Hot Topics in Contemporary Crystallography 6

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Advanced macromolecular crystallography workshop

Programme book

Zagreb, 2024.

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Programme at a glance

Time slots	7/4/2024	HT1 8/4/2024	HT2 9/4/2024	HT3 10/4/2024	HT4 11/4/2024
8:30–9:30		**L1: Basics** T. Bergfors	**L1: Data Collection on Laboratory Diffractometers** K. Molčanov	**L1: Structure Solution** E. Krissinel	**L2: Introduction to PDB** G. Evans
9:30–9:40		LSB	LSB	LSB	LSB
9:40–10:40		**L2: Techniques** A. Abuhammad	**L2: Data Collection at the Synchrotron** A. Héroux	**T1: Molecular Replacement** E. Krissinel & L. Catapano	**T1: PDBe & PDBe-KB** G. Evans
10:40–11:00		Coffee break	Coffee break	Coffee break	Coffee break
11:00–11:45		**L3: Sample Preparation** P. Shaw Stewart	**L3: Serial Crystallography at XFELs** H. Chapman	**T2: Phasing** E. Krissinel & L. Catapano	**T2: Programmatic Access for PDBe, PDBe-KB & more** G. Evans & P. Magaña
11:45–11:55		LSB	LSB	LSB	LSB
11:55–12:40		**L4: LCP Crystallization** M. Caffrey	**L4: Liquid Jet Developments** S. Bajt	**L2: Validation & Deposition** E. Krissinel	**L3 & T3: AlphaFold** P. Magaña
12:40–14:00		Lunch break	Lunch break	Lunch break	Lunch break
14:00–15:00		**D1: LCP Crystallization** M. Caffrey	**L5: Data Processing for Serial Crystallography** D. Oberthür	**T3: Model Building** L. Catapano	**L3 & T3: AlphaFold** P. Magaña
15:00–15:10		LSB	LSB	LSB	LSB
15:10–16:10		**L5: Cryo-cooling** E. Garman	**T1: SSX Experiment** D. Oberthür	**L3: Refinement** R. Nicholls	**T4: CSD-Crossminer** CCDC team
16:10–16:20		LSB	LSB	LSB	LSB
16:20–17:00		**L6: RadDam** E. Garman	Poster session	**T4: Refinement** R. Nicholls	**T5: CSD + PDB** CCDC team

Time slots	7/4/2024	HT1 8/4/2024	HT2 9/4/2024	HT3 10/4/2024	HT4 11/4/2024
17:00–17:20	**Registration**	Coffee break	Coffee break	Coffee break	Coffee break
17:20–18:20		**T1: Cryo-cooling** E. Garman	**L6: Home-lab Data Collection and Processing Using PROTEUM5** A. Luebben	**T4: Refinement** R. Nicholls	**T6: GOLD**
18:20–18:30				LSB	
18:30–19:15	Opening ceremony			**HT4 L1: Discovery INTRO** CCDC team	Closing ceremony
19:15–21:00	Dinner	Dinner	Dinner	Dinner	Dinner
21:00–23:00	Get together around posters & **A1: Bring your own sample**	**A1: Bring your own sample**	**DTP**	Farewell drink around posters	

* **A** stand for activity, **D** for demonstration, **DTP** for “Discussion Triggering Presentation”, **L** for a lecture, **LSB** for “leg stretching break”, **S** for the session, and **T** for a tutorial.

Sunday, 7th April 2024

17:00 – 18:30	**Registration**
18:30 – 19:15	**Opening ceremony**
19:15 – 21:00	**Dinner**
21:00 – 23:00	**Get together around posters** & **A1: Bring your own sample**

Monday, 8th April 2024
Hot Topic 1: Crystallization

	Morning session	*chair: Aleksandar Višnjevac*
8:30 – 9:30	**Terese Bergfors**: Back to Basics: How Phase Diagrams Can Help You	
9:30 – 9:40	**Leg stretching break**	
9:40 – 10:40	**Areej Abuhammad**: Mastering Protein Crystallization: Proven Tips and Tricks for Success	
10:40 - 11:00	**Coffee break**	
11:00 – 11:45	**Patrick D. Shaw Stewart**: Sample Preparation for Routine Structural Biology	
11:45 – 11.55	**Leg stretching break**	
11:55 – 12:40	**Martin Caffrey**: Crystallizing Membrane Proteins in Lipidic Systems (*lecture*)	
12:40 – 14:00	**Lunch break**	

	Afternoon session	*chair: Zoran Štefanić*
14:00 – 15:00	**Martin Caffrey**: Crystallizing Membrane Proteins in Lipidic Systems (*demonstration*)	
15:00 – 15:10	**Leg stretching break**	
15:10 – 16:10	**Elspeth Garman**: Cryo-Cooling: Why and How?	
16:10 – 16:20	**Leg stretching break**	
16:20 – 17:00	**Elspeth Garman**: Radiation Damage in Macromolecular Crystallography	
17:00 – 17:20	**Coffee break**	
17:20 – 19:15	**Elspeth Garman**: Cryo-Practical session	
19:15 – 21:00	**Dinner**	
21:00 – 23:00	**Patrick D. Shaw Stewart**: *A1: Bring your own sample*: Sample Preparation - Introduction + Demo	

Tuesday, 9th April 2024
Hot Topic 2: Data collection

	Morning session	*chair: Alen Bjelopetrović*
8:30 – 9:30	**Krešimir Molčanov**: Data Collection on Laboratory Diffractometers: Basics, Strategies, Data Reduction	
9:30 – 9:40	**Leg stretching break**	
9:40 – 10:40	**Annie Héroux**: Data Collection at the Synchrotron	
10:40 – 11:00	**Coffee break**	
11:00 – 11:45	**Henry Chapman:** Advancements and Future Directions in Serial Crystallography at XFELs	
11:45 – 11.55	**Leg stretching break**	
11:55 – 12:40	**Saša Bajt**: Liquid Jet Developments for Efficient Sample Delivery at XFELs	
12:40 – 14:00	**Lunch break**	

	Afternoon session	*chair: Elspeth Garman*
14:00 – 15:00	**Dominik Oberthür**: Data Processing for Serial Crystallography, Automation, and Further Outlook	
15:00 – 15:10	**Leg stretching break**	
15:10 – 16:10	**Dominik Oberthür**: How to Carry Out an Experiment	
16:10 – 16:20	**Leg stretching break**	
16:20 – 17:00	**Poster session**	
17:00 – 17:20	**Coffee break**	
17:20 – 18:20	**Anna Luebben**: Data Collection on Laboratory Diffractometers II: Home-lab data collection and processing using PROTEUM5	
19:15 – 21:00	**Dinner**	
21:00 – 23:00	**Discussion Triggering Presentations**	

Wednesday, 10th April 2024
Hot Topic 3: Processing & Structure determination

	Morning session	*chair: Ana Šantić*
8:30 – 9:30	**Eugene Krissinel**: Structure Solution in CCP4 Cloud	
9:30 – 9:40	**Leg stretching break**	
9:40 – 10:40	**Eugene Krissinel** & **Lucrezia Catapano**: Automatic and Fundamental Molecular Replacement	
10:40 – 11:00	**Coffee break**	
11:00 – 11:45	**Eugene Krissinel** & **Lucrezia Catapano**: Automatic and Fundamental Experimental Phasing	
11:45 – 11.55	**Leg stretching break**	
11:55 – 12:40	**Eugene Krissinel**: Model Validation and Deposition	
12:40 – 14:00	**Lunch break**	

	Afternoon session	*chair: Jasminka Popović*
14:00 – 15:00	**Lucrezia Catapano**: Automatic Model Building, Refinement and Ligand Fitting	
15:00 – 15:10	**Leg stretching break**	
15:10 – 16:10	**Rob Nicholls**: Macromolecular Refinement in CCP4	
16:10 – 16:20	**Leg stretching break**	
16:20 – 17:00	**Rob Nicholls**: CCP4 Cloud Tutorial – Refinement with REFMAC5, Ligand Dictionary Generation with ACEDRG	
17:00 – 17:20	**Coffee break**	
17:20 – 18:20	**Rob Nicholls**: CCP4 Cloud Tutorial - Interactive Model Building, Ligand Fitting and Refinement with COOT	
18:20 – 18:30	**Leg stretching break**	
18:30 – 19:15	**HT4 CCDC team**: Introduction to the CSD, CSD-Core and CSD-Discovery	
19:15 – 21:00	**Dinner**	
21:00 – 23:00	**Farewell drink around posters**	

Thursday, 11th April 2024
Hot Topic 4: Databases

	Morning session	*chair: Terese Bergfors*
8:30 – 9:30	**Genevieve Evans**: Introduction to PDB	
9:30 – 9:40	**Leg stretching break**	
9:40 – 10:40	**Genevieve Evans**: Access, Visualization & Evaluation at PDBe & PDBe-KB	
10:40 – 11:00	**Coffee break**	
11:00 – 11:45	**Genevieve Evans** & **Paulyna Magaña**: Harnessing PDBe-KB	
11:45 – 11.55	**Leg stretching break**	
11:55 – 12:40	**Paulyna Magaña**: AlphaFold Database: A Powerful Tool for Accelerating Scientific Research	
12:40 – 14:00	**Lunch break**	

	Afternoon session	*chair: Areej Abuhammad*
14:00 – 15:00	**Paulyna Magaña**: AlphaFold Database: A Powerful Tool for Accelerating Scientific Research	
15:00 – 15:10	**Leg stretching break**	
15:10 – 16:10	**CCDC team**: Mining Crystallographic Databases Using CSD-Crossminer	
16:10 – 16:20	**Leg stretching break**	
16:20 – 17:00	**CCDC team**: Exploring Interactions in the CSD and PDB Using ISOSTAR and SUPERSTAR	
17:00 – 17:20	**Coffee break**	
17:20 – 18:20	**CCDC team**: How to Perform Ensemble Docking with GOLD?	
18:30 – 19:15	**Closing Ceremony**	
19:15 – 21:00	**Dinner**	

Tuesday, 9th April 2024
Discussion Triggering Presentations

21:00 – 21:10	**DTP1**	**B. Tosun**, İ. Yapici, S. Wakatsuki, H. DeMirci: *Unlocking Efficiency: Structural Exploration of Burkholderia ambifaria's CO_2- Fixing Enzymes*
21:10 – 21:20	**DTP2**	**H. Çetinok**, H. DeMirci: *Human Serum Albumin's Unutilised Potential as a Cancer Targeting Agent*
21:20 – 21:30	**DTP3**	**G. Karakadıoğlu**, H. DeMirci: *Development of New Generation of Imatinib Using Structural Biology Techniques at Ambient Temperature*
21:30 – 21:40	**DTP4**	**A. Polák**, K. Meskova, K. Tomkova, O. Cehlar, S. Njemoga, E. Kontsekova, R. Skrabana: *Co-crystallization of tau proteins mediated by monoclonal antibodies*
21:40 – 21:50	**DTP5**	**B. Gomaz**, A. Pandini, A. Maršavelski, Z. Štefanić: *Computational analysis of purine nucleoside phosphorylase dynamics*
21:50 – 22:00	**DTP6**	M. Z. Mišković, M. Wojtys, A. Bzowska, Z. Štefanić, **I. Leščić Ašler**: *Location is everything: Influence of His-tag fusion site on properties of adenylosuccinate synthetase from Helicobacter pylori*
22:00 – 22:10	**DTP7**	**L. Goedl**, L. Riegler-Berket, M. Oberer: *Structure-based search for inhibitors of monoacylglycerol lipase of Mycobacterium tuberculosis*
22:10 – 22:20	**DTP8**	**L. Costello Heaven**, J. Schönfeld, C. Monteiro, J. Whitchurch, N. Mongan, H. Collins, M. Mazzorana, R. Flaig, D. Heery: *Characterising chromatin-binding domains in KAT6A histone acetyltransferase*
22:20 – 22:30	**DTP9**	**Z. Štefanić**, A. Pandini, A. Maršavelski, B. Gomaz: *Protein allostery - a programmatic approach*

Posters

P01 **M. Bhardwaj**, A. Kumar Patel:
Deciphering SARS-CoV-2 3CL Protease: Exploring Structural, Functional Aspects, and Therapeutic Perspectives

P02 **Ö. Güven**, H. Demirci:
The Unique Function and the Structural Studies of FK506 Binding Protein 1A

P03 **B. Tosun**, İ. Yapici, S. Wakatsuki, H. DeMirci:
Unlocking Efficiency: Structural Exploration of Burkholderia ambifaria's CO_2- Fixing Enzymes

P04 **D. Biçer**, S. Skou Thirup, J. Preben Morth, D. Otzen:
Fragment Based Active Site Exploration of Thermoset Degrading Enzymes

P05 **H. Çetinok**, H. DeMirci:
Human Serum Albumin's Unutilised Potential as a Cancer Targeting Agent

P06 **S. Njemoga**, P. Kadeřávek, E. Barrera, O. Cehlár:
Modeling of AD-related conformation of monomeric tau protein

P07 **N. Todorovic**, C. Grininger, T. Pavkov-Keller, W. Keller:
Structural characterization of the domestic cat allergen Fel d 4

P08 **C. P. Wren**, R. J. Flood, N. M. Mockler, M. Savko, Q. Shi, P. B. Crowley:
Symmetry Matched Protein – Macrocycle Assembly

P09 **S. Müller**, L. Rivera, M. Uhart, E. Barrera, D. M. Bustos, V. Pevala, R. Skrabana:
Exploring β-Carbolines as Allosteric Modulators of 14-3-3 Proteins: A Comprehensive Approach from Virtual Screening to Crystallography

