >200,000 atomic-level, experimentally-determined, three-dimensional (3D) structures of proteins and nucleic acids archived in the Protein Data Bank (PDB), the RCSB.org research-focused web portal of the RCSB Protein Data Bank (RCSB PDB) provides open access to ~1 million Computed Structure Models (CSMs) generated using AlphaFold2 (from AlphaFold DB) and RoseTTAFold (from Model Archive). Both of these artificial intelligence/machine learning software tools built on decades of methodological research in de novo protein structure prediction and relied on open access to the immense number of sequences in genomic sequence databases and the PDB archive. For the avoidance of doubt, experimentally-determined PDB structures and CSMs delivered on by the RCSB PDB are clearly identified as to provenance and reliability.

RCSB.org users can query, organize, visualize, analyze, and compare experimental structures and CSMs side-by-side by utilizing powerful tools:

- Search: User queries can be applied to all PDB structures and CSMs; PDB structures only; and can exclude either PDB structures or CSMs from the search results. CSMs are not included in the search results by default.
- View and Organize Results: By default, search results are ordered using a query-based relevancy score. Results can be resorted using different criteria (e.g., listing experimental PDB structures first, per-residue confidence score (pLDDT)).
- Explore Similar Proteins: "Group" summary pages and search results simplify exploration of PDB structures with similar sequence identity/UniProt ID or were deposited as part of the same study.
- Explore Individual Structures: Structure Summary Pages offer details of experimental PDB structures and CSMs.
- Assess quality: Analogous to the validation slider for experimental structures, all CSMs report global and local confidence levels as pLDDT scores.
- Visualize in 3D: View experimental PDB structures and CSMs in Mol*. Use the standalone Mol* 3D Viewer to upload single or multiple data files, align structures, and run Structure Motif Search.
- Download: From Structure Summary Pages, download the ModelCIF data file hosted by the corresponding external archive.

RCSB PDB will continue to develop resources to support exploration of experimentally-determined PDB structures alongside CSMs, scaling operations to accommodate growth in the PDB archive and increased availability of predicted structures.

RCSB PDB Core Operations are funded by National Science Foundation (DBI-1832184), US Department of Energy (DE-SC0019749), and National Cancer Institute, National Institute of Allergy and Infectious Diseases, and National Institute of General Medical Sciences of the National Institutes of Health under grant R01GM133198.

Pervasive A-T and G-C Hoogsteen base pairs in duplex DNA bound to proteins

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Watson-Crick base pairs (bps) serve as the fundamental genetic elements and the building blocks of the DNA double helix. However, it has been discovered that A-T and G-C bps can also form alternative 'Hoogsteen' conformation, which contribute to the structural and functional complexity of DNA. Notably, recent evidence suggests that many Hoogsteen bps in crystal structures of DNA duplexes have been mismodeled as Watson-Crick bps. Identifying such mismodeled Hoogsteen bps presents a significant challenge as it requires manual examination of the electron density for thousands of DNA bps deposited in the Protein Data Base.

Here, we developed an automated approach for identifying mismodeled Hoogsteen bps. This approach is demonstrated by analyzing 1418 bps previously reported to have reduced C1'-C1' distance, a characteristic signature of mismodelled Hoogsteen bps. Through our analysis, we have discovered 31 A-T and 8 G-C mismodelled Hoogsteen bps associated with 15 different proteins, including nucleosome remodeling protein HRP3, MarR family proteins, polymerase I in translocation conformation, and mitochondrial transcription factor A. Additionally, we have identified several bps which were best modeled with fractional populations of Hoogsteen and Watson-Crick bps. These findings suggest broader roles for Hoogsteen bps protein-DNA binding specificity and in the biological function of DNA, expanding our understanding of DNA's functional complexity.

Protein - membrane Interactions in Huntingtin Aggregation and Transmission

Nora Jaber*, Yun-Kyung Lee*, Jennifer Jiang, Bruna Favetta, Benjamin Schuster, Wei Dai
** co-presenting*

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Many proteins associated with neurodegeneration are intrinsically disordered proteins (IDPs). There is currently little knowledge of the molecular factors that influence how organization of their microenvironment controls IDP function. Evidence shows IDP-membrane interactions modulate the formation of IDP inclusions, dynamics, and functions. However, the role that these interactions play in the context of neurodegeneration, specifically how it influences aggregation and transmission of IDPs, is poorly understood. The goal of this research is to characterize the functional roles of membranes in transmission of IDPs, using mutant huntingtin (mHTT) as a model protein. We take an interdisciplinary approach by combining correlative light and electron microscopy (CLEM), cryo-electron tomography (cryoET) and biochemical and biophysical assays to elucidate molecular mechanisms of huntingtin transmission and to understand how interactions with membranes affect mHTT aggregation and transmission. Deciphering the fundamental characteristics that influence neurodegeneration will allow for making more informed decisions for therapeutic approaches down the line.

