APS Workshop 11: APS-U-enabled Advanced Tools for Structural Biology: Advancing Enzymatic Catalysis and Drug Discovery through Synchrotron Serial Crystallography

Thursday, May 9, Morning

8:30 – 8:40 Workshop Organizers
Introduction

8:40 – 9:15 Alke Meents (Deutsches Elektronen-Synchrotron DESY)
High-throughput Pharmaceutical Screening with Serial Crystallography at Room Temperature

9:15 – 9:50 Simon Vecchioni (New York University)
Metal-mediated DNA Nanotechnology: A Structure Screening Platform Using Self-assembled Crystals

9:50 – 10:40 Break – Beamline Posters

10:40 – 11:15 John Rose (University of Georgia)
Using AlphaFold2 at SER-CAT for Structure-function Analysis

11:15 – 11:50 Zhong Ren (University of Illinois at Chicago)
Spin-coupled Electron Densities of Iron-sulfur Cluster Imaged by In-situ *Serial Laue Diffraction*

11:50 – 1:30 Lunch Break

Thursday, May 9, Afternoon

1:30 – 2:05 Vadim Cherezov (University of Southern California)
Serial Crystallography of Membrane Proteins

2:05 – 2:40 Kara Zeilinski (Cornell University)
A New Approach to Mix-and-inject Serial Synchrotron Crystallography Resolves the Function of DJ-1

2:40 – 3:10 Break – Beamline Posters

3:10 – 3:45 Marius Schmidt (University of Wisconsin-Milwaukee)
Ten Years of TR-S(F)X Versus the Onset of TR Cryo-EM

3:45 – 4:20 Meng Yuan (The Scripps Research Institute)
Structural and Mechanistic Insights into Disease-associated Endolysosomal Exonucleases PLD3 and PLD4

4:20 – 4:55 Rebecca Jernigan (Arizona State University)
The Dynamics of Drug Discovery: Exploring NendoU from SARS-CoV-2 through Serial Crystallography

4:55 – 5:00 Workshop Organizers
Final Remarks

5:00 Adjourn

This workshop is generously supported by the following:

High-throughput Pharmaceutical Screening with Serial Crystallography at Room Temperature

Alke Meents[1], Sebastian Guenther[1], and Pontus Fischer[1]

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Structure-based drug discovery is a very powerful technique for drug development. In contrast to biological assay-based screening techniques, in structure-based drug development potential binders are designed based on the 3-dimensional atomic structure of a target protein. Whereas it has recently become possible to reliably predict the 3-dimensional structure of proteins, prediction of binding interactions between a target protein target and smaller chemical compounds such as fragments and drugs still rely on experimental validation [1]. A certain limitation of most x-ray screening experiments conducted so far arises from the fact that they have been performed at cryogenic temperatures, whereas ligand binding in the living world takes place at physiological temperatures [2].

However, x-ray structure analyses of protein crystals at near physiological temperatures present a certain challenge. On the one hand, the resolution that can be achieved in such experiments is usually significantly lower and, on the other hand, the moisture-sensitive crystals tend drying out very quickly. Using serial crystallography, it should at least theoretically be possible to achieve a similar resolution at room temperature as with conventional rotational measurements at cryogenic temperatures. To test this hypothesis, we performed the same fragment screening experiments on the fosfomycin resistance protein FosAKP once at cryo-temperature and once with fixed-target serial crystallography at room temperature under otherwise identical conditions. The results of the different methods will be compared and a possible strategy for a combination of cryo- and room temperature measurements for pharmaceutical screening experiments will be presented. If the measurements planned for the beginning of April this year are successful, we will also present additional measurements from XFEL.

[1] S. Guenther et al, Science, 372, 642-646, (2021).
[2] T. Skaist Mehlman et al., *eLife* 12, e84632 (2023).