P10 **N. Pal**, B. Daniel, K. Gruber, S. Schmidt, D. Tischler:
Structure-function relationships of N-N bond forming enzymes

P11 **K. Pokrywka**, M. Grzechowiak, P. Worsztynowicz, J. Śliwiak, J. Loch, M. Gilski, M. Jaskolski:
Exploring the role of active site residues of Rhizobium etli L-asparaginase in catalysis

P12 **L. Goedl**, L. Riegler-Berket, M. Oberer:
Structure-based search for inhibitors of monoacylglycerol lipase of Mycobacterium tuberculosis

P13 **T. Paradžik**:
Unveiling Hub Protein Properties in Bacteria

P14 **P. Stanić**, K. Molčanov:
In Depth Crystallographic Study of Pancake Bond Between Radical Cations in Crystals – Novel $TMPD^+$ Chloride Salt Co-crystal with 2,5-Dichlorohydroquinone

P15 **M. Graczyk-Kokosza**, M. Gilski:
Influence of N7G modification on RNA structure

Posters

Sponsor

P16 **F. Szubert,** A. Bebel, E. Biterova, D. Dawidowski, M. Kumar, B. Mrugala, M. Siuda, N. Stach, B. Zieba, K. Zrubek, C. Cukier, B. Lupa:
Development of Structural Analysis Services of Protein-ligand Complexes in 'fast track' Mode

Sponsor

P17 A. Bebel, F. Szubert, K. Zrubek, B. Mrugala, M. Siuda, E. Biterova, D. Dawidowski, M. Kumar, N. Stach, **B. Zieba**, C. Cukier, B. Lupa:
Development of novel X-ray crystallography services at Selvita

Prof. Terese Bergfors

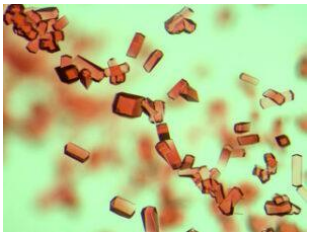
Crystallization Platform, Dept. of Cell and Molecular Biology
Uppsala University
Sweden

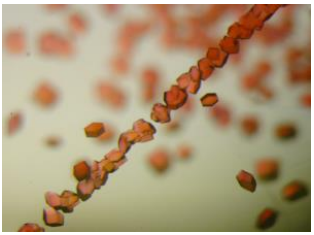
HT1 L1: Back to basics: How phase diagrams can help you

X-ray crystallography accounts for most of the structures in the Protein Data Bank. But to crystallize a particular protein may require screening hundreds of conditions. Although crystals might appear in the initial screens, usually further optimization is needed. How does one recognize which results from these kits are worth optimizing?

Understanding the phase diagram can help answer that question. Phase diagrams are maps of the events (phase transitions) that occur in the crystallization setups. The first part of this talk will give an overview of the steps in a crystallographic project and then look at:

- two critical events in the phase diagram: supersaturation and nucleation
- how precipitants drive the protein into a state of supersaturation





In the second part, the lecture will look at the following questions, from the perspective of an academic laboratory. Examples of what worked and what did not will be presented for our protein targets from *Mycobacterium tuberculosis.*

- What is the single most important parameter in screening?
- How many conditions should the initial screen contain: 15, 150 or 15,000?
- Are all crystallization kits equally successful?
- Which is more critical: the choice of precipitant or the kinetic pathway?

Suggested readings:

- Bergfors, T. (editor) Protein Crystallization, Third Edition 2022. International University Line, La Jolla, 670 pages.
- McPherson, A. and Gavira, JA. Introduction to protein crystallization. 2014. Acta Cryst F, vol. 70, 2-20.
- Asherie, N. Protein crystallization and phase diagrams. 2004. Methods vol. 34, 266-272.

Prof. Areej Abuhammad

School of Pharmacy,
University of Jordan, Amman,
Jordan

HT1 L2: Mastering Protein Crystallization: Proven Tips and Tricks for Success

Navigating the complexities of protein crystallization can be daunting, given its historically empirical nature. Although the traditional methods often involve selecting crystallization agents through trial-and-error, the results can vary drastically. From non-diffracting large crystals to the often-disheartening absence of crystallization, the challenges are manifold. Various factors, such as pH, ionic strength, temperature, and the choice of additives, play pivotal roles in influencing the outcomes.

Additionally, the significance of the protein construct, often overlooked, is paramount. Drawing from extensive experience in the field, this presentation will shift the spotlight onto actionable tips and tricks. These practical insights will guide attendees in harnessing the multitude of factors impacting protein crystallization, empowering them to achieve more predictable and successful outcomes.

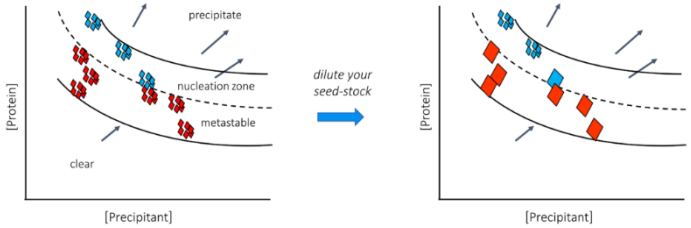
Dr. Patrick D. Shaw Stewart

Douglas Instruments Ltd,
Douglas House, East Garston, Berkshire, RG17 7HD, UK

HT1 L3: Sample Preparation for Routine Structural Biology

Serial data collection usually requires relatively small crystals that are well-ordered. Microseeding is an effective way to generate such samples. During the ten years since the random microseed matrix-screening (rMMS) method was published, understanding of the theoretical advantages of the method has increased [2 – 4], and several practical variations of the method have emerged. Moreover seeding can be carried out in a microbatch-under-oil setup, which is easy to scale up, volume-wise, and allows easy interpretation of phase diagrams. By combining these techniques, control can be increased and sample quality for both routine and advanced data collection improved.

Protein structure determination by cryoEM requires expensive equipment that has low throughput. It is therefore wasteful to examine samples that can be shown in advance to be aggregated, since such samples are unlikely to be suitable. We used a high-throughput screening approach with dynamic light scattering to explore 96 chemical conditions with as little as 10 µL of protein solution to identify conditions with reduced aggregation.



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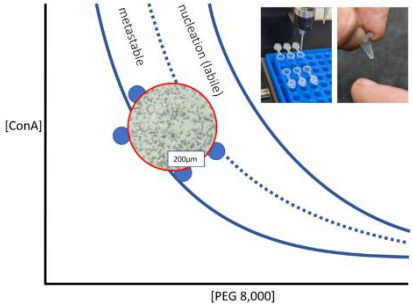
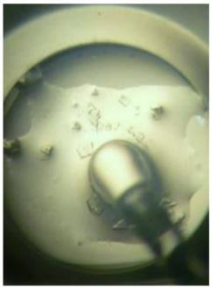
- D'Arcy, Allan, Frederic Villard, and May Marsh. "An automated microseed matrix-screening method for protein crystallization." Acta Crystallographica Section D: Biological Crystallography 63.4 (2007): 550-554.
- Shaw Stewart, Patrick D., et al. "Random microseeding: a theoretical and practical exploration of seed stability and seeding techniques for successful protein crystallization." Crystal Growth & Design 11.8 (2011): 3432-3441.
- D'Arcy, A., Bergfors, T., Cowan-Jacob, S. W., & Marsh, M. (2014). Microseed matrix screening for optimization in protein crystallization: what have we learned? Acta Crystallographica Section F: Structural Biology Communications, 70(9), 1117-1126.
- Shaw Stewart, P., & Mueller-Dieckmann, J. (2014). Automation in biological crystallization. Acta Crystallographica Section F: Structural Biology Communications, 70(6), 686-696.
- Obmolova, G., Malia, T. J., Teplyakov, A., Sweet, R. W., & Gilliland, G. L. (2014). Protein crystallization with microseed matrix screening: application to human germline antibody Fabs. Structural Biology and Crystallization Communications, 70(8).
- Abuhammad, Areej, et al. "Structure of arylamine N-acetyltransferase from Mycobacterium tuberculosis determined by cross-seeding with the homologous protein from M. marinum: triumph over adversity." Acta Crystallographica Section D: Biological Crystallography 69.8 (2013): 1433-1446.
- Kolek, S. A., Bräuning, B., & Shaw Stewart, P. D. (2016). A novel microseeding method for the crystallization of membrane proteins in lipidic cubic phase. Acta Crystallographica Section F: Structural Biology Communications, 72(4), 307-312.
- Falke, S., Dierks, K., Blanchet, C., Graewert, M., Cipriani, F., Meijers, R., Svergun, D. and Betzel, C., 2018. Multi-channel in situ dynamic light scattering instrumentation enhancing biological small-angle X-ray scattering experiments at the PETRA III beamline P12. Journal of synchrotron radiation, 25(2).
- https://www.douglas.co.uk/cryoem.htm

A1: Bring your own sample: Sample Preparation - Introduction + Demo

Advanced crystallographic methods require diffracting crystals of specific sizes – very small for microED, small and uniform for serial data collection, and very large for neutron diffraction. Microseeding into the metastable zone of the crystallization phase diagram allows the number and size of crystals to be controlled. We recommend the same fundamental work-flow for preparing crystal samples for these three data-collection methods: (1) identify metastable conditions in a microbatch-under-oil format where microseeding is effective; (2) construct a simple phase diagram in microbatch; (3) scale up experiments in a microbatch format (this is much easier than scaling up in vapor diffusion). Case studies will be shown together with suggestions for tailoring the approach to the individual data-collection methods.





Dr. Martin Caffrey

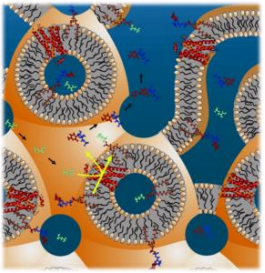
Membrane Structural and Functional Biology Group, Trinity College Dublin, Ireland

HT1 L4 & D1: Crystallizing Membrane Proteins in Lipidic Systems

One of the primary impasses on the route that eventually leads to membrane protein structure through to activity and function is found at the crystal production stage. Diffraction quality crystals, with which an atomic resolution structure is sought, are particularly difficult to prepare when a membrane source is used.

The reason for this lies partly in our limited ability to manipulate proteins with hydrophobic/amphipathic surfaces that are usually enveloped with membrane lipid. More often than not, the protein gets trapped as an intractable aggregate in its watery course from membrane to crystal.

As a result, access to the structure and thus function of tens of thousands of membrane proteins is limited.



The health consequences of this are great given the role membrane proteins play in disease; blindness and cystic fibrosis are examples. In contrast, a veritable cornucopia of soluble proteins have offered up their structure and valuable insight into function, reflecting the relative ease with which they are crystallized. There exists therefore a pressing need for new ways of producing crystals of membrane proteins. In this presentation, I will review the field of membrane protein crystallogenesis.

Emphasis will be placed on crystallization approaches which make use of the lipidic systems. In my talk I will describe these methods and our progress in understanding how they work at a molecular level. The practicalities of implementing these methods in low- and high-throughput modes will be examined. A practical demonstration of the lipid cubic phase or in meso method will be provided.

References:

- Caffrey, M. 2015. A comprehensive review of the lipid cubic phase or in meso method for crystallizing membrane and soluble proteins and complexes. Acta Cryst. F71, 3-18. https://doi.org/10.1107/S2053230X14026843
- Caffrey, M. 2021. Membrane protein crystallization. In Bergfors T. M. (ed.) Protein Crystallization. 3rd Edition (Ebook), IUL Biotechnology Series, 10. Chapter 19, pp 373-410. ISBN: 978-0-9720774-7-7. eBook
- Caffrey, M., Cherezov, V. 2009. Crystallizing Membrane Proteins In Lipidic Mesophases. Nature Protocols. 4:706-731. (PMID: 19390528) https://www.nature.com/articles/nprot.2009.31
- Caffrey, M., Porter, C. 2010. Crystallizing membrane proteins for structure determination using lipidic mesophases. Vis. Exp. 45: www.jove.com/index/details.stp?id=1712, (doi: 10.3791/1712)
- Lab Publications: https://www.tcd.ie/Biochemistry/research/caffrey/publications/

Prof. Elspeth Garman

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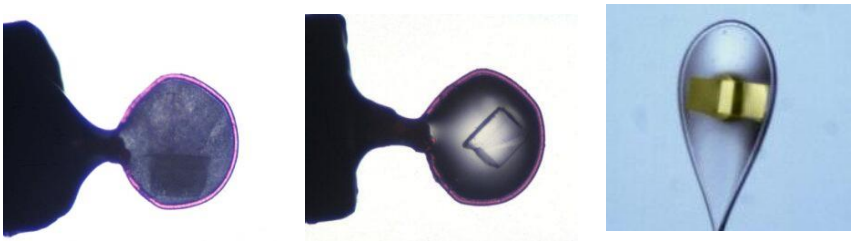
South Parks Road, Oxford
OX1 3QU, U.K.

HT1 L5: Cryo-Cooling: Why and how?

When a crystal has finally been obtained, the next stage in the structure determination pipeline is to prepare it for a diffraction test either at room temperature or 100 K. Often this step is given little thought, and the deleterious effects of various unfavorable crystal handling and cryo-protection protocols are not appreciated. In many laboratories a 'standard' route is used, which has worked for other protein crystals in the past. However, entrenched and accepted practices are not necessary the optimum ones for a particular problem, and may not work at all for your particular protein. The main aim is to avoid the formation of any ice both within the crystal and around it (see Figure below).