A Multidisciplinary Approach Towards Visual Membrane Proteomics of the Human Pathogen Candida glabrata

Jennifer Jiang, Yun-Kyung Lee, Mikhail V. Keniya, Anusha Puri, Gigi Lin, Jeff Cheng, Xueying Zhan, Sang-Hyuk Lee, Min Xu, David S. Perlin, Wei Dai

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Fungal infections have been on the rise and increasing clinical use of antifungal agents has led to the emergence of antifungal drug resistance. Integral membrane proteins localized to the fungal plasma membrane have shown great potential for the development of more efficacious antifungal agents. The most recently approved antifungal drug class, echinocandins, inhibits β-(1,3)-glucan synthase, a resident plasma membrane protein complex that is responsible for synthesizing β-(1,3)-glucans, the most abundant and important structural polysaccharide of fungal cell wall. Other membrane proteins, including chitin synthase and the yeast proton pump H+-ATPase (Pma1), are currently being explored for development of novel antifungal therapeutics. While the structures of these membrane protein complexes have been characterized in recent years, information on the spatial arrangement of plasma membrane constituents and their interactions have been lacking. By integrating quantitative mass spectrometry, cryo-electron tomography (cryo-ET), and unsupervised deep-learning based techniques, we characterized the molecular landscape of the fungal plasma membrane proteome in situ. Interestingly, echinocandin treatment of plasma membranes showed that Pma1 undergoes spatial reorganization, suggesting that these antifungals may work by altering the lipid microenvironment of membrane proteins. Future work on building comprehensive molecular maps of these fungal-specific structures in context of their cellular environment will provide critical structural and spatial insights that will guide the development of next-generation antifungals.

Analyzing protein complexes at micromolar concentrations with mass photometry: The MassFluidix HC microfluidics system

Benjamin Jumper

Refeyn Inc.

To achieve a mechanistic understanding of biological processes, it is essential to investigate the biomolecular interactions responsible for their regulation and execution. Mass photometry, a versatile mass measurement technology that operates in solution without labels, is ideal for this application. It can be used to identify and characterize the biomolecules involved, and their oligomerization and complex formation. Like other techniques with single-molecule resolution, mass photometry is best suited to analyzing samples with a low concentration of biomolecules (~100 pM – 100 nM), but in some cases, it is informative to measure more concentrated samples. Here, we present a method that enables the mass photometry analysis of higher-concentration samples (up to the tens of µM).

The method combines mass photometry with a novel, rapid-dilution microfluidics system, MassFluidix. With this microfluidics system, users can maintain a sample at a desired higher concentration, and then dilute it to the nM range immediately prior to the mass photometry measurement. Due to the speed of the dilution (<20 ms), the data captures the state of the system at the higher concentration of interest. We apply the technique to measure interactions between an IgG antibody and the neonatal Fc receptor, concluding that it enables characterization of samples with up to µM concentrations.

EmrE Molecular Basis of Drug Recognition by NMR and MD Simulations

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New York University, New York, NY

The multidrug efflux pump EmrE requires anionic residues in the substrate binding pocket for coupling drug transport with the proton motive force. Here, we show how protonation of a single membrane embedded glutamate residue (Glu14) within the homodimer of EmrE modulates the structure and dynamics in an allosteric manner using NMR spectroscopy. The structure of EmrE in the Glu14 protonated state displays an occluded conformation that is inaccessible for drug binding by the presence of tyrosine and tryptophan residues in the binding pocket. Ionization of a single Glu14 residue in one monomer induces an equilibrium shift toward the open state by altering its side chain position and that of a nearby tryptophan residue. The structural change creates a population of open conformations that bind to drugs via a conformational selection mechanism, thereby increasing the binding affinity by about 2000-fold. The ubiquity of proton-coupled exchange in efflux pumps suggests a mechanism that may be shared in other secondary active multidrug efflux pumps.

Cryo-EM of Human Na+-Dicarboxylate Cotransporters

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Metabolic intermediates, including succinate, malate and citrate, are important precursors and regulators of energy metabolism and fatty acid synthesis. Members of the SLC13 gene family are Na+-coupled di-/tricarboxylate transporters that import these nutrients across the plasma membrane. The underlying molecular mechanism of transport is not completely understood. We used eukaryotic expression systems to produce the Na+-di/tricarboxylate cotransporters NaCT, and NaDC3. By optimizing the detergent in sample preparation and the ice thickness in the data collection process, we were able to obtain cryo-EM maps of these 120-130 kDa membrane proteins at a resolution of 2.1-2.6 Å. Structures determined in the presence of various substrates and inhibitors yielded conformations in both the inward-facing (Ci) and outward-facing (Co) states. Such information revealed the structural basis for substrate specificity and the inhibition mechanism. In addition, we obtained an asymmetric dimer conformation with one protomer in a Ci state and the other in a Co state, providing evidence that the two protomers work independently. Our research shows the combination of biochemistry and cryo-EM can yield high resolution structures of membrane transporters in various states of the reaction cycle.

Regulation of GPCR function by its disordered C-terminal domain

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Weill Cornell Medcine, New York, NY

G protein-coupled receptors (GPCRs) interact with a variety of intracellular molecules that can initiate signaling events, post-translationally modify (PTM), and desensitize the receptor. All GPCRs contain an intracellular, intrinsically-disordered C-terminal domain (CTD) that regulates the function of the receptor through interactions with binding partners and PTMs. Here we have investigated two inhibitory functions

of the CTD of metabotropic glutamate receptors (mGluRs); dimeric, neuronal GPCRs that is important for synaptic modulation and plasticity. First, we discovered a novel interaction between mGluR CTDs and the plasma membrane using a combination of in vitro, in silico, and in cell approaches. Second, we used a combination of binding experiments and cryo-EM to decipher the determinants of mGluR interactions with transducer proteins; especially beta-arrestins. Together, this work elucidates the biophysical underpinnings of distinct, underexplored roles of the disordered CTDs of GPCRs and highlights the complexity of functional regulation of proteins in cellular contexts.