Metal-mediated DNA Nanotechnology: A Structure Screening Platform Using Self-assembled Crystals

Simon Vecchioni[1], Brandon Lu[1], William Livernois[2], Arpan De[2], Lara Perren[1], Yoel Ohayon[1], Karol Woloszyn[1], Chengde Mao[3], M.P. Anantram[2], James W. Canary[1], and Ruojie Sha[1]

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DNA as a vehicle for molecular programming relies on the predictability and information storage capability of the canonical Watson-Crick base pairs, A:T and G:C. Three-dimensional crystals formed by Watson-Crick DNA self-assembly have been known for 15 years, but have yielded poor diffraction results from guest molecules. Here we present a generalized method for the structure screening of metal-mediated DNA base pairs using self-assembled crystals. By introducing pyrimidine:pyrimidine mismatches into DNA tiles, we enable the specific coordination of metal ions (Ag^{+}, Au^{+}, Hg^{2+}, Cd^{2+}, Co^{2+}) into the double helix. These non-Watson-Crick interactions are amenable to structure determination, even at low resolution, using careful f'' calibration techniques. Primarily using APS beamline 17-ID, we have published over 100 structures of metal base pairs from a diverse pool of nucleotides; and we employ this comprehensive structural library to elucidate fundamental design rules for an expanded DNA metal coding system [1].

We further manipulate our self-assembled DNA crystals to capture a series of reversible, pH-driven chemical reactions via cryogen quenching [2]. Importantly, we identify titration points at which mmDNA pairs exist in a heterobimetallic state, where a single DNA base pair binds to both Ag^{+} and Hg^{2+} in tandem. Our self-assembly system was further able to capture the intermediates in pH-driven transmetallation, and modeling identifies the conductance modulation that arises from ionic reconfiguration. The precision self-assembly of bioinorganic DNA chemistry at the sub-nanometer scale will drive atomistic design frameworks for nanodevices and nanotechnologies based on semantomorphic architectures.

[1] Vecchioni, S., et al, "Metal-Mediated DNA Nanotechnology in 3D: Structural Library by Templated Diffraction." (2023) Advanced Materials, 35, 2210938.
[2] Lu, B., et al, "Heterobimetallic Base Pair Programming in Designer 3D DNA Crystals." (2023) JACS, 145 (32).

Using AlphaFold2 at SER-CAT for Structure-function Analysis

John P. Rose[1,2], Zheng-Qing (Albert) Fu[1,2], John Chrzas[1,2], and Bi-Cheng "B.C." Wang[1,2]

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Advances in structure prediction by Alphafold2 significantly improves model accuracy such that the most Alphafold2 models resemble the refined crystal structures and can be superimposed with sub 1Å RMSDs [1, 2]. The precision of the Alphafold2 model makes it a first-choice search model for molecular replacement phasing that significantly increases the efficiency of MX structure determination since phasing is not dependent on isomorphous heavy atom derivatives or anomalous scatterers (*e.g.*, selenomethionine) being present in the crystal.

SER-CAT has recently developed a client/server system consisting of AlphaFold2-multi and an AI-based protein interaction identification method [3] that could significantly speed up the process of identifying protein-protein binding partners *in silico* by prioritizing a long list of potential binding partners, extending the application of AlphaFold2 far beyond structure predictions.

The presentation will describe the SER-CAT AlphaFold2 client-server setup and the use of Alphafold2 to produce models of the protein-receptor complexes understudy which are then analyzed using an in-house developed comparator that ranks the complexes on strength of interaction and identifies those complexes that look promising and should further analyzed *in vitro* in the lab.

Work is supported by the SER-CAT Member Institutions (see www2.ser-cat.org), the University of Georgia Research Foundation, and the Georgia Research Alliance.

[1] Jumper *et al.*: Nature 596:583-589, July 15, 2021.
[2] Evans *et al.*: Protein complex prediction with AlphaFold-Multimer. bioRxiv 2021, https://doi.org/10.1101/2021.10.04.463034.
[3] Fu *et al.*: Int. J. Mol. Sci. 2022, *23*, 11685.

Spin-coupled Electron Densities of Iron-sulfur Cluster Imaged by *In-situ* Serial Laue Diffraction

Zhong Ren[1,3,5], Fan Zhang[4], Weijia Kang[1], Cong Wang[1], Heewhan Shin[1], Xiaoli Zeng[1], Semini Gunawardana[1], Kalinga Bowatte[1], Norbert Krauß[4], Tilman Lamparter[4], and Xiaojing Yang[1,2]