This lecture will follow the crystal from growth drop to goniometer, through the identification of a suitable stabilizing buffer, a benign cryo-protectant buffer and an effective cooling protocol, emphasizing the physical principles which affect the outcome (resolution of diffraction and mosaic spread: with the aim of maximizing the former and minimizing the latter) [1,2,3]. The practical aspects will be covered during the hands-on session.



Left: Protein crystal in a 1mm diameter mohair fiber loop. Buffer film is opaque with ice due to inadequate concentration of cryoprotectant. **Centre**: Same crystal with transparent film – a necessary but not sufficient condition for optimum diffraction. **Right**: 200µm sized crystal after optimizing the cryo-cooling protocol.

References:

- [1] Macromolecular Cryocrystallography. Garman, EF & Schneider, TR. J.Appl.Cryst. (1997) 30, 211-237.
- [2] Cool Data: Quantity and Quality. Garman, EF. Acta Cryst. (1999) D55, 1641-1653.
- [3] Practical Macromolecular Cryocrystallography. Pflugrath, JW. Acta Cryst. (2015) F71, 622-642.

HT1 L6: Radiation Damage in Macromolecular Crystallography

The three-dimensional structures of biological macromolecules are largely determined by crystallography: intense synchrotron produced X-ray beams of around 13 keV energy are used to measure the diffracted intensities of reflections from crystals of the molecule of interest, and the structure is solved by obtaining phase information by a variety of methods. The crystals typically contain between 30% and 70% solvent, confined in channels between the macromolecules.

For protein crystals at room temperature, radiation damage during the diffraction experiment is rapid even on a laboratory X-ray source. In the past, the required data had to be collected from several different crystals and merged together. The intense X-ray beams produced by third and fourth generation synchrotrons such as the EBS, ESRF, France and the NSLSII, U.S.A. destroy crystalline order in a matter of seconds. Over the last 30 years, the use of cryo-cooling techniques which allow X-ray data to be collected with the sample held in an open stream of cooled nitrogen gas at 100K, has become the norm; at 100K crystals can withstand around 70 times [1] the dose (energy lost/mass = J/kg = Gy) compared with room temperature, and the necessary data can usually be obtained from a single crystal.

However, observations of degradation of crystal diffraction with increasing radiation dose at 100K are commonplace at modern synchrotrons. Researchers have characterised the effects on the data in reciprocal space and on the resulting models in real space in order to understand the physical and chemical processes involved in this damage (reviewed in [2,3]). It manifests itself in a number of different ways, including: changes in crystal colour, decreasing diffraction power with dose, a small but measurable linear increase in unit cell volume, and specific structural damage to covalent bonds in the amino acids of the protein molecules (see Figure below). The bonds are broken in a reproducible order: firstly delocalisation of the sulphur atoms in disulphides, secondly decarboxylation of glutamate and aspartate residues, followed by the breaking of the C-S bond in methionines [4-6]. Enzyme active sites seem particularly sensitive to damage, so this phenomenon can lead to incorrect conclusions on biological mechanisms being drawn. Thus, the issue of radiation damage during diffraction experiments is a concern for all structural biologists.

An outline of our current understanding of radiation effects and some current lines of investigations, including a recent survey of damage in deposited PDB structures [7] will be presented.

±4s-level difference maps Fo4-Fo1
L to **R**: broken disulphide bond, decarboxylated glutamate, damaged methionine.
Red/green is -ve/+ve electron density

References:

- [1] Nave, C., Garman, EF (2005) Towards an understanding of radiation damage in Cryo-cooled crystals. J. Synch. Radiat. 12, 257-260
- [2] Garman, EF, Weik, M (2018) Radiation Damage in Macromolecular Crystallography. Chapter 4 in 'Protein Crystallography: Challenges and Practical Solutions' Eds: Konstantinos Beis & Gwyndaf Evans, Published by the Royal Society of Chemistry, Pp 88-116

- [3] Garman, EF, Weik, M (2023) Radiation damage to biological macromolecules, Curr.Opin.Struct.Biol. 82:102662
- [4] Weik, M, Ravelli, RGB, Kryger, G, McSweeney, S, Raves, ML, Harel, M, Gros, P, Silman, I, Kroon, J, & Sussman, JL (2000) Specific chemical and structural damage to proteins produced by synchrotron radiation. PNAS 97, 623-628
- [5] Burmeister, WP (2000) Structural changes in a cryo-cooled protein crystal owing to radiation damage. Acta Cryst. D56, 328-341
- [6] Ravelli, RGB, McSweeney, S (2000) The 'fingerprint' that X-rays can leave on structures. Structure 8, 315-328.
- [7] Shelley, KL, Garman, EF (2022) Quantifying radiation damage in the Protein Data Bank. Nature Comms 13:1314- 1325

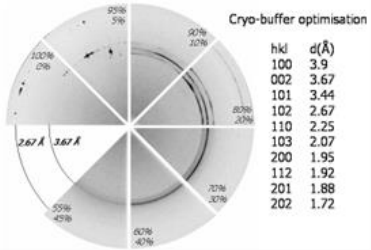
HT1 T1: Cryo-Practical Session

Background:

The aim is to flash-cool (NOT 'freeze'!) crystals in order to reduce the rate of radiation damage during exposure to X-rays. Protein crystals contain up to 80% solvent (water) and water can form ice crystals upon freezing, or alternatively, if the cooling process is fast enough, can solidify as an amorphous (non-crystalline) glassy solid. Any ice crystals in and around the protein crystal will 1) perturb the crystalline lattice due to the 7% volume increase of ice as compared to water and 2) give rise to diffraction during exposure to X-rays, which will be parasitic on the diffraction pattern generated by the protein crystals. To increase the time available to obtain the desired vitreous solid on cooling and thus avoid the formation of ice crystals, the crystal is usually soaked in a cryo-protectant. Cryoprotectants lower the freezing point of water (just as 'anti-freeze' is added to the cooling water of a car in winter) and more importantly they also slow down the kinetics in the solution by increasing the mean free path of water molecules being able to meet other water molecules to form ice. With luck, crystals might grow from crystallization solutions which already have cryo-protectant properties. In this case the crystal can be transferred directly from the crystallization droplet to the nitrogen gas stream (or liquid nitrogen), a procedure called "fish-and-cool".

In practice, a crystal usually has to be soaked for a few seconds in a solution of the same composition as your crystallization solution complemented with the cryo-protectant. However, as such a solution is different from the crystallization solution, the crystal might not tolerate the new environment and crack (osmotic shock) or lose its sharp edges (solubility change). The ideal soaking solution has the water of the crystallization buffer replaced by the cryoprotectant, rather than being made by just diluting the mother liquor with the cryoprotectant.

The object is :**1)** to find the nature of cryo-protectant tolerated best by the crystal and **2)** to find the suitable concentration of cryo-protectant. This concentration should be low enough such that the cryo-solution is as similar as possible to the crystallization solution and does not harm the crystal, and high enough such that ice crystals do not form.



This figure shows the effect on the diffraction pattern of water with different percentages of glycerol added before flash-cooling. Amorphous solid is achieved by adding 45% (v/v) glycerol.

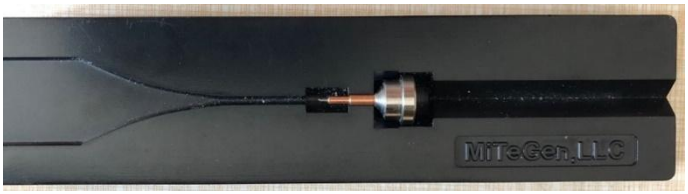
Demonstration/Practical

Demonstration A: Mounting for a room temperature testing

Room temperature mounting equipment will be demonstrated using the MiTeGen system: a metal 'top hat' with loop attached which has two different diameters of stem over which the plastic sleeve fits snuggly, and black plastic sleeve guide ('aligner', shown below) for threading polyamide sleeve onto pin with containing the looped crystal so that the crystal is not deposited on the inside of the sleeve instead of staying in the loop. The size of the loop is matched to the size of the crystal before 'fishing' so that the crystal cannot move around in the liquid across the loop during the RT data collection.

The polyamide sleeve is sealed at one end and contains a small volume of the crystallization buffer which has been previously pipetted into the end of the sleeve. This prevents crystal dehydration during RT data collection.

Each student will practice threading the plastic sleeve onto the pin using the sleeve guide.



Demonstration B: Optimum crystal fishing strategies

'Soup spoon' versus 'minimize surrounding film' crystal fishing techniques will be shown. Keep the plane of the loop at 90° to the surface of the drop at all times – achievable with practice! This technique will equalize the surface tension forces on each side of the loop and give a much thinner film of liquid than the 'soup-spoon' method.

Demonstration C: Storage of crystals in pucks for transport and data collection

The crystal holding pucks and tools for loading them will be explained.

Demonstration D: Keep crystal moving under liquid nitrogen

When flash-cooling a crystal into a Dewar of liquid next the microscope, the Dewar lid should be used to flap away the cold gas layer above the liquid nitrogen before plunging the crystal in. This avoids 'slow-cooling' by the crystal travelling through a temperature profile of warming gaseous nitrogen

coming off the 77 K liquid. Also, very importantly, immediately after plunging, gently move the crystal on the wand around in the liquid nitrogen in order to leave behind the insulating bubbles of gas that form round the crystal/loop. This ensures a fast cooling rate. The Dewar should be kept as full as possible to avoid having a layer of warmer gas above the surface, since this will give a temperature gradient through which the crystal must travel to reach the liquid, and thus it will cool more slowly (and thus not as well).

Demonstration E: Cryovial transfer to canes

If any using canes and vials to store crystals, there will be a demonstration of a good way to handle the vials.

With gloves on (both insulating and plastic lab gloves) hold palm UPWARDS and FLAT with a gap between the index finger and other 3 digits. Hold vial in pincer movement keeping fingers FLAT (people tend to curl their fingers up and then this method doesn't work well).

Each person using canes should practice loading and unloading a vial in this way from a warm cane.

N.B. NEVER have the 2 liter tall tin Dewar full of liquid nitrogen on the bench. It should ALWAYS be on the floor, ideally in a simple stand to stop it being kicked over.

Below are two exercises that will be outlined and participants will carry out some of the steps. There will not be a goniometer or liquid nitrogen at HTCC6, but improvisation will portray the ideas, and then all the steps can be carried out at home!

Exercise 1: Fishing crystals from Linbro plates and flash-cooling them in a 100 K nitrogen stream

Method:

1. Under the microscope look at the Linbro-plate and find a drop with well-formed crystals. The crystals should be good for data collection: single with clean edges.

2. Under the microscope look at different cryo-loops (Hampton or MiTeGen) and select one of the size comparable to the size of the crystal you decided to fish. This is very important, because the following steps have to be carried out quickly and there is no time to waste selecting a suitable loop once you have opened up the tray.

3. Place the cryo-loop on the goniometer head and make sure that the center of the loop aligns with the trajectory of the X-ray beam (center of loop in the center of the camera). Adjust the goniometer axes if necessary.

4. Verify that the cryo-stream is well aligned with the center of the cryo-loop. IMPORTANT.

5. Take the coverslip off from the drop you selected in step 1 (you may need to use a scalpel blade to lever up the coverslip) and place it on the cover of the Linbro plate or on the plate lid if there is one (obviously up-side-down).

6. Pipette 2 ml of the cryo-solution into a nearby empty well or, if there are only 2 or 3 crystals in your well, place it next to the droplet containing the crystals.

Note: if there are a lot of crystals in the well, DO NOT put the cryo-solution on top of them! The crystals might degrade in the time it takes to fish them all, and the cryo-solution may very likely dehydrate the crystals, shrinking the unit cell of the crystals left in there for the longer times. This will make it more challenging to merge data collected from different crystals at the data analysis stage.

7.Attach the cryo-loop you selected in step 2 on the crystal wand, fish a crystal, bath it for a few seconds in the cryo-solution, fish it again, and very importantly, BLOCK THE CRYOSTREAM. Put the cryo-loop onto the goniometer head. Once it is there safely, unblock the stream as quickly as possible (i.e. remove the blocker, usually a strip of cardboard, very fast to 'flash-cool' the crystals).

Note: DO NOT refocus the microscope to check that the crystal is in the loop, as the crystal will dehydrate during that time and the mosaicity may increase.

8. Do not forget to put the coverslip back over the reservoir to prevent the remaining crystals from dehydration.

Exercise 2: Fishing crystals from 96-well plates and flash-cool them in liquid nitrogen

Method:

1. Under the microscope look over the 96-well plate and find a drop with well-formed crystals. The crystals should be good for data collection: single with clean edges. Write down the well number.

2. Under the microscope look at different cryo-loops (Hampton or MiTeGen) and select one of the size comparable to the size of the crystal you decided to fish.

3. If using cryo-vials, with the help of the tweezers transfer the empty vial of the cryo-loop to within the sample holder basket placed in the Dewar containing liquid nitrogen.

4. With the plate under the microscope, use the scalpel to cut the tape, which covers the drop of choice.

5. If the crystal of choice is attached to other crystals (see drop on the very right in the scheme below), try to carefully separate the crystals with an acupuncture needle. If there is a skin over the crystals, use two needles, one in each hand. Hold the skin down with one needle (keep your hand still) and chop round the skin with the other needle.

6. Pipette 1 ml of the cryo-solution next to the droplet containing crystals. **Scenario 1**: If only one of the crystallization wells contains a crystallization drop (blue), use one of the empty wells to add the cryo-solution (red). **Scenario 2**: If all of the crystallization wells contain crystallization drops, put the cryo-solution on the shelf between the crystallization drops. Be careful and make sure that the drop does not move towards the crystallization well and join with the crystallization drop (see point **6** in **Exercise 1** above).