VitroJet: Moving Sample Preparation into the New Era

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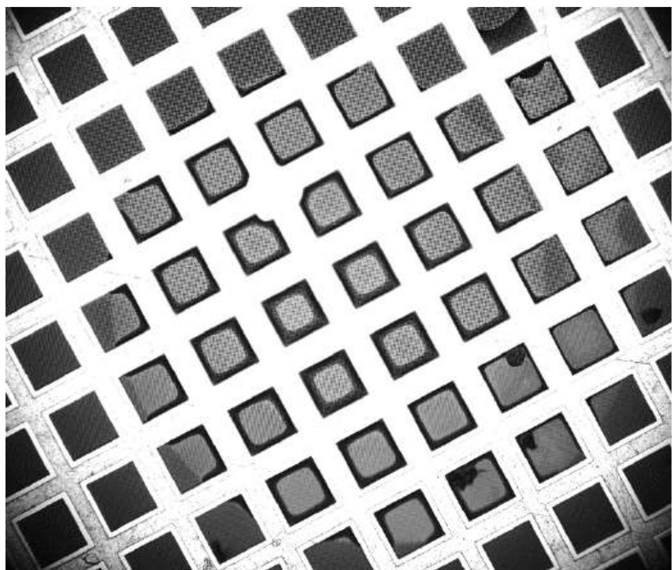
In structural biology, cryo-electron microscopy (cryo-EM) is increasing in popularity as a method for biomolecular structure determination.[1,2] Once a good quality grid is prepared for the microscope, data collection and biomolecular processing are streamlined. Within the cryo-EM workflow, the main bottleneck is sample preparation. Optimizing the combination of biochemistry and grid preparation needs many and long iterations. Making reproducible grids of which the quality can be determined before microscope usage can limit the number of iterations significantly. This makes the cryo-EM infrastructures more efficient, enabling structure determination in a shorter timeframe.

For this reason, the VitroJet was developed. The Vitrojet focuses on controlling the grid preparation process, enabling to investigate protein behavior in a structured manner.[3] An integrated plasma treatment makes grids reproducibly hydrophilic. Sample deposition through pin-printing decreases shear forces in comparison to the regular blotting method and reduces the required volume extensively. Furthermore, it allows for reproducible control of layer thickness, of which the resolution is highly dependent.[4] Visual feedback from two implemented cameras enables sample quality inspection and layer thickness estimation on a nanoscale before electron microscope screening. The visual feedback of the grid camera compares well with the atlas taken on the electron microscope, giving a clear indication on the number of usable holes before electron microscopy screening (Figure 1). Jet vitrification enables vitrification of autogrids, limiting manual handling and removing the need for the tedious post-clipping process. Since any grid type and only sub-nanoliter volumes are used, the Vitrojet can be used for all single particle analysis applications. In this manner, the electron microscopes can be fed with high quality samples to investigate protein behavior.

In collaboration with different labs over the world, we have exciting results to show. Currently, the focus lies on investigating how to assess and control thin ice layer production. By adapting the parameter settings of the VitroJet, ice thickness and gradients can be adjusted. Prediction of ice thicknesses with the implemented VitroJet camera saves microscope screening time tremendously. Ice thicknesses were determined by using the energy filter method in the electron microscope (Figure 2, ref [5]), and the outcome was correlated with VitroJet settings and camera feedback. A high number of different samples have been processed together with our collaborators and the results will be shown. Overall, we show the high control and reproducibility of the samples prepared on the VitroJet.



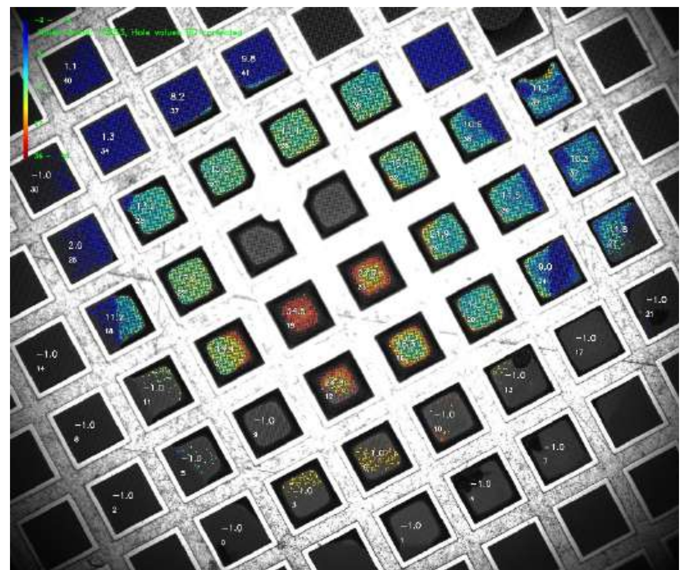
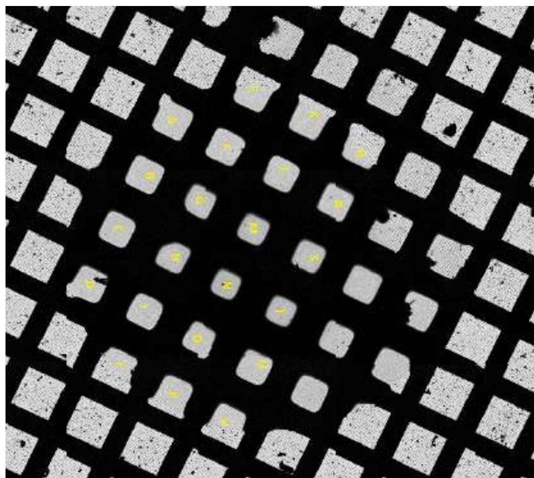