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Iron-sulfur clusters are inorganic cofactors found in many proteins involved in fundamental biological processes including DNA processing. The prokaryotic DNA repair enzyme PhrB, a member of the protein family of cryptochromes and photolyases, carries a four-iron-four-sulfur cluster ([4Fe4S]) in addition to the catalytic flavin and a second ribolumazine cofactors. Two interdependent photoreductions of the flavin and ribolumazine cofactors are mediated by the [4Fe4S] cluster functioning as an electron cache to hold a fine balance of electrons. We apply the more traditional temperature-scan cryo-trapping technique in protein crystallography and the newly developed technology of *in-situ* serial Laue diffraction at room temperature. These diffraction methods in dynamic crystallography enable us to capture strong signals of electron density changes in the [4Fe4S] cluster that depict quantized electronic movements. The mixed valence layers of the [4Fe4S] cluster due to spin coupling and their dynamic responses to light illumination are observed directly in our difference maps between its redox states. These direct observations of the quantum effects in a protein bound iron-sulfur cluster have thus opened a window into the mechanistic understanding of metal clusters in biological systems.

Serial Crystallography of Membrane Proteins

Vadim Cherezov[1]

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The advancements of X-ray Free Electron Lasers (XFELs) and the recent upgrades at synchrotron sources have opened up new opportunities in structural biology by mitigating radiation damage and enabling time-resolved studies with unprecedented temporal resolution. Serial crystallography and related approaches accelerated structural studies of radiation-damage-prone macromolecules and membrane proteins which have historically presented challenges with crystallization. This talk will summarize key technology advancements, focused on crystal preparation and delivery for serial crystallography, and provide a brief outlook on future developments in the field.

A New Approach to Mix-and-inject Serial Synchrotron Crystallography Resolves the Function of DJ-1

Zielinski, K.A.[1], Dolamore, C.[2], Dalton, K.M.[3], Hekstra, D.R.[3,4], Henning, R.[5], Srajer, V.[5], Wilson, M.A.[2], and Pollack, L.[1]

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Time-resolved crystallography is an evolving technique for both X-ray Free Electron Lasers (XFELs) and synchrotrons. Its goal is to capture snapshots of intermediate states of biological reactions to obtain direct structural evidence of reaction mechanisms. Both light-activated and chemically-triggered approaches have been successfully demonstrated, but recent advances in Mix-and-Inject Serial Crystallography (MISC) hold particular promise due to its ability to visualize a protein interacting with a small ligand over many time scales. This is achieved by utilizing a flow-focused diffusive mixer to rapidly mix small molecules into a centrally flowing solution of protein crystals to initiate the reaction. Here, we present a new microfluidic device that couples a flow-focused diffusive mixer to a Kapton observation region to perform MISC experiments at synchrotrons. After passing through the mixer, protein crystals undergoing a reaction continue to co-flow with a sheath into the x-ray interaction region to reliably achieve high quality diffraction patterns. By changing the flowrates and the position of the x-ray beam relative to the tip of the mixer, timepoints ranging from ~50 ms – 30 s are reachable. This flow cell was first used at BioCARS at the APS, and datasets were collected in about 1-4 hours, due in part to enhanced information from the polychromatic beam relative to a conventional monochromatic still diffraction pattern. We used this new technology to study DJ-1, an important protein in oxidative stress response. Interestingly, DJ-1's enzymatic function has been heavily disputed, but we directly observed DJ-1 acting on its substrate, methylglyoxal, confirming its role as a glyoxalase rather than a deglycase. Additionally, a few of the intermediate states of the reaction are clear, giving insight into how this important reaction progresses.

Ten Years of TR-S(F)X Versus the Onset of TR Cryo-EM

Marius Schmidt[1], Tek Malla[1], and Emina A. Stojkovic[2]

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Time-resolved crystallography has been rejuvenated once Free Electron Laser X-ray sources (XFELs) became available. Substantial technical improvements enabled the first successful, high-resolution experiments with time-resolved serial femtosecond crystallography (TR-SFX). In the following years, synchrotron light sources also feature serial crystallography (SSX) capabilities with sub-millisecond time-resolution. One example is ID-29 at the European Synchrotron Radiation Facility (ESRF), which was recently upgraded with a multi-bend achromat lattice to an 'extremely brilliant source.' On the other hand, cryo-EM experienced a 'resolution revolution' and allows now the determination of macromolecular structures with near atomic resolution. We will compare results from traditional crystallography, SFX, and SSX with results from recent single particle cryo-EM investigations on the same biological molecule, either crystalline at ambient temperatures or at cryogenic temperatures in solution, respectively. A subjective outlook is drawn what to expect and what not to expect from either method.