Scenario 1 Scenario 2

7. Attach the cryo-loop you selected in step 2 onto the crystal wand, fish a crystal, bath it for a few seconds in the cryo-solution and transfer it quickly into the liquid nitrogen. KEEP THE CRYSTAL MOVING GENTLY after plunging it, in order to leave behind gaseous bubbles of nitrogen which insulate the crystal from the cold liquid and which can prevent efficient cooling. Then once the bubbling has ceased, place it within the empty vial placed under liquid nitrogen. Feed the crystal pin straight into a puck by looking directly down the wand held perpendicularly to the plane of the puck (i.e. put your face over the Dewar while wearing safety glasses to line up the pin carefully not to lose the crystal on the inside of the puck).

8. If the crystal won't be fished because it is stuck to the surface of the crystallization well, try first to lift it by waving the cryo-loop near it in the crystallization well. If this procedure fails, scratch the well near the crystal with the acupuncture needle to stress the plastic. This sometimes releases the crystal. If this does not work, try to detach it directly with the acupuncture needle and repeat step **7**.

9. Do not forget to seal the open well with sealing tape.

Questions:

- How can you find the best suitable cryo-protectant?
- Why is it important to put the crystal in the center of the cryo-stream?
- Did you find it more difficult to fish crystals from a Linbro plate or from a 96-well plate?

Some additional background explanations:

How can we cool the crystal to 100 K without destroying the ordered lattice? Small changes in procedure can have a significant impact on the resulting resolution of the data and thus impact the quality of the biological information obtained. The crystal must be cooled so fast that the solvent in the intermolecular cavities becomes amorphous glassy solid, rather than as ordered ice crystallites. The latter disrupt the internal ordered lattice when they form, since their volume is greater than that of liquid, and also give powder diffraction rings that obscure reflections at particular resolutions (3 intense powder rings between 3.5 and 3.9 Å, see Figure above). Thus, it is often necessary to add an organic solvent, a 'cryo-protectant' such as glycerol, ethylene glycol or light polyethylene glycols to the crystal buffer and soak the crystal in it prior to swiftly plunging it into liquid nitrogen (77 K).

Optimizing the cryoprotection step is very important for the final outcome, and typically the experimenter takes the crystallization buffer and dilutes it with the cryo-protectant. Sometimes the crystal (imagine YOU are the crystal!) does not take kindly to this treatment, since the protein solubility might increase and then the sharp edges of the crystal start to become rounded, or at the other extreme, the osmotic pressure of this cryoprotectant buffer might be much larger/smaller than prior to soaking, and small 'crazy paving' cracks will appear on the crystal surface. If either of these problems is encountered, it is worth making up the cryoprotectant buffer by replacing the water in the original crystallization buffer with the cryoprotectant agent (i.e., making up the latter at double concentration and then adding water and cryoprotectant agent to reproduce the original crystallization buffer concentrations). In problematic cases this method has often given very good results. Also, if osmotic shock is the issue, sequential in situ soaking in increasing concentrations of cryoprotectant buffer can have a huge positive impact (e.g., the resolution of subtype N6 influenza neuraminidase crystals, which required 40% glycerol for optimum protection, routinely increased from 3.2 Å to 1.6 Å resolution after using this protocol).

For two reasons, harvesting just one crystal at a time for soaking in the cryo-buffer is recommended, rather than pipetting the buffer onto a whole drop containing many crystals. Firstly, many cryo-protectant agents cause dehydration and shrinkage of the unit cell, and since fishing them takes time for each crystal, they will spend varying times soaking and thus have a range of unit cells, which will give increased systematic errors if data from different crystals is to be merged. Secondly, many crystals will remain stable in the cryoprotectant buffer for a limited time, but will then start to degrade, so it is unwise to leave them in it for too long while cooling the other crystals.

The next step is to 'fish' the crystal into a cryo-loop under a microscope, first checking that there is no skin over the drop and that it is not stuck to the bottom of the well, by stirring the drop with a tool (acupuncture needle, cat's whisker etc.). To minimize the amount of liquid round the crystal (and thus minimize the background-ray scattering) the loop can be brought out of the drop with its plane perpendicular to the drop surface. To avoid ice forming in the liquid nitrogen, the Dewar should be kept as full as possible and have an easily removable lid on top of it during 'fishing', and the nitrogen should be regularly renewed.

If the cryo-cooled crystal doesn't diffract, testing at RT can be very worthwhile as then it can be determined whether the crystal lattice was destroyed by the cryo-cooling protocol which should then be altered (soak time, soak temperature, cryoprotectant) or whether it was never well ordered in the first place, and new crystallization trials are then required.

Crystals for data collection at 100 K at synchrotrons are typically now cooled into the pucks that fit straight into the beamline mounting robot, and then shipped to the synchrotron in special transport Dewars. These Dewars should be allowed to dry out after every trip, in order to disperse water vapor that has condensed and been trapped inside when they are opened and closed.

Dr. Krešimir Molčanov

Ruđer Bošković Institute
Zagreb, Croatia

HT2 L1: Data Collection on Laboratory Diffractometers: Basics, Strategies, Data Reduction

Basics of data collection and data reduction on laboratory diffractometers are presented and strategies for data collection are laid out. Preliminary measurements for determination of unit cell, Laue group and exposure time are important, and relations between crystal symmetry, reciprocal lattice and Ewald sphere are explained. Symmetry of the diffraction pattern is linked to the crystal symmetry; further, Friedel's law adds (an approximate) inversion symmetry to the diffraction pattern. Thus, Laue group can be determined from preliminary measurement.

Different geometries of modern goniometers are laid out: Eulerian, kappa and three-circle; relation between diffractometer axes and reciprocal lattice of the crystal are explained.

Strategies for data collection are laid out. A good choice for strategy considers symmetry of the crystal and type of goniometer. Terms such as multiplicity, redundancy, symmetry-inequivalent part of Ewald sphere and measurement of Friedel pairs are explained.

After the data collection, the raw diffractometer data are processed (or 'reduced') into a reduced form, a .hkl file, which contains a list of reflections with Miller indices, intensities and their standard deviations. Multiple measurements of the same reflection (or its symmetry equivalents) are merged, and random errors are thus cancelled out. Different types of absorption correction and their (dis)advantages. are discussed.

Dr. Annie Héroux

Beamline scientist
Elettra, NSFL, Italy

HT2 L2: Data Collection at the Synchrotron

Even before the pandemic, most of the beamlines accepting MX proposals were moving towards remote access. This mode can be intimidating for new users as concerns of "being on your own", fear of not having enough beam time and wasting precious samples come to mind. During this talk I will walk through the steps and address some of the concerns involved before, during and after your beam time in order to optimizing your results from a synchrotron visit.

The speed at which MX data can be acquire has been steadily increasing with new brighter sources, better and faster detectors and automatization at the beamlines. One of the most challenging aspect of data collection is actually being able to manage all the information obtained and be able to use it during the visit, while you still have available samples.

A concerted effort amongst facilities, especially in Europe is pushing to converge towards similar web interfaces at beamlines in order to provide a familiar environment to users. Web tools to access data analysis results and meta data are also evolving to catch up with new experiments for MX such as serial crystallography and time resolve crystallography. A better understanding of what is available and how to take advantage of these tools is crucial to harvest as much info from your samples.

Hopefully we will be able to apply some of the concepts reviewed here with some hands-on remote data collections at one or more facilities.

Links of interest:

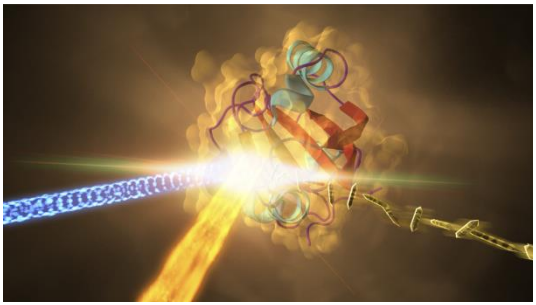
- Oscarsson, M. et al. 2019. "MXCuBE2: The Dawn of MXCuBE Collaboration." J. Synchrotron Rad. 26 (Pt 2): 393–405. New feature https://github.com/mxcube/mxcubeweb
- Gabadinho, J. et al. 2010. MxCuBE: a synchrotron beamline control environment customized for macromolecular crystallography experiments. J. Synchrotron Rad. 17, 700-707
- S. Fisher et al., 2015 SynchWeb: a modern interface for ISPyB J. Appl. Cryst.. 48, 927-932
- S. Delageniere et al., (2011) SPyB: an information management system for synchrotron macromolecular crystallography Bioinformatics. 27 (22): 3186-3192

Prof. Henry Chapman

Center for Free-Electron Laser Science, DESY
Hamburg, Germany

HT2 L3: Advancements and Future Directions in Serial Crystallography at XFELs

In this presentation, we aim to provide an insightful overview of advancements and future directions in serial crystallography (SX) utilizing X-ray Free Electron Lasers (XFELs). The discussion will commence with a brief exploration of the background of XFEL serial crystallography, highlighting its unique capabilities and applications in structural biology.



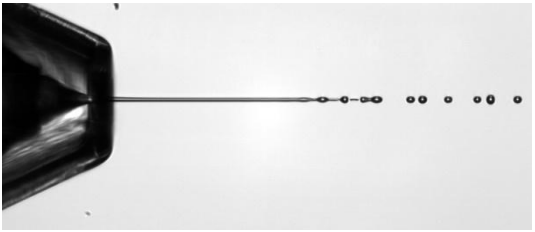
We will present compelling examples from our ongoing work as well as notable contributions from the existing literature, showcasing the potential and versatility of this technique. Furthermore, the presentation will delve into exciting possibilities for advancing XFEL-based research towards single-particle imaging, offering a glimpse into the promising future of this cutting-edge field.

Dr. Saša Bajt

Center for Free-Electron Laser Science
DESY and CUI (Center for Ultrafast Imaging)
Germany

HT2 L4: Liquid Jet Developments for Efficient Sample Delivery at XFELs

A significant focus will be placed on the evolution of liquid jet technologies, a vital component for efficient sample delivery in XFEL experiments. We will review and provide updates on recent developments in liquid jet methodologies, shedding light on their critical role in achieving successful experiments.



Dr. Dominik Oberthür

Center for Free-Electron Laser Science CFEL,
Deutsches Elektronen-Synchrotron DESY,
Germany

HT2 L5: Data Processing for Serial Crystallography, Automation, and Further Outlook

Serial crystallography, powered by X-ray Free Electron Lasers (XFELs) and synchrotrons, has revolutionized the study of biomolecular structures. The data deluge generated by these advanced techniques necessitates efficient and automated data processing pipelines. In this lecture, we will delve into the intricate process of handling large volumes of data produced in serial crystallography. We will explore cutting-edge methods and technologies that automate data processing, reducing human intervention and enhancing the speed and accuracy of structural determination. The discussion will extend to emerging trends and future prospects in data processing for serial crystallography. Additionally, we will highlight the role of automation in expediting the transition from data collection to structural insights, enabling faster decision-making and a more comprehensive understanding of biological macromolecules.

HT2 T1: How to Carry Out an Experiment?

We will give a tutorial on how to carry out an SSX experiment at P11 of DESY. We will go through all the steps, explaining the set-up in a virtual demo, highlighting how one can get from crystals to several structures within the duration of the session.

Dr. Anna Luebben

Bruker AXS
Karlsruhe, Germany

HT2 L6: Data Collection on Laboratory Diffractometers II: Home-lab data collection and processing using PROTEUM5

PROTEUM5 is a comprehensive suite of programs for protein crystallography enabling crystal characterization, data collection, reduction, and analysis. The resulting output files can be seamlessly input into the CCP4 suite of programs for structure determination.

This lecture will outline the similarities and differences between beamlines and home-lab systems to be considered when collecting diffraction data and will walk-through a real-life example to highlight best practices for collecting and processing data on laboratory diffractometers using PROTEUM5.

Dr. Eugene Krissinel

Science and Technology Facilities Council,
CCP4 core group
Oxford, UK

HT3 L1: Structure Solution in CCP4 Cloud

CCP4 Cloud is a front-end for the CCP4 Software Suite, which represents a conceptually new approach to organizing and maintaining crystallographic projects in CCP4 and running CCP4 tasks. Unlike conventional type of front-end, which usually keeps projects and runs tasks on the user's machine, CCP4 Cloud exploits the paradigm of remote computing, when all projects and data are kept online and tasks are executed on remote servers, "in cloud". CCP4 Cloud is accessible through any web browser, making it easy to use and accessible from anywhere with internet connection. CCP4 maintains a public instance of CCP4 Cloud at RAL Harwell (https://cloud.ccp4.ac.uk). If necessary, CCP4 Cloud servers may be placed on the user's machine, in which case all data and projects are kept locally and CCP4 Cloud behaves as a

desktop GUI (so-called “desktop mode”). Starting from CCP4 series 7.1, CCP4 Cloud is included in the distributed package and can be started out-of-the-box by clicking on icon launchers.

A few important CCP4 components: data processing software, Coot, CCP4 Molecular Graphics and some others, can be used only on users’ machines. They are linked with CCP4 Cloud automatically once CCP4 is installed on the user’s computer.

CCP4 Cloud’s key benefits include high platform compatibility, access to significant computational resources and suitability for team work. CCP4 Cloud projects are accessible from any geographic location and computing platform; with Cloud, one can solve structures even from tablet devices and mobile phones. A number of internet resources are seamlessly integrated; structures from PDB, UniProt, AlphaFold and ESM databases can be imported instantly, 3rd party software such as PDB-REDO and Buster from Global Phasing are made available as well. Time-consuming tasks, such as structure auto-solvers and auto-builders, can be conducted in shorter time without the need for specialised hardware installed locally.