Figure 1a. *the feedback of the grid camera after deposition, where the lighter area indicates the deposition layer of the sample, in this case apoferritin. The image is rotated to correlate it to the atlas image and the used grid type is a quantifoil grid, 200 mesh, R2/1.*

Figure 1b. *color overlay of the grid camera feedback, where the color corresponds to the intensity variation. Red corresponds to thick layers, and dark blue corresponds to thin layers.*



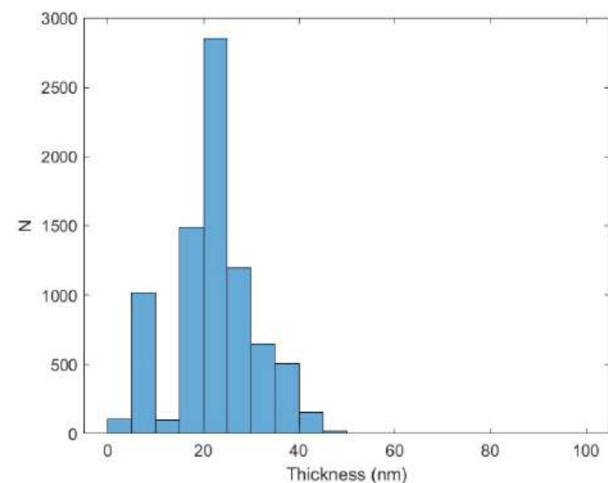


Figure 2a*. the atlas overview of the grid on which an apoferritin sample is deposited, taken in the electron microscope. The grid type is a quantifoil grid, 200 mesh, R2/1.* The cryo-EM micrograph is recorded by Saba Shazad in the Ernst-Ruska Centre / Forschungszentrum Jülich as part of the Vitrojet acceptance procedure.

Figure 2b. the corresponding histogram of the atlas, in which the relative thickness in each hole containing sample is determined by using the energy filter method.

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Using Cryo-EM to Interrogate How UvrA Identifies Damaged DNA

Erica Milia, Devon Semoy, Silas Hartley, David Jeruzalmi

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Nucleotide excision repair is a method of DNA repair that removes DNA damage, prototypically caused by UV rays. The first protein in this pathway is UvrA, which can identify a startlingly wide variety of DNA damages. The mechanism for how it does so remains unclear. By examining UvrA bound to different DNA damages using cryo-electron microscopy, we hope to elucidate the mechanism of DNA damage detection. So far, there are at least two conformers of the UvrA dimer bound to DNA. In one conformer, the DNA appears stretched, indicating that UvrA may apply torque to the DNA to check if it is damaged.

Integrating native MS analysis into cryo-EM workflows applied to the characterization of SARS-CoV-2 replication-transcription assemblies

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for the COVID-19 pandemic, has killed millions of people and has disrupted healthcare systems worldwide. Viral propagation and infection depend on the replication and transcription of the viral genome. In SARS-CoV-2, both processes utilize the RNA-dependent RNA polymerase (RdRp) enzyme to synthesize all viral RNAs within the host and has been a proven target for antiviral drugs. To faithfully replicate its unusually large genome (~30 kb) and efficiently generate mRNA transcripts, SARS-CoV-2 has evolved an arsenal of nonstructural proteins (nsps) that act in concert with RdRp, and thereby exhibit functionalities and interactions that are also prime targets for structure-based drug development.

In this work, we show how we integrated our native mass spectrometry (nMS) platform into a structural biology lab's workflow to facilitate optimized sample preparation and obtain relevant structural information for successful single-particle, cryo-EM analysis. We have been routinely using nMS to obtain rapid feedback on sample integrity, purity, and homogeneity as well as extract information on subunit stoichiometry, connectivity, ligand/drug binding and protein modifications.

We used our nMS-based diagnostic and screening platform to determine which SARS-CoV-2 nsps and RNA constructs co-assemble with RdRp in distinct functional states. The SARS-CoV-2 replication-transcription complex (RTC) is comprised of nsp12 that harbors the RdRp domain together with protein cofactors nsp7 and nsp8 bound to an RNA duplex. We determined high-resolution structures of the RTC associated with the essential nsp13 helicase that unwinds RNA duplexes in a nucleotide-dependent manner. Furthermore, we used nMS to assay various conditions in capturing the RTC in a pre-incorporation state and obtained structures of RTCs bound to each of the natural nucleotide substrates as well as with the antiviral nucleotide analogue remdesivir revealing insights into substrate recognition and remdesivir selectivity.