We thank APS Sector 19 SBC for beamtime. We thank Shibom Basu and Daniele de Sanctis as well as the beamline scientists at ESRF ID-29 for experimental support. We also thank the New York Structural Biology Center for collecting the cryo-EM data, and the colleagues at SACLA and LCLS for the opportunity to conduct experiments there.

Structural and Mechanistic Insights into Disease-associated Endolysosomal Exonucleases PLD3 and PLD4

Meng Yuan[1], Linghang Peng[2], Deli Huang[2], Amanda Gavin[2], Fangkun Luan[2], Jenny Tran[2], Ziqi Feng[1], Xueyong Zhu[1], Jeanne Matteson[1], Ian A. Wilson[1,3], and David Nemazee[2]

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Phospholipases D3 and D4 (PLD3 and PLD4), located in endolysosomal compartments, exhibit exonuclease activity, digesting single-stranded nucleic acids in a 5′-to-3′ manner. PLD3 and PLD4 are associated with autoinflammatory and autoimmune diseases. We determined the crystal structures of PLD3 and PLD4 in apo, intermediate, and product states. Both enzymes form a dimer of pseudo-dimers, where the intra-chain dimer topology forms a basic active site at the interface. The structures reveal a two-step 'link-and-release' catalytic mechanism involving a covalent histidine-linked intermediate. We also demonstrate phosphatase activity of PLD3 and PLD4 against 5′-phosphorylated oligonucleotides, where the substrate phosphorylates a histidine at the enzyme's active site, and the resulting phosphohistidine hydrolyzes extremely slowly. Therefore, 5′-phosphorylated oligonucleotides act as blockers of catalysis of the exonucleases. Compared to PLD3, PLD4 contains an extra hydrophobic clamp that stabilizes the substrate and could affect oligonucleotide substrate preference and product release. Additionally, the disease-associated mutants reduce enzyme activity and thermostability, while the structures provide insights into disease association. Overall, our biochemical and structural analyses deepen our understanding of PLD catalytic mechanism and provide insights into therapeutic design.

The Dynamics of Drug Discovery: Exploring NendoU from SARS-CoV-2 through Serial Crystallography

Rebecca Jernigan[1], Dhenugen Logeswaran[1], Nirupa Nagaratnam[1], Diandra Doplar[1], Mokul Sonker[1], Manashi Sonowal[1], Nirmali Siriwardhana[1], Gihan Ketawala[1], Megan Shelby[2], Mimi Cho Young[2] , Matthew Coleman[2], Julius Chen[1], Matthew Coleman[2] , Emily Kaschner[1] , Sebastien Boutet[3] , Mark Hunter[3], Meng Liang[3], Ray Sierra[3], Valerio Mariani[3], Thomas Grant[4], Shibom Basu[5], Daniele de Sanctis[5], Julien Orlans[5], Jay-How Yang[1], Raimund Fromme[1], Michelle Zacks[1], Matthias Frank[2], Jose Martin Garcia[1,6], Marius Schmidt[7], Alexandra Ros[1], Debra Hansen[1], Sabine Botha[1], and Petra Fromme[1]

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The uridine-specific endoribonuclease, NendoU, from SARS-CoV-2, enables viral evasion of the innate immune system by degrading pathogen associated molecular patterns (PAMPs) unique to RNA virus infections. Understanding the structural dynamics, biological mechanisms, and binding of inhibitory drugs is of importance to NendoU's role in coronavirus infections and the subsequent development of therapeutics. Using serial femtosecond crystallography (SFX) at an X-ray Free Electron Laser (XFEL), the 2.6 Å structure of NendoU provided key insights into its flexibility, dynamics, and intrinsic properties. Functional studies of NendoU support its cleavage specificity in solution and in its crystalline form. Through the use of serial crystallography at synchrotron light sources, conditions have been optimized for new microcrystallization and ligand binding conditions of NendoU and continue to help optimize for mix-and-inject time-resolved experiments. These experiments provide room-temperature data using microcrystals that can provide a more comprehensive understanding of the variations in NendoU mutants, new ligands, and new crystallization conditions while also preparing for more specific time-resolved studies. NendoU is a unique protein among the *Nidovirales* order, and insight into the biological mechanism could provide additional benefit in therapeutic options in treatment not just in health care, but veterinary medicine as well.