CCP4 Cloud projects can be exported as single files and reimported back in Cloud as necessary. When several researchers need to work on a project simultaneously, it can be shared between them online, regardless of their location, allowing for efficient communication and collaboration in real-time.

Retaining structure-solution projects is as important as retaining experimental data. This can be useful for the subsequent understanding of ambiguous structural features, revisiting and revising structural studies, education, facilitating the peer-review publication process, training and software development. Starting from 1st January 2023, CCP4 Cloud projects can be archived and referenced in publications or similar reports by a unique Archive ID. Archived projects represent a missing link between experimental data and the final structure, deposited in the wwPDB. This is a new initiative on keeping information complementary to PDB depositions and facilitating the exchange of knowledge and structure solution practices in the structural biology community.

The lecture will give a practical introduction in CCP4 Cloud:

- principles of CCP4 Cloud interface
- project types and their initiation
- principles of project development
- importing data and exporting results
- forming and running crystallographic tasks
- use of documentation resources and tutorials for self-education
- overview of typical projects for molecular replacement and experimental phasing
- automation facilities
- overview of available crystallographic tasks
- project export/import and sharing
- and more…

HT3 L2: Model Validation and Deposition

Macromolecular structure determination is a complex process that involves a series of steps, including data processing, model building, and refinement. The accuracy and reliability of the final structure model depend on the quality of data and methods used in each of these steps and is often difficult to assess, partially because there is no single score which would unambiguously indicate structure quality. A number of quality indicators need to be checked before concluding that the structure represents the best or next-to-the-best fit to given experimental observations, i.e., represents a valid result and accurately reflects the true nature of the macromolecule.

The assessment procedure, commonly called “structure validation”, should always be performed before depositing results to the Protein Data Bank (the PDB). CCP4 offers a number of validation utilities, most of which are executed at the end of refinement jobs. Model validation also helps to identify errors and inconsistencies in the model, which should be corrected before deposition. Validation of macromolecular structures is critical, as inaccuracies or errors in the models can lead to incorrect interpretation of the biological function of the protein.

CCP4 Cloud interface provides an easy-to-use platform for protein structure determination and validation. Most task reports in CCP4 Cloud come with the Verdict section, which represents a validation summary for the task’s results. Verdicts come with few quality assessment sections that provide more detailed information on various quality indicators, for example, per-residue B-factor analysis and electron density fit scores, Ramachandran outliers, and full Molprobity analysis. The Verdict includes information on potential errors or issues in the model and suggestions on parameters that may be used to improve the results. The Verdict also provides a quick overview of the quality of the model and can be used to identify potential issues that need to be addressed before deposition.

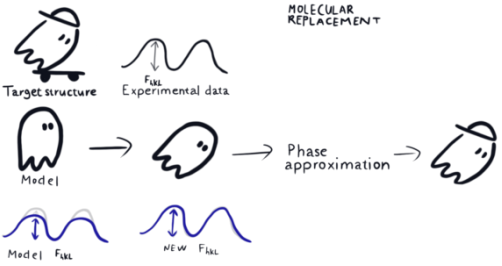
PDB deposition requires mmCIF-formatted files with experimental observations or structure factors and model coordinates. They are prepared with CCP4 Cloud’s deposition task, which also acquires PDB validation reports containing detailed quality analysis and scores. These reports represent a copy of what will be examined by PDB curators when the structure is considered for deposition, and they will also be available to referees when results are submitted for publication in a journal.

Dr. Eugene Krissinel & **Dr. Lucrezia Catapano**, tutor

CCP4 core group, UK
&
MRC Laboratory of Molecular Biology
Cambridge, UK

HT3 T1: Automatic and Fundamental Molecular Replacement

Molecular Replacement (MR) is the most popular method to solve the Phase Problem nowadays. MR is based on the assumption that target structure has a highly similar structural homologue, which may be found by, e.g. matching target's sequence to sequences of known structures. In this method, a sufficiently close molecular model is positioned (i.e., rotated and translated) in crystal coordinates such that calculated structure amplitudes reproduce experimental results in the best possible way. Then, calculated phases are considered as first approximations to real phases, which allow for subsequent model building, refinement and phase improvement.



For the success of Molecular Replacement, the MR model should be sufficiently similar to the target structure. In this tutorial, we will discuss how to find and prepare suitable MR Search Models (including working with Alphafold2 predicted models) for use in molecular replacement programs (Phaser and Molrep). We will also cover the basic principles of MR and the software available in CCP4 for each step of MR, including both fundamental tools and automatic pipelines that combine all needful steps and can deliver phased structure starting from experimental observation and sequence – or even without the later.

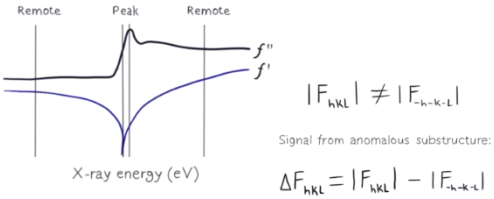
HT3 T2: Automatic and Fundamental Experimental Phasing

MR assumes that a close structure homolog does exist. So, for solving a structure we need a similar structure solved. How was the first ever structure solved then? In principle, structure can be found from reflection data by so-called Direct Methods, based on phase relationships between structure factors. Direct Methods were developed by Herbert Hauptman and Jerome Karle,

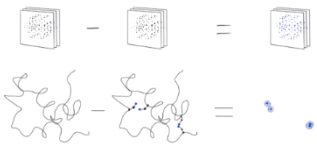
for which they won the 1985 Nobel Prize in Chemistry (https://www.nobelprize.org/prizes/chemistry/1985/summary/). Direct Methods have been implemented in several software packages (e.g., Shelx).

As appears, for purely technical reasons, such as natural presence of noise in experimental data, Direct Methods can be used only for limited-size structures, depending on the resolution (usually limited by 10-100 atoms; as a maximum, ~1000 atom structures have been reported solved by the method). Therefore, Direct Methods are not generally suitable for determining macromolecular structures. However, if we could have diffraction data from a small subset of atoms in the crystal, then we can find their positions with Direct Methods, calculate their phases and use them for the estimation of the overall structure's phases. A set of phasing techniques, based on this idea, is called Experimental Phasing.

In this workshop, we will focus on the most popular Single-wavelength Anomalous Diffraction (SAD) phasing. SAD approach exploits the anomalous scattering from a set of selected atoms in the crystal, which can be identified in diffraction images (such a set is commonly called as heavy-atom substructure). We will discuss what an anomalous signal is and how we can estimate its properties for a given anomalous scatterer, such as Se, S, Zn, Mg and others.



Next, we will learn how to use various software available in CCP4 to find the heavy-atom substructure. Since diffraction from a substructure is not distinguishable from diffraction of its inverse, the software produces coordinates and phases for both of them. Such sets of substructures and phases are commonly called "original" and "inverted" hands.



However, only one hand gives the correct protein phases. We will see how the correct hand can be identified with CCP4 software, and solve a structure with SAD phasing using prepared data containing a signal from anomalous scattering. We will learn how this can be done in CCP4 using both fundamental programs or sophisticated pipelines. As a result we shall get the phased structure from just reflection data and sequence – or give you a protein backbone if sequence is not known.

Dr. Lucrezia Catapano, tutor

MRC Laboratory of Molecular Biology, Cambridge, UK

HT3 T3: Automatic Model Building, Refinement and Ligand Fitting

Automatic Model Building (AMB) is recommended when large parts of structure need to be built (e.g., after Experimental Phasing) or re-built (e.g., after Molecular Replacement, especially if MR search model has low sequence similarity to the target structure). AMB is expected to deliver 70% to 90% of structure residues fit correctly in the density, depending on the difficulty of the case, and results should be always inspected visually, corrected and completed manually with Coot. AMB usually saves considerable time and effort and is particularly useful when dealing with large and complex protein structures, where manual model building would be time-consuming and error-prone.

CCP4 provides several AMB tools, which include automatic pipelines and project workflows for model building, refinement, and ligand fitting. AMB software generates an initial trace of the macromolecule (protein or RNA/DNA) based on the electron density map obtained after Experimental Phasing (EP) or Molecular Replacement (MR). While the need for AMB is rather obvious in case of EP (note that AMB is included in Crank-2 pipeline for automatic EP), it is often required also in ME even if MR search model was calculated by AlphaFold-2 or obtained from AFDB with 100% sequence homology. This is because using highly-homologous search models does not guarantee correct conformations, low-confidence residues (equivalent to high B-factors) are removed before MR, and the structure may be partly disordered.

CCP4 Cloud gives access to the following AMB tools: Buccaneer (fast protein building), ARP/wARP (thorough protein building in resolutions up to 2.5A), CCP4Build (thorough protein building), Modelcraft (thorough protein and RNA/DNA building) and awNUCE (RNA building without phase modification). Appreciating the confusing range of tools and their specifications, we tend to recommend Modelcraft as a first attempt in most cases, although performance of each tool depends on the case.

In this tutorial, we will cover steps involved in automatic model building, and get practical experience of using the corresponding CCP4 Cloud tasks.

Step 1: Density modification

Density modification is used to improve the phase quality and make electron density more suitable for AMB. This step is especially needed after EP, which often results in phases of insufficient quality. Note, however, that Modelcraft and CCP4Build include density modification steps, therefore, it is not recommended before running these tasks. Density modification uses several techniques, equivalent to incorporating additional information into the electron density map, which helps auto-builders to start from better assumptions and produce higher quality structures in the end.

Step 2: Automatic Model building

The next step is to generate an initial trace of the protein using Modelcraft, ARP/wARP, Buccaneer, CCP4Build and Automatic RNA/DNA Building with Arp/wArp (awNUCE).

Step 3: Automated Model Refinement

After generating the initial trace, the next step is to improve structure quality and phases by refining with chosen parameters. Suitable parameters for Refmac may be found in the course of multiple trials with examination of Verdict section in Refmac report pages. Alternatively, good results can be usually achieved with the automatic refinement and ligand fitting workflow wREL. In case of low-resolution data, using the LoRESTR pipeline may be required.

Step 4: Automatic Ligand Fitting

Once the protein model has been refined, the next step is to fit ligands into the model. Make sure to remove water molecules that can be put by model builders into ligand density blobs. Also keep in mind that auto-builders may build protein backbone into ligand density by mistake, in which case residues in wrong positions must be removed with Coot before ligand fitting. We will consider ligand fitting techniques using automatic tools in CCP4 Cloud.

Step 5: Model Validation

The final step in automatic model building is model validation. This involves checking the quality and accuracy of the final model with reference to acceptable range of quality scores and structures of similar resolution found in the PDB. Here we will demonstrate a range of validation tools available in CCP4 Cloud.

Dr. Rob Nicholls

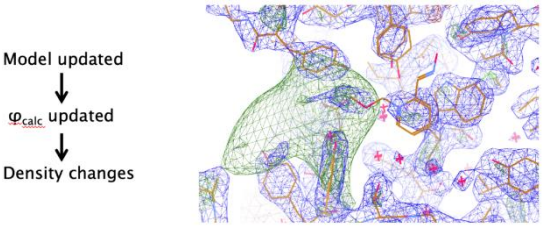
MRC Laboratory of Molecular Biology
Cambridge Biomedical Campus
Cambridge, UK

HT3 L3: Macromolecular Refinement in CCP4

Refinement of atomic structural models against crystallographic experimental data is an integral part of crystal structure analysis. Since crystallographic observations are intensities of the corresponding structure factors, and there is no direct way of observing the phases, most crystallographic computations revolve around recovering the lost phases. Hence refinement in general has two purposes:

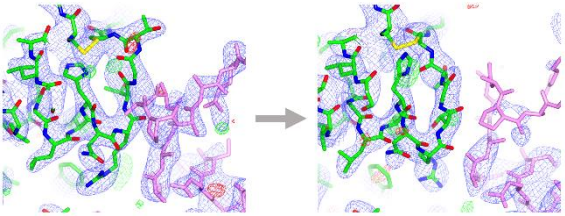
1. To derive as accurate atomic structural models as possible, and

2. To improve model phases thus generating the best possible electron density maps.



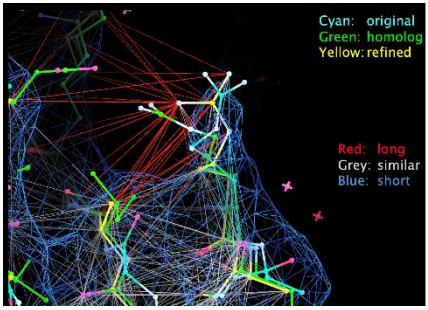
Although the main aim of macromolecular crystallography (MX) is to derive accurate atomic models in order to answer specific biological questions, the importance of improving phases and the resulting electron density maps should not be underestimated. Such maps help in automatic and manual model building, affecting the quality of final atomic models.

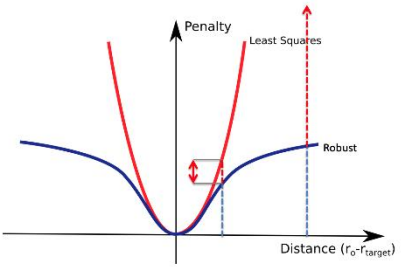
Effects such as crystal mosaicity and disorder lead to poor diffraction quality and weak intensities, resulting in only low-resolution data being available. Such high-resolution information loss worsens the observation-to-parameter ratio, which results in unstable refinement, overfitting, and ultimately an unreliable model. Consequently, regularisers are used in order to stabilise MX refinement, and ensure consistency between the derived model and available prior knowledge. Using the Bayesian framework, such regularisers are implemented in the form of restraints, which are often referred to as “geometry terms”. Regularisers are typically used at all resolutions, although more may be required at lower resolutions in order to achieve an acceptable effective parameter-to-observation ratio. Indeed, challenges that are encountered during model parameterization and refinement may vary depending on the high-resolution limit of the collected diffraction data.