In addition, nsp12 contains an enigmatic Nidoviral RdRp-associated nucleotidyltransferase (NiRAN) domain that is essential for viral propagation and possesses three distinct activities associated with the N-terminal modification of nsp9 namely nucleotidylation, RNAylation and RNA capping. We employed nMS to assay each of these activities, specifically the covalent attachment of a 5'-triphosphorylated RNA (pppRNA) to the N-terminus of nsp9, forming a covalent RNA-nsp9 intermediate (RNAylation) and then transfer of the RNA from RNA-nsp9 to GDP (deRNAylation) to generate the core cap structure GpppN-

RNA. We also used nMS to determine if this cap is subsequently methylated by a guanine-N7-methyltransferase (nsp10/14) and a 2′-O-methyltransferase (nsp10/nsp16) to generate the final 5'-capped mRNA.

Exploring Trends in Cryo-EM Sample Preparation Databases for Optimized Freezing Conditions

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SPT Labtech

Sample preparation is a key determinant in the success of a cryo-EM study. However, sample preparation workflows remain largely unchanged since the 1980s [3][4], and despite the resolution revolution enabling protein structure determination at atomic detail by cryogenic electron microscopy (cryo-EM) [1], most samples require extensive optimization and approximately ten different methods in combination will be tried before improvement is seen [2].

Chameleon® is an automated vitrification device that uses a picolitre piezo electric dispenser coupled with nanowire self-wicking grids to achieve blot free and controlled thin film formation at millisecond time scales. Our poster will summarize our most recent work around expanding the Quantifoil Active® nanowire grid range including novel combined multi-hole geometries and highlighting new developments including graphene oxide modified grids and software to enable the exploration of trends in chameleon sample preparation databases.

Quantifoil® Active multi-hole grids are designed to include four different and commonly used hole diameters in a single grid square, arranged in a regular pattern. To assess the effect of hole size on particle distribution and orientation, data was collected across all hole sizes and the processing was segmented based on particle provenance. Reconstructions are shown for each hole size from a single data collection.

Modifying conventional grids with graphene oxide has been extensively used in cases where sample distribution or orientation and angular distribution are problematic. We have now established graphene oxide modification protocols that can be applied to nanowire Quantifoil® Active grids and present data that shows improved reproducible outcomes when incorporated with Chameleon®.

The chameleon allows sample information, dispense-to-plunge times, and glow discharge parameters as well as environment and chameleon specific parameters to be captured and stored in a database. Here, we present the Chamelogator software, which provides chameleon users with a tool to visualize and analyze this captured data. The information that can be collated includes system specific information, allowing the performance of a chameleon to be monitored over time. Sample based analysis might include how buffer components such as detergents affect parameters such as glow discharge strength or plunge parameters. Our study aims to showcase some of the analyses possible with chamelogator and how they can be utilized by users to optimize the sample preparation workflow.

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Exploring the Effects of Post Translational Modifications in Histone Tails Using Molecular Dynamics Simulations

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The nucleosome core particle (NCP) is the basic unit of chromatin consisting of 147 bp of DNA wrapped around the histone octamer. Each histone protein has intrinsically disordered tails that protrude from the histone core. On the histone tails, multiple epigenetic changes occur through Post-Translational Modifications (PTMs), such as histone acetylation. Epigenetic changes are ubiquitous in disease development, such as cancer. It has been shown H2B histone tails play an essential role in cancer associated with p14ARF. Our previous study has shown that H2B is related to DNA unwrapping in nucleosomes. H2B histone tails play a critical role in NCP stability and biological processes, yet not well understood, therefore, it is essential to understand the stability and dynamics of these tails that lead to epigenetic changes. Here, we demonstrate with all-atomistic MD simulations of the NCP that acetylation of the histone tails changes their conformational space and interaction with the DNA. We perform simulations of the H2B tails, a critical regulator in gene regulation, in both the lysine-acetylated (ACK) and unacetylated (WT) states. We use two different NaCl concentrations of 0.15M and 2.4M to perform simulations at microseconds timescale as salt can modulate the effects of electrostatics. The aim is to explore the effects of acetylation upon charge neutralization in nucleosome stability and plasticity through tail dynamics. The preliminary results have shown that the ACK H2B tails shift their secondary structure helical propensity of tails. The number of contacts of the DNA- tail decreases upon acetylation of lysine residues. The acetylation makes the tail more flexible and dynamic, characterized by principal component analysis (PCA). Mainly, H2B acetylation may make chromatin less compact, which can aid in gene regulation and NCP stability.