Restraints representing chemical information are commonly used at all resolutions (e.g. bond and angle restraints), which help local structure adopt chemically reasonable conformations. At medium resolution, TLS, local NCS, and B-value restraints may be used. At lower resolutions, additional supplementary “external restraints” may be needed in order to encourage consistency with models of homologous structures, formation of hydrogen bonding networks, nucleotide base pairing and stacking. Notably, jelly-body restraints stabilise MX refinement without injecting externally derived information.

When external restraints are used, an anharmonic penalty function is used to control robustness to any outliers caused by inconsistencies between data and prior information. These external restraints and robust estimation procedures are also useful during model building, where necessary to increase real-space refinement convergence radius and stability.





Where several datasets and models are available for a macromolecule, restraints can facilitate information transfer between structures, potentially improving refinement and thus resultant model quality. Pragmatically, determining suitability of reference structures and refinement parameters can be challenging; the automated pipeline LORESTR facilitates this process by trialing multiple protocols.

Additional consideration must be given in cases where a model contains a novel ligand/compound for which a description does not yet exist in the CCP4 Monomer Library, or if a compound is covalently linked to the macromolecule (post-translation modifications). In such cases, a bespoke restraint dictionary corresponding to the compound and/or linkage is required in order to that the model maintains sensible chemistry and acceptable geometry. Such dictionaries can be generated using AceDRG. Following refinement in REFMAC5, visualization and validation tools in Coot facilitate manual inspection, critique and improvement of the protein-ligand complex model.

In this session, we will discuss techniques to facilitate the refinement of high-quality MX models, including more challenging cases where only low-resolution data are available. We shall focus on implementations within the CCP4 suite, specifically using the software tools: REFMAC5, Coot, AceDRG, ProSMART, LibG and LORESTR.

Low-resolution MX refinement with REFMAC5, ProSMART, LibG & LORESTR:

- Nicholls et al. (2017) Low Resolution Refinement of Atomic Models Against Crystallographic Data. Protein Crystallography, 565-93.
- Nicholls et al. (2013) Recent Advances in Low Resolution Refinement Tools in REFMAC5. Methods for Bio. Xtallography, 231-58.
- Nicholls et al. (2012) Low Resolution Refinement Tools in REFMAC5. Acta Cryst. D68, 404-17.

Tools for ligand fitting & validation with CCP4/Coot:

- Nicholls (2017) Ligand fitting with CCP4. Acta Cryst. D73, 158-70.
- Emsley (2017) Tools for ligand validation in Coot. Acta Cryst. D73, 203-10.
- Debreczeni & Emsley (2012) Handling ligands with Coot. Acta Cryst. D68, 425-30.

Modeling covalent linkages:

- Nicholls et al. (2021) Modelling covalent linkages in CCP4. Acta Cryst D77, 712-26
- Nicholls et al. (2021) The missing link: covalent linkages in structural models. Acta Cryst D77, 727-45.

Primary software references:

REFMAC5	Murshudov et al. (2011) REFMAC5 for the refinement of macromolecular crystal structures. Acta Cryst. D67, 355-67.
Coot	Emsley et al. (2010) Features and development of Coot. Acta Cryst. D66, 486-501.
AceDRG	Long et al. (2017) AceDRG: a stereochemical description generator for ligands. Acta Cryst. D66, 486-501
ProSMART	Nicholls et al. (2014) Conformation-Independent Structural Comparison of macromolecules with ProSMART. Acta Cryst. D70, 2487-99.
LibG	Brown et al. (2015) Tools for macromolecular model building and refinement into electron cryo-microscopy reconstructions. Acta Cryst. D71, 136-53.
LORESTR	Kovalevskiy et al. (2016) Automated refinement of macromolecular structures at low resolution using prior information. Acta Cryst. D72, 1149-61.

Additional reading relevant to refinement with REFMAC5 and associated tools:

Effect of Twinning on R-factors:

- Murshudov GN (2011) Some properties of Crystallographic Reliability Index – Rfactor: Effect of Twinning. & Comp. Math., 10, 250-61.

Cooperative utilisation of information from MX and NMR:

- Kovalevskiy et al. (2018) Overview of refinement procedures within REFMAC5: Utilising Data from Different Sources. Acta Cryst. D74, 215-27.
- Carlon et al. (2016) How to tackle protein structural data from solution and solid state: An integrated approach. Progress in nuclear magnetic resonance spectroscopy. 92, 54-70.

Tools for cryo-EM model fitting & refinement:

- Casanal et al. (2020) Current developments in Coot for macromolecular model building of electron cryo-microscopy and crystallographic data. Protein Science 29(4), 1055-64.
- Nicholls et al. (2018) Current approaches for the fitting and refinement of atomic models into cryo-EM maps using CCP-EM. Acta Cryst. D74, 492-505.
- Murshudov (2016) Refinement of atomic structures against cryo-EM maps. Methods in Enzymology, 277-305.
- Brown et al. (2015) Tools for macromolecular model building and refinement into electron cryo-microscopy reconstructions. Acta Cryst. D71, 136-53.

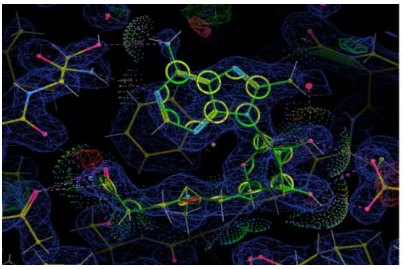
HT3 T4: Interactive Model Building, Ligand Fitting and Refinement with Coot

CCP4 Cloud (Krissinel et al., 2022) includes three in-built MX refinement tutorials:

1. Simple refinement – which focusses on adjustment of data-geometry weighting and inspection of refinement results.

2. Twin refinement – which demonstrates how to extract information about twinning from REFMAC5 output.

3.Ligand Dictionary Generation, Fitting and Refinement – which uses AceDRG to generate a ligand dictionary and conformer, Coot to fit the ligand into the macromolecular model, REFMAC5 to refine the protein-ligand complex model, and finally Coot to validate the refined ligand binding site.



It should be noted that the CCP4 suite only includes these tutorials if they were selected during installation. Otherwise, the CCP4 Cloud tutorials will only be available when using the "remote" version of CCP4 Cloud (ccp4cloud-remote), which uses the CCP4 server for project management and processing.

In this session we shall focus on working through the third tutorial and discussing the procedure of ligand dictionary generation, fitting, refinement and validation in CCP4. More information on this topic can be found in Nicholls (2017).

- Krissinel, E. et al. (2022) CCP4 Cloud for structure determination and project management in macromolecular crystallography. Acta Cryst. D78, 1079-89.
- Nicholls (2017) Ligand fitting with CCP4. Acta Cryst. D73, 158-70.

HT4 L1: Introduction to the CSD, CSD-Core and CSD-Discovery

CCDC Team

This talk will give participants an introduction to small molecule structure data in the Cambridge Structural Database (CSD) and how CSD-Discovery tools can be used to discover new molecules. It will provide an overview of a range of scientific applications within CSD-Core and CSD-Discovery that can be used in the drug discovery field, from knowledge driven conformer generation to the search and analysis of protein-ligand complexes.

Dr. Genevieve Evans

PDBe Biocurator
(& Structural Biologist)
Protein Databank in Europe

HT4 L2: Introduction to PDB

This talk will give participants an introduction to 3D macromolecular structure data in the Protein Data Bank (PDB).

The Protein Data Bank (PDB) archive is managed by the Worldwide PDB (wwPDB) consortium (wwpdb.org), who together collaborate on the development and support of the PDB archive. All experimentally determined structural biology data that is published is deposited at wwPDB. wwPDB member sites from around the world work together and coordinate data processing and weekly release of the world's structural biology data into one single PDB repository. This repository can be accessed from any of the wwPDB member websites.

Protein Data Bank in Europe (PDBe) is one of the founding members of the wwPDB and provides unique tools and enrichment of data to support understanding of structural biology data in the PDB archive.

This introductory talk will explore the type of data in the PDB archive, trends and changes, metrics to understand this data, the underpinning file formats, as well as some of the tools to explore the data and analyse structures.

Part of this session will be hands-on utilisation of Mol*.

Visualisation of molecular structure data is important when making assessment of structures. The focus will be on the Mol* (molstar.org) visualisation software, which allows users to view models and experimental maps within the browsers.

Learning outcomes:

- How to navigate Mol* and rapidly generate presentation-ready images.
- How to retrieve and assess the quality of structural data from the PDB and EMDB archives to find the best structure for your needs.
- Relevant metadata & validation metrics to consider.
- Pre-populated assessment of structural features in a structure,

for example:

- Protein-protein interactions
- Protein-ligand interactions

HT4 T1: Access, Visualization & Evaluation at PDBe & PDBe-KB

Part I:

The PDB archive is a freely-available database that predates the internet and reflects a generous spirit and foresight from the structural biology community. Analysis of this database has often yielded new insights, and these insights have driven the development of new tools and improvements to the process of determining new structures.

This hands-on session will focus on how to find an interesting data cohort for comparison / analysis using PDBe search tools and the PDBe webpages.

Learning outcomes:

- How to download a sets of coordinate files
- Tools for navigating coordinate files in mmCIF file format
- Search the PDB for different cohorts of data
- Step towards programmatic access
    - introduction to Solr-based queries

Part II:

PDBe-KB is a community-driven resource managed by the PDBe team, collating functional annotations and predictions for structure data in the PDB archive from the bioinformatic community. PDBe-KB is a flagship of the ELIXIR 3D-BioInfo Community (elixir-europe.org/communities/3d-bioinfo), an international group of researchers and software developers working on improving use of macromolecular structure data in life science research.

Learning outcomes

- Become aware of pre-populated analysis & information provided via PDBe-KB
    - Visualise and analyse sets of structures, pre-grouped
    - Structural superposition data, pre-determined
    - Sequence-based knowledge mapped onto a sets of structures
- Integrating PDBe-KB with alternative search approaches:
    - *g.* fold-centric search

Dr. Genevieve Evans & **Dr. Paulyna Magaña**

Protein Databank in Europe

HT4 T2: Harnessing PDBe-KB

If you are looking to access large volumes of PDB data for your own analyses, the best way for quick and automated access is to do it programmatically, instead of going through the website.

This session will introduce how to programmatically access data in the PDB. Various options for API (Application Programming Interface) access available from PDBe will be explored.

Dr. Paulyna Magaña

AlphaFold DB Bioinformatician,
Protein Databank in Europe

HT4 L3 & T3: AlphaFold Database: A Powerful Tool for Accelerating Scientific Research

AlphaFold has been shown to predict protein structures with high accuracy, rivalling or even surpassing experimental methods. The AlphaFold Protein Structure Database (AlphaFold DB), a collaboration between DeepMind and EMBL-EBI, enables free and open access to over 200 million protein structure predictions by AlphaFold. This includes nearly all catalogued proteins known to science.

Explore stories of AlphaFold's impact: unfolded.deepmind.com/

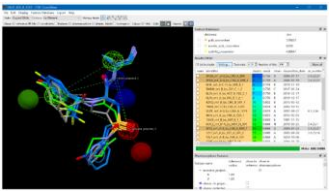
This presentation will provide an overview of the AlphaFold Database, including its background, how to access the predicted structures, how to interpret the data, and current developments. It will also highlight how the AlphaFold Database can be used to generate new hypotheses for testing, using specific case studies and hands-on examples.

HT4 T4: Mining crystallographic databases using CSD-CrossMiner

In this hands-on session participants will learn how to perform pharmacophore searches across the CSD and the PDB using CSD-CrossMiner.

Learning Outcomes:

- Familiarity with the CSD-CrossMiner interface and how it can be used in your research.
- How to interact with a pharmacophore query.
- How to perform pharmacophore searches across the CSD and PDB.
- How to analyse and interact with your results.



HT4 T5: Exploring interactions in the CSD and PDB using IsoStar and SuperStar

This hands-on session will give participants experience of exploring interactions in crystal structures. We will see how IsoStar and SuperStar uses crystallographic information about non-bonded interactions to generate interaction maps within protein binding sites or around small molecules, i.e. to predict 'hot-spots' where a chosen interaction type is particularly favourable.

Learning Outcomes:

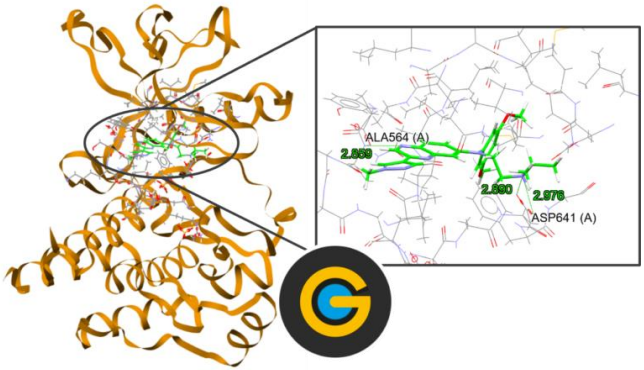
- How knowledge-based approaches can be used to understand interactions within a solid form.
- How to generate interaction maps within protein binding sites or around small molecules.
- Familiarity with CSD-Discovery tools that can help to understand interaction in the solid form.