A high resolution cryoEM structure of VcDnaB/ssDNA reveals insights into helicase loading

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The DnaB helicase is perhaps the best-studied and ubiquitous bacterial helicase. The assembled complex is a homo-hexamer consisting of two tiers, containing a tiers of C-terminal domains (CTD) and N-terminal domains (NTD) connected by linking helices between the tiers. In order to carry out its function, DnaB is first either self-loaded or loaded by helicase loaders such as DnaC onto DNA. It is postulated that DnaB then translocates along DNA using a “hand-over-hand” mechanism. Despite numerous crystallographic and cryoEM studies characterizing the structure of DnaB in complex with various different helicase loaders, the precise mechanism of DnaB loading and its translocation along DNA remain murky. In this poster we report the high-resolution cryoEM structure of a novel conformation of Vibrio cholerae DnaB (VcDnaB; a putative self-loader) in complex with ATPgammaS and ssDNA. This loading intermediate exhibits a high degree of coplanarity between the individual subunits of the CTD and NTD tiers, giving it more resemblance with T4 bacteriophage gp41 helicase than with the more closely related EcDnaB from E. coli. Furthermore, we report that the structure is in a conformation unforeseen in a DnaB/ssDNA complex, the NTD tier is arranged in a dilated conformation and the CTD tier is arranged in a constricted conformation.

Role of Conformational Penalty in HIV-1 TAR-Tat Interaction

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Despite advances in understanding the structure of RNA-protein complexes, our knowledge of these interactions remains largely descriptive, not predictive. This hinders our ability to deduce binding affinities from RNA sequences. The formation of RNA-protein complexes demands changes in the conformation of the interacting molecules, such as the breaking or forging of new interactions within the molecules or between the biomolecules and their surroundings. Consequently, binding affinity is influenced not only by the vigor of the newly established contacts but also by the inherent tendencies of the molecules to adopt the conformational states competent for binding. The free energy associated with the binding can be broken down into two distinct energetic components: (a) the formation of favorable intermolecular contacts and (b) the energetic cost incurred in altering the RNA conformation from its stable ground state to a binding-competent higher-energy state, referred to as the ""conformational penalty.""

While the formation of intermolecular contacts is well characterized by the expanding database of structures of RNA-protein complexes, our knowledge regarding the conformational penalty remains incomplete and limited. Technical challenges have impeded our ability to quantitatively measure this particular energetic cost of binding. This is primarily because the high-energy binding-compatible states are often present in very low populations, rendering them beyond the detection limit of traditional methods. The lack of quantitative insight into this aspect poses a significant obstacle to a comprehensive understanding of the RNA-protein binding process that calls for a deeper, more quantifiable approach to harness its potential implications for science and medicine truly.

In this context, we outline specific experiments to measure the conformational penalty. These studies focus on determining the atomic resolution ensemble of HIV-1 TAR (Transactivation Response Element) variants in both their free and Tat (Trans-activator of transcription) arginine-rich motif (ARM) peptide-bound states. By integrating data from NMR (Nuclear Magnetic Resonance) with MD (Molecular Dynamics) simulations and FARFAR (Fragment Assembly of RNA with Full Atom Refinement) RNA empirical structure prediction, we aim to provide detailed insights into this critical aspect of RNA-protein interaction.

Kinetic resolution of the atomic 3D structures formed by ground and excited conformational states in an RNA dynamic ensemble

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Knowing the 3D structures formed by the various conformations populating the RNA free-energy landscape, their relative abundance, and kinetic interconversion rates is required to obtain a quantitative and predictive understanding of how RNAs fold and function at the atomic level. While methods integrating ensemble-averaged experimental data with computational modeling are helping define the most abundant conformations in RNA ensembles, elucidating the kinetic rates of interconversion between the different conformations remains challenging as is the determination of the 3D structures formed by sparsely-populated short-lived RNA excited conformational states (ESs). Here, we developed an approach integrating Rosetta FARFAR RNA structure prediction with NMR residual dipolar couplings and relaxation dispersion that simultaneously determines the 3D atomic structures formed by the ground-state (GS) and ES sub-ensembles, their relative abundance, and kinetic rate of interconversion. The approach is demonstrated on HIV-1 TAR RNA, which has a six-nucleotide apical loop

previously shown to form a sparely populated (~ 13%) short-lived (lifetime ~ 45 μs) ES. In the GS, the apical loop forms a broad distribution of open conformations, which interconvert on the pico-to-nanosecond timescale. Most residues are unpaired and pre-organized to bind the Tat-super-elongation protein complex. The apical loop zips up in the ES, forming a narrow distribution of closed conformations, which alter the shape and sequester critical residues required for protein recognition. Our work introduces a general approach for determining the 3D ensemble models formed by sparsely populated RNA conformational states, provides a rare atomic view of an RNA ES and how it differs from the GS, and kinetically resolves the atomic 3D structures of RNA conformational sub-states, which inter-change on timescales spanning six orders of magnitude in time, from picoseconds to microseconds.

PDB-Dev: A prototype system for archiving integrative structures

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Structures of many complex biological assemblies are increasingly determined using integrative approaches, in which data from multiple experimental methods are combined. Based on recommendations from the worldwide Protein Data Bank (wwPDB) integrative/hybrid methods task force, a standalone prototype system, called PDB-Dev, has been built for archiving integrative structures and making them publicly available. Data standards and software tools have been developed for collecting, curating, validating, visualizing, archiving, and disseminating integrative structures that span diverse spatiotemporal scales and conformational states. Mechanisms have been created to validate integrative structures based on the experimental data underpinning the structure. Building upon the foundational framework, PDB-Dev has been further expanded to handle large dynamic macromolecular ensembles and integrative structures that combine experimental restraints with initial structural models computed by deep learning algorithms. Data standards and supporting tools have been extended to capture information regarding conformational dynamics and related kinetic data derived from biophysical methods. Following the FAIR (Findable, Accessible, Interoperable and Reusable) principles, PDB-Dev ensures that the results of integrative structure determination are freely accessible to everyone.

Uncovering the Mechanisms of Novel PAS Domain Chemosensory Transcriptional Regulators

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One-component signal transduction comprises most of the cellular communication in prokaryotes. Within this interesting class of signaling proteins lies the Per-ARNT-Sim (PAS) domain, a superfamily of domains that sense and respond to environmental stimuli such as light, oxygen, metals, fatty acids, or temperature by binding ligands within their cavity. When found within helix-turn-helix (HTH) transcriptional activators, these PAS-HTH proteins can become powerful tools for control over gene expression. Our lab has previously engineered one of these proteins into a successful blue light- driven optogenetic system, leading us to wonder if we can similarly develop PAS-HTHs into chemosensory transcriptional regulators that can be activated via small molecule recognition. Using a combination of bioinformatic and computational methods, we have discovered PAS-HTHs that have the potential for unique cofactor binding abilities. By combining NMR spectroscopy, mass spectrometry, differential scanning fluorimetry, and X-ray crystallography we aim to better understand the structure-function relationship of two novel PAS-HTH bacterial transcription factors with a varying range of ligand preference and dynamics. Uncovering critical information regarding multimerization state will lend insights into requirements for activation, like we have seen with our blue-light driven system, EL222, activation triggers changes in intramolecular domain interactions allowing for DNA binding. This work will lead to a deeper understanding of one- component PAS domain signal transduction and allow for the development of tools to function as inducible gene expression systems and reporter assays through bioengineering.

Structural basis of allosteric modulation of metabotropic glutamate receptor activation and desensitization

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The metabotropic glutamate receptors (mGluRs) are neuromodulatory family C G protein coupled receptors, which assemble as dimers and allosterically couple extracellular ligand binding domains (LBDs) to transmembrane domains (TMDs) to drive intracellular signaling. Pharmacologically, mGluRs can be targeted either at the LBDs by glutamate and synthetic "orthosteric" compounds or at the TMDs by allosteric modulators. Despite the potential of allosteric TMD-targeting compounds as therapeutics, an understanding of the functional and structural basis of their effects on mGluRs is limited. Here we use a battery of approaches to dissect the distinct functional and structural effects of orthosteric versus allosteric ligands. We find using electrophysiological and live cell imaging assays that both agonists and positive allosteric modulators (PAMs) can drive activation and desensitization of mGluRs. The effects of PAMs are pleiotropic, including both the ability to boost the maximal response to orthosteric agonists and to serve independently as desensitization-biased agonists across mGluR subtypes. Conformational sensors reveal PAM-driven inter-subunit re-arrangements at both the LBD and TMD. Motivated by this, we determine cryo-electron microscopy structures of mGluR3 in the presence of either an agonist or antagonist alone or in combination with a PAM. These structures reveal PAM-driven re-shaping of intra- and inter-subunit conformations and provide evidence for a rolling TMD dimer interface activation pathway that controls G protein and beta-arrestin coupling.

Investigating Dynamics and Function of a Non-Canonical Sensor Histidine Kinase

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Bacteria sense and adapt to the environment using two component systems (TCSs), comprised of a sensor histidine kinase (SHK) and cognate response regulator (RR). Signal input to the SHK sensor domain, such as the blue light sensing light-oxygen-voltage (LOV), modulates catalytic domain autophosphorylation and transfer of the phosphoryl group to the RR. The canonical SHK model fails to describe the lesser-studied HWE/HisKA2 family, which can adopt non-dimeric architectures and are known to participate in the general stress response (GSR) networks of Alphaproteobacteria. Here, we focus on RT-HK, a novel HWE/HisKA2 family HK with a LOV sensor domain from a thermophilic Alphaproteobacteria. Signaling dynamics and function were assessed using a combination of size exclusion chromatography multi-angle light scattering (SEC-MALS), hydrogen deuterium exchange mass spectrometry (HDX-MS), nuclear magnetic resonance (NMR), limited proteolysis, and autophosphorylation/phosphotransfer profiling. Results suggest that RT-HK has typical signaling dynamics, yet it uses an inverted logic (the only known naturally occurring LOV-HK that does so). The protein is also in equilibrium between monomeric and dimeric states that can be shifted by nucleotide binding and light exposure. Finally, we show that RT-HK signals to a key regulator of the GSR. These results contribute to mounting evidence that HWE/HisKA2 family members are unique in their oligomeric states and functional activity, offering new perspectives on SHK function and dynamics.