HT4 T6: How to perform ensemble docking with GOLD?

In this hands-on session participants will learn how to perform ensemble docking using the CCDC's docking software GOLD.

Learning outcomes:

- The basics of ensemble docking.
- How to use the Hermes interface, the CCDC's 3D visualizer for proteins.
- Step-by-step set-up of an ensemble docking simulation in GOLD, including:
  - Superimposing the protein structures;
  - Binding site definition;
  - Ligand selection and ligand flexibility;
  - Functional waters.
- Visualization and analysis of ensemble docking results.



Abstracts

Deciphering SARS-CoV-2 3CL Protease: Exploring Structural, Functional Aspects, and Therapeutic Perspectives

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bhardwaj.mohit4@gmail.com

Recently, the world has confronted the emergence of SARS-CoV-2, part of the Coronaviridae family, which are +ssRNA viruses with a 5' Cap and 3' poly-A tail, capable of infecting animals and humans. SARS-CoV-2's life cycle depends on the primary role of its main protease (3CLpro) in cleaving viral polyproteins. 3CLpro operates as a catalytically active dimer, with each protomer housing a catalytic dyad (His41/cys145). Essential N- and C-terminal domains regulate 3CLpro's monomer/dimer equilibrium and catalytic function. Researchers are actively exploring novel methods to develop treatments targeting 3CLpro's, exploiting its highly preserved genetic sequences, (A) compounds aiming at inhibiting the catalytic site directly, and (B)reducing activity by focusing on the dimerization interface. Understanding the mechanisms governing dimerization is vital for the development of structure-based therapeutics against coronavirus. Our research endeavors to elucidate the role of interactions at the dimer interface as well as distant interactions within the protease and to determine whether dimerization is essential for activating 3CLpro's catalytic functions. Our investigation reveals the enzymatic structural alterations in response to compromised dimerization, resulting in the deactivation of catalytic activity. To further analyze these changes, we introduced mutations in crucial amino acid residues at the dimer interface, as well as those distanced from this interface and the catalytic site, assessing their impact on both oligomerization and enzyme activity. To evaluate the consequences of these mutations, we applied analytical methodologies, including GFC and Native-PAGE, to confirm alterations in oligomerization. Additionally, we assessed their impact on enzyme activity. Furthermore, we employed DSC and CD thermal analyses to compare the influence of these mutants on thermal stability. We further validated these findings through MD simulation analysis using RMSD, RMSF, ROG, and hydrogen bond analysis. Our preliminary results highlight the critical role of a specific amino acid in maintaining dimerization, demonstrated by its mutation leading to an immediate shift of the enzyme into an inactive monomeric state. The structural examination of these mutants will elucidate the vital function of a particular amino acid in maintaining the internal structure of the active site and aligning essential residues involved at the dimer interface and substrate catalysis.

Keywords: 3-Chymotrypsin-like protease; Allosteric Site; Gel Filtration Chromatography; Severe Acute Respiratory Syndrome Coronavirus

The Unique Function and the Structural Studies of FK506 Binding Protein 1A

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All proteins are composed of monomers called aminoacids, which are bound together by peptide bonds. These bonds have two conformations, cis- bonds where the side chains of adjacent aminoacids facing the same direction, and trans- peptide bonds where the side chains are facing opposite direction. Under normal circumstances, most aminoacids prefer trans- peptide bonds, as they are geometrically more feasible, with the exception of Proline. This aminoacid has a unique structure, where the side chain joining together with the N-terminal amino group, forming an 'imino' group instead, and thus, it can form both cis- and trans- peptide bonds. This ability of Proline is important, as isomerisation between two types of peptide bonds can cause shifts in the conformation and the function of the protein. Therefore, the isomerase protein families with such a unique function are highly important in the study of many cellular processes.There are three families of cis- / trans- peptide bond isomerases, cyclophilins, FK506-Binding Proteins and Parvulin.

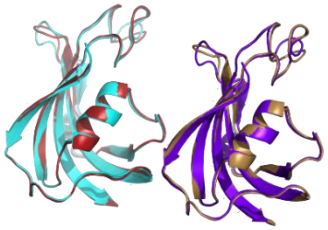

Figure 1. Structural alignment of FKBP1A cryogenic (100K) and ambient (300K) temperatures. We see a shift in the loop regions including a proline residue. There is no significant shift in the drug binding pocket.

FK506 Binding Protein (FKBP) is a family of proteins with a wide variety of cellular functions, including immune system regulation and proline-rich protein folding. In this study, we are looking into FKBP1A, one of the proteins in this family. FKBP1A is generally defined as a target for premier immunosupressants, like Rapamycin and FK506, in addition to its roles shared by its respective family. FKBP1A regulates two different cellular process, depending on the bound drug; FK506 binding is affiliated with Calcium-dependent Calmodulin pathway, resulting in regulation of Nuclear Factor of Activated T Cells (NF-AT), while Rapamycin binding is affiliated with mTOR complex, a key component in virtually every cellular process.

Here we are showing a atomic resolution (1.05 A) cryogenic structure of FKBP1A, determined with a home source X-ray, with our efforts to come up with a new and effective inhibitor for such a unique protein and structural studies of this protein at different temperatures, in an attempt to see meaningful differences in flexible regions and drug binding pocket.

Keywords: FKBP1A, Proline Isomerase, Immune System Regulation

Unlocking Efficiency: Structural Exploration of *Burkholderia ambifaria*'s CO_2- Fixing Enzymes

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Reductive enoyl-CoA carboxylases (ECRs) stand as the most efficient CO_2 fixing enzymes known, surpassing RubisCo by up to 100 times. Having unraveled the catalytics steps of ECRs, we are now on a quest to demystify their CO_2 binding mechanism, suspecting a novel pocket marked by an Asn-Phe/Tyr-Glu triade. My colleague and I are working on an ECR from *Burkholderia ambifaria,* which is a homotetramer. Until this point, we were able to determine the novel structures of *B. ambifaria* ECR in apo, NADP+ bound, and ethylmalonyl-CoA bound forms and try to understand the reaction mechanism by examining the different steps of the enzymatic reaction. However, our crystals are thin and small, and we are trying to get bigger crystals. In this collaborative project with Soichi Wakatsuki from Stanford University, we aim to enhance our crystals and our study to gain a deeper understanding of these highly efficient C-cycling enzymes.

Keywords: reductive enoyl-CoA carboxylase; carbon fixation; B. ambifaria; crystallography; enzymatic reaction mechanism

Fragment Based Active Site Exploration of Thermoset Degrading Enzymes

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Thermoset plastics are the one of main polymers that are widely used in almost every area from construction materials to kitchen sponges. The main advantages of thermosets are high resistance to physical and chemical factors due to highly crosslinked structures however, besides the very durable characteristics of thermosets, they are also very hard to recycle indeed less than 10 % are recycled in sophisticated and expensive ways. An alternative way of thermoset recycling would be the biocatalytic degradation and recycling by enzymes. Enzymatic Degradation of Thermosets Plastics is a multidisciplinary project in which biochemistry, structural biology, biophysics, computational biology, and synthetic chemistry fields collaborate to identify plastic degrading enzymes from various sources including soil-dwelling bacteria and fungi and then characterize enzyme kinetics and enzyme-ligand interaction by crystallography. Finally, polymer-enzyme interactions are investigated by biophysical methods and computational techniques. The important step in determining the structure of an enzyme-substrate complex is that soluble fractions of plastic polymer are used for crystallization and structure determination. Then, structural data from various soluble fragments are used to map active sites on the enzyme surface by using fragment-based approaches. Furthermore, due to the low affinity of fragments, weak electron density regions are refined by computationally averaging by using apo and ligand-bound enzymes. Also, the determination of structures generates lots of data from dozens of crystals. To handle with this, a Database will be designed to store all the necessary information about enzymes, crystallization conditions, and diffraction data. The database would ease the process of automated structure determination and ligand detection in active sites.

Keywords: Fragment Screening, Crystallography, Thermoset Degrading Enzymes

Human Serum Albumin's Unutilised Potential as a Cancer Targeting Agent

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Cancer research is not only about developing new drugs but also overcoming resistance mechanisms cancer tissues equip, no matter how effective a drug is, if the cancer becomes resistant to it, it is useless. As of date, a major focus of the medicine research is developing new delivery methods to combat resistance. Human Serum Albumin has required properties to combat resistance based on efflux pumps in addition to enhancing drug effectiveness via improving drug uptake to the tissue. Human Serum Albumin is the main transporter protein of the blood, In addition to most fat-soluble metabolites, it is also responsible for the transportation of neutral and acidic drugs, and their bioavailability hence absorption into the tissues. From a structural biology perspective, deciphering how a drug binds to the Human Serum Albumin can eliminate many of the derivatives to investigate new active ingredient development pipelines by accurately predicting which of the potential derivatives do not have appropriate binding to the carrier protein. Another property of Human Serum Albumin is that it can transcytose in the endothelial cells to the surrounding tissue to carry metabolites hence drugs, anything bonded to the Human Serum Albumin also gets carried to tissue. One interesting is that to siphon more nutrients from the bloodstream, cancer tissues increase the Human Serum Albumin receptors in their endothel resulting in uneven absorption of both nutrients and in our case drugs. This unevenness results in better targeting of cancer tissues. This transcytosis ability and increased transcytosis to cancer tissue has made Human Serum Albumin an excellent carrier. It is the most abundant protein in the blood serum hence the immüne system is indifferent to it and it has a half-life of 3 weeks, Its properties allow Human Serum Albumin to protect its cargo and the repetition is being taken advantage of nanoparticle formation for drug delivery methods. Regarding protein-structured drugs, some of them come genetically conjugated to Human Serum Albumin increasing both their absorption and half-life. Similarly, there are attempts to develop Human Serum Albumin nanoparticles with the potential of carrying a large number of drug molecules making the concentration increase of the drugs drastic enough to overwhelm efflux pumps.

Keywords: Cancer targeting, Humam Serum Albumin, Drug delivery, Transcytosis

Development of New Generation of Imatinib Using Structural Biology Techniques at Ambient Temperature

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Chronic myeloid leukemia (CML) is a kind of blood cancer and a group of life-threatening malignant myeloproliferative disorders that present with increased white blood cells in the bone marrow. Most CML patients have been associated with a chromosomal anomaly with the BCR-ABL fusion oncogene, which occurs as a result of a translocation between the Abelson murine leukemia (ABL1) gene on chromosome 9 breakpoint cluster region (BCR) gene on chromosome 22. Imatinib mesylate is the first development small molecule target that is a type of non-receptor tyrosine kinase targeted to the BCR-ABL fusion protein. Since 2001, it has quickly become the frontline therapy for treating CML worldwide. Despite a high response rate in CML patients with imatinib therapy, almost one-third of patients still have an inadequate response to Imatinib. Imatinib reduces BCR-ABL activity by binding to the inactive conformation of tyrosine kinases. However, due to mutations in various regions of the BCR-ABL kinase, resistance to Imatinib has begun to occur in CML patients. Therefore, there is a need to develop a more potent new molecule with an imatinib function that is more resistant to mutations. This study aims to re-evaluate the inactive conformation of the BCR-ABL fusion protein kinase domain, which is in the imatinib binding domain. Rosetta-2 E. coli strain has been used to express the gene encoding our target protein. Purified proteins are then used for crystallization and co-crystallization. Then, the 3D structures of the best crystals are revealed by using X-ray crystallography diffraction, which is a more dynamic and more disruptive way with the high-resolution. These diffraction dates which are from the best crystals will be collect from the XtalCheck module using Terasaki plates (Rigaku Oxford Diffraction) at ambient temperature. Finally, we will obtain the apo crystal structure of ABL kinase and complex with Imatinib and also the mutant version. The ultimate aim of this research project is to produce a new generation of Imatinib to treat CML disease and improve patients' lives.

Keywords: Chronic myeloid leukemia, Imatinib, BCR-ABL, drug resistance, X-ray crystallography

Modeling of AD-related conformation of monomeric tau protein

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A group of proteins that lack a stable three-dimensional structure under physiological conditions, called intrinsically disordered proteins (IDPs), has been the subject of increasing attention over the past few decades. Such proteins retain their structural flexibility in unbound form and prevalently undergo folding process upon binding to their partners. Thanks to these properties, IDPs play key roles in cell proliferation and signaling processes, but also cause misfolding, missignaling and/or oligomerization processes and are often associated with several human diseases. The representative of IDPs is the microtubule-associated protein tau, which forms insoluble deposits in the brain of Alzheimer's disease (AD) patients [1]. Abundant posttranslational modifications drive the dissociation of tau monomers from microtubules and the gain of toxic function. Therefore, inhibiting aggregates' formation seems like a promising therapeutic strategy for fighting AD. In our study, we are focusing on truncated tau protein comprising residues 321-391, recognized by conformational AD-specific monoclonal antibody DC11 allowing us to study specific tau conformation.

Monoclonal antibody DC11 discriminates very strictly between physiological and truncated tau proteins, implying the presence of a conformational epitope that carries pathological functions in the pathogenesis of AD [2]. So far, we have obtained a DC11 Fab fragment crystals with a diffraction limit of 1.29. Å. Structure of DC11 was solved by molecular replacement, and further used in molecular docking to investigate local motifs of the tau (321-391), which initial conformers were generated by AlphaFold 2. From NMR spectroscopy epitope mapping experiments we were able to identify tau residues involved in the interaction with the antibody. In order to conduct complex structural study of the DC11 tau epitope, a combination of experiments and computational methods is the prefential approach to be used. [1] Braak H. et al., Journal of Neuropath. Exp. Neur. 70, 960–969 (2011) [2] Vechterova L., et al., NeuroReport 14, 87–91 (2003) This work was supported by grant no. APVV 21-0479, VEGA 2/0125/23 and MSCA-RISE no. 873127.