Structural analysis and inhibition of human LINE-1 ORF2 protein reveals novel adaptations and functions

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The LINE-1 (L1) retrotransposon is an ancient genetic parasite that has written around one third of the human genome through a “copy-and-paste” mechanism catalyzed by its multifunctional enzyme ORF2p. ORF2p reverse transcriptase (RT) and endonuclease activities have been implicated in the pathophysiology of cancer, autoimmunity, and aging, making ORF2p a potential therapeutic target. However, a lack of structural and mechanistic knowledge hampers efforts to rationally exploit it. Here, we report structures of the human ORF2p ‘core’ (residues 238-1061, including the RT domain) by X-ray crystallography and cryo-EM in multiple conformational states. Our analyses reveal two novel folded domains, a dynamic closed ring conformation with extensive contacts to RNA template, and associated adaptations that contribute to unique aspects of the L1 lifecycle and insertion mechanism. We characterize ORF2p biochemical activities and inhibition by RT inhibitors and find that L1 RT is efficiently primed by short RNAs. While LINE-1 expression and activity have been associated with cytosolic double stranded nucleic acids, their origin is controversial. We demonstrate via imaging and biochemical

experiments that non-canonical cytosolic ORF2p RT activity can produce inflammatory RNA:DNA hybrids which activate innate immune signaling via cGAS/STING. We further compute integrative structural models of full-length ORF2p, allowing us to propose an updated L1 lifecycle model. Finally, we present an evolutionary analysis that both reveals ORF2p structural conservation and uncovers relationships of specific domains to other RNA- and DNA-dependent polymerases. These data provide key mechanistic insights into L1 polymerization and insertion, shed light on the evolutionary history of L1 elements, and enable rational L1 RT inhibitor development.

Leveraging AlphaFold, Phenix and Rosetta for Automatic Crystal Structure Determination and Enhanced Refinement at Ultralow Resolutions

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In the realm of macromolecular structural biology, AlphaFold has emerged as a groundbreaking tool for predicting protein structures from amino acid sequences. Despite its prowess, certain discrepancies between its predictions and experimental crystal structures have been observed. Addressing this, our research has seamlessly integrated AlphaFold with the mr_rosetta molecular replacement procedure in Phenix. This synergy has paved the way for a fully automated crystal structure determination process, utilizing only the protein sequence and reduced diffraction data. The method has shown promise in tackling challenges arising from significant differences between predicted and experimental structures, especially in scenarios of moderate resolution where conventional autobuild procedures might falter.

We also delve into the challenges of refining crystal structures at ultralow resolutions. Such structures often present complications due to high solvent content, weak lattice contacts, and other factors. To circumvent these challenges, we introduced the quasi-rigid-body AlphaFold/Rosetta-aided low-resolution structure (QARLS) refinement procedure. This innovative approach was applied to structures like the ryanodine receptor RyR1, achieving remarkable improvements in Rfree values and overall geometry. The QARLS procedure, validated across multiple structures, stands as a robust strategy for refining ultralow-resolution crystal structures, with potential extensions to cryo-EM and cryo-ET analyses.

Using the integrative structural biology approach to gain insights into the SARS-CoV-2 polyproteins

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SARS-CoV-2, a human coronavirus, is responsible for the COVID-19 pandemic. Like many RNA viruses, it employs a “polyprotein strategy” for gene translation, which not only allows for a compact genome but also regulates the activity of the virus through a precise spatial and temporal cleavage pattern. However, the study of polyproteins remains an understudied aspect of the viral life cycle. In SARS-CoV-2, the ~30 kb genome is translated into two large polyproteins, subsequently cleaved by viral papain-like protease and main protease (Mpro). In this study, we have provided structural insights into the Mpro-mediated processing of the nsp7-11 polyprotein, whose mature products include cofactors of the viral replicase, and identified the order of cleavages. Integrative structural modeling based on mass spectrometry (including hydrogen-deuterium exchange and crosslinking) and small angle X-ray scattering yielded a nsp7-11 structural ensemble for the first time. These integrative models support the notion that the interplay between conformation and accessibility of the cleavage junctions determines the preference

and order of cleavage of the polyproteins. Additionally, the processing results along with the integrative models suggest that nsp7-8 intermediate cleaves the last. Its functional role in the viral cycle is unknown, but disruption of the nsp7-8 junction site in coronaviral reverse genetics systems is lethal, suggesting a unique drug target site. Overall, the findings from this study have advanced the understanding of coronaviral polyprotein processing and revealed potential new targets for drug discovery.

Cryo-EM structure of Botulinum neurotoxin type C

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Botulinum neurotoxins (BoNTs) are produced by anaerobic bacteria of the Clostridium Botulinum and are responsible for the serious disease called botulism. BoNTs are a family of bacterial toxins with seven major serotypes (BoNT/A to BoNT/G). BoNTs are among the most widely used therapeutic proteins, however, only two subtypes BoNT/A1 and BoNT/B1 are currently used for medical and cosmetic applications. BoNT/C is associated with outbreaks of animal botulism, particularly in birds. BoNT/C has been shown to be a good alternative for current BoNT therapeutic proteins because it exerts a very long-lasting neuronal inhibitory effect as well as the absence of neuromuscular damage. Currently, the full-length structure of BoNT/C has not been reported. Here we solved the cryo-EM structure of BoNT/Ci (BoNT/C catalytically inactive version) and compared it with BoNT/C structures predicted by AlphaFold. Our results by cryo-EM show that BoNT/C adopts a more ‘closed butterfly’ organization compared with the ‘open butterfly’ BoNT/C structures predicted by AlphaFold. Our high-resolution cryo-EM structure of BoNT/C also reveals a bent translocation domain, which might play a role to enhance the binding toward the target neuronal cell membrane.

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