Keywords: IDP; protein tau; crystallography; docking

Structural characterization of the domestic cat allergen Fel d 4

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Throughout the years pets have been recognized as one of the major sources of indoor allergens posing a great risk for the development of IgE-mediated diseases such as allergenic rhinitis and asthma.[1] In particular, cats (*Felis domesticus*) are a significant source of allergens found in their fur, saliva, serum and urine. To date, the WHO/IUIS database reports eight cat allergens, designated as Fel d 1 to Fel d 8. Among these allergens, the frequency of IgE recognition and allergen-specific IgE reactivity is found to be the highest for Fel d 1, Fel d 4 and Fel d 7 making them clinically most important cat allergens.[2] Fel d 4 belongs to the lipocalin protein family from which more than 20 proteins of different animals are listed as allergens. Allergic sensitization towards a specific pet is rather uncommon due to a high cross-reactivity found among different pet allergens. Fel d 4 shares high sequence identity with the lipocalin allergens predominantly from dog (Can f 6) and horse (Equ c 1). These allergens contribute to polysensitization triggering allergic responses caused by contact with different animals.[3] The structural analysis of these allergens and linear and conformational epitope studies are valuable assets for the improvement of diagnostic approaches as well as the development of specific immunotherapies. We generated a recombinant protein and employed X-ray crystallography to reveal the structure of the lipocalin cat allergen Fel d 4. We confirmed that Fel d 4 has a typical lipocalin fold, consisting of 8-stranded β-barrel and α-helix, and a high structural similarity to Can f 6 and Equ c 1. Based on the obtained structural data we performed in silico structure-based investigation of the allergen epitopes. Our work aims to understand the specific immunoglobulin binding sites of the Fel d 4 and contribute to the understanding of the polysensitization related to pet allergy.

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Keywords: allergy; cat; Fel d 4; X-ray crystallography

Symmetry Matched Protein – Macrocycle Assembly

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Anionic water-soluble macrocycles, such as calixarenes, are adept at protein recognition and assembly.[1,2] A derivative of cyclotriveratrylene (**CTV**), **WPCTX** is a phosphate-containing C_3 symmetric macrocycle (Fig 1A).[3] We report three distinct co-crystal structures of **WPCTX** with the C_3 symmetric, 6-bladed β-propeller *Ralstonia solanacearum* lectin (RSL). Each of the three structures is mediated by a tetrameric cluster of **WPCTX**. This tetramer has been previously reported in **CTV** derivatives.[4] Three ligands in the cluster encapsulate the N-terminus of RSL in their cavity. The cluster also forms exo interactions with either Lys34 or Lys25. One of the structures is a metal-mediated protein – macrocycle assembly, in which zinc coordinates both the protein and macrocycle (Fig 1B). These structures present a new type of macrocycle available to aid protein crystallisation while also suggesting how molecular building blocks can be used to design and control protein-based frameworks.

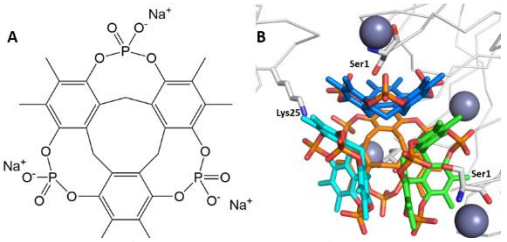

Figure 1. **A)** Structure of **WPCTX** **B)** Tetrameric cluster of **WPCTX** in RSL – **WPCTX** *H*32 form. Three macrocyclic units encapsulate a Zn bound N-terminal serine. A fourth Zn coordinates three phosphates of **WPCTX**. Lys25 forms exo interaction with the cluster.

Keywords: Protein; Macrocycle; Assembly

Co-crystallization of tau proteins mediated by monoclonal antibodies

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Tau protein involved in the pathogenesis of Alzheimer's disease and related tauopathies is intrinsically disordered protein (IDP). IDP tau, being devoid of stable 3-D structured domains, still can exhibit local propensities to adopt transient structural motifs, which can dictate its physiological fate. These motifs of tau are not amenable to X-ray crystallography in monomeric form. However, transient conformation can be captured in crystallisable complexes with tau binding partners. Antigen-binding regions (Fab) of monoclonal antibodies serve as chaperones in complex with tau and facilitate conformational folding and thus crystallization of the complex (Skrabana et al., 2010).

A monoclonal antibody, MN423, is able to bind only to the filament core of tau that terminates at Glu391. This fact enabled to solve the structure of tau 386TDHGAE391 region at 1.65A resolution by co-crystallization of MN423 antibody in complex with tau306-391 fragment. The solved region of tau protein proposed its role in PHF assembly (Sevcik et al., 2007). The portfolio of monoclonal antibodies suitable to study tau transient conformations includes DC25 with epitope 347–353, DC190 with epitope 368-376 (Weisova et al., 2019) and others.

The aim of my project is to prepare novel tau-Fab complex and focus on small structural motives included in the amyloid misfolding of tau. As the prevalence of Alzheimer's disease is not declining, on the contrary – it is rising, this project has a great importance and potential.

This project is supported by APVV-21-0479, VEGA 2/0141/23, 2/0125/23 and EU Grants ADDIT-CE, InterTau.

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Keywords: disordered proteins, tau protein, co-crystallization, monoclonal antibodies

Exploring β-Carbolines as Allosteric Modulators of 14-3-3 Proteins: *A Comprehensive Approach from Virtual Screening to Crystallography*

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The 14-3-3 protein family, consisting of seven distinct mammalian paralogs, has emerged as a pivotal mediator in numerous cellular processes. These proteins function by binding to phosphorylated Ser and Thr residues of partner proteins through a conserved amphipathic groove on their surface (Aitken, 2006). Consequently, 14-3-3 proteins have garnered significant attention as attractive targets for drug discovery (Aghazadeh & Papadopoulos, 2016). In order to detect distinct druggable hot- spots, we decided to make a virtual screening of 20 million compounds using ZINC database over a monomer of 14-3-3. For this, we used Autodock 4.0 for molecular docking. We detected a list of 50 compounds that belong to a family of β-carbolines with high affinity binding to a specific region located on the opposite face of the main binding groove of 14-3-3. We decided to use parental β-carboline – Norharmane (NH) as a first approach for further *in silico* assays. Molecular dynamic simulations demonstrated that the amino acids that conform such cavity drive the dynamics of the whole protein. The presence of NH induced a conformational shift, causing the protein to transition into a closed state. This conformational change obstructs partner accessibility to the primary groove. Further on, to obtain paralog specific compounds, a set of endogenous beta-carbolines were studied with a combination of pharmacophore-based docking and enhanced molecular dynamics simulations, selecting candidates by binding affinities and residence times. Collectively, our findings underscore the existence of small molecules like members of the beta-carboline family with the potential to modulate 14-3-3 proteins through a novel allosteric site. To validate the NH binding site on 14-3-3 proteins, we have co-crystallized NH with two 14-3-3 paralogs. The paralogs were expressed in BL21(DE3)pLysS bacteria and purified using His Trap columns and size-exclusion chromatography. In a primary crystallization screening we identified one condition (0.1 M HEPES at pH 7.5 and 20% w/v PEG 10000) giving promising crystals of NH putative complexes. The crystals diffracted to 6 Å resolution at synchrotron X-ray source. Now we are focusing on obtaining better diffracting crystals allowing for structure solution. The convergence of *in silico*, *in vitro*, and *in vivo* methods promises to discover and optimize agents targeting this site in diverse 14-3-3 paralogs.

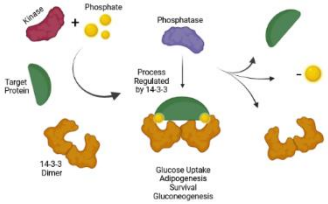

Figure 1. Mechanism of Action of 14-3-3 Proteins in the Regulation of its Target Proteins

Grant: MSCA-RISE InterTAU No 873127
Keywords: 14-3-3 Proteins; β-Carbolines; Allosteric Modulation; Drug Discovery.

Structure-function relationships of N-N bond forming enzymes.

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Molecules containing an N-N bond are widespread in Nature. Many of these compounds are known to have potent biological activities like anti-malarial, anti-apoptotic, and anti-bacterial activity. One example and the focus of our project is piperazic acid, the only known amino acid with N-N bonds. The biocatalytic synthesis of piperazic acid derivatives starting from L-ornithine involves two enzymes: a FAD-dependent monooxygenase and a heme/flavin-dependent enzyme. The FAD-dependent monooxygenase is responsible for hydroxylating the d-amino group of ornithine, whereas the heme-dependent enzyme catalyzes the formation of an N-N bond, resulting in a 6-membered cyclic structure of piperazic acid (1, 2, 3).

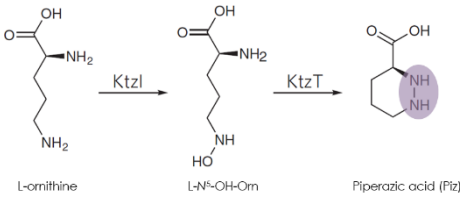

Figure 1. Formation of N-N bond resulting in piperazic acid synthesis.

In this study, we are trying to understand the structure, characteristics, and significant interactions involved in substrate binding for the heme-dependent enzyme. The crystallographic structure of the FAD-dependent monooxygenase is already known (4). We further plan to develop engineered variants of these enzymes and discover other substrates and enzymes that form piperazic acid-containing products.

Keywords: FAD-dependent monooxygenase; Heme-dependent piperazate synthase; N-N bond;

Exploring the role of active site residues of *Rhizobium etli* L-asparaginase in catalysis

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Asparaginases, which catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia, are grouped into three structural Classes. Some Class 1 (bacterial-type) L-asparaginases have been used as very effective antileukemic drugs. However, their administration is often associated with serious side effects. Therefore, alternative sources of therapeutic L-asparaginases are critically needed. From among the three Classes of L-asparaginases, Class 3 enzymes (also called *Rhizobium etli*-type) are the least structurally characterized. The recently solved crystal structure of ReAV, the inducible L-asparaginase from *R. etli* [1], shows no similarity to known enzymes with this activity, but has curious resemblance to some serine β-lactamases or glutaminases. This makes ReAV potentially interesting as a novel antileukemic agent. The putative active site region of ReAV comprises two Ser-Lys tandems (S48-K51 and S80-K263) and a nearby zinc coordination center formed by C135, K138, C189, and a water molecule. There is also a puzzling water triad tightly H-bonded around the S48 hydroxyl group, possibly indicating the catalytic nucleophile. A recent pan-genomic analysis of L-asparaginase distribution within the bacterial kingdom [2] highlighted the philogenetically strictly conserved residues, including the residues involved in zinc coordination (C135, K138, C189), residues from the Ser-Lys tandems (S48, K51, S80, K263), and residues located in the close vicinity of the metal binding site (R47, H139, Y156, D187, C249). We applied site-directed mutagenesis coupled with enzymatic assays and X-ray structural analysis to decode the role of the selected residues in catalysis. In most cases, substitution of the highly conserved residues with alanine completely abolished the L-asparaginase activity. Interestingly, modification of K138 from the metal coordination sphere rendered the mutant protein partially active and its K_m value highly sensitive to the presence of zinc cations, making it an excellent target for co-crystallization with the substrate molecule. We have been able to crystallize 10 ReAV variants and solve their crystal structures, revealing some intriguing variations in the active site area as a result of the mutations, and providing useful hints about the catalytic mechanism. Work supported by National Science Centre (NCN, Poland) grant 2020/37/B/NZ1/03250.

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Keywords: hydrolase, L-asparaginase, leukemia, site-directed mutagenesis, X-ray crystallography

Computational analysis of purine nucleoside phosphorylase dynamics

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Proteins, the fundamental participants in all cellular processes, exhibit intricate dynamics crucial for their diverse functionalities. This study delves into understanding the dynamic behaviour of proteins using advanced molecular dynamics simulations and computational analysis techniques. Focusing on hexameric purine nucleoside phosphorylase (PNP) from *Helicobacter pylori*, a key enzyme in nucleotide metabolism, [1] we investigate the allosteric communication pathways within its oligomeric structure.

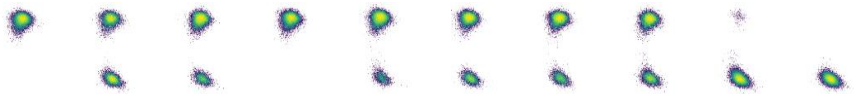

Figure 1. Time dependent Ramachandran diagram

By employing a combination of structural bioinformatics, molecular dynamics simulations, and data mining algorithms, [2] we aim to unravel the complex interplay of conformational changes and correlated motions governing PNP function. Our research not only contributes to elucidating the allosteric regulation mechanisms in PNP enzymes but also provides insights into general principles of protein dynamics and function.

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[2] https://alokomp.irb.hr/ - funded by Croatian Science Foundation, Grant number IP-2019-04-6764 (access date 13th of February 2024)

Keywords: Helicobacter pylori, Purine nucleoside phosphorylase, Python

Location is everything: Influence of His-tag fusion site on properties of adenylosuccinate synthetase from *Helicobacter pylori*

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Protein purification procedure normally has to be custom-tailored for each protein, as it depends on protein properties, as well as the purpose of protein production[1]. Optimization of the procedure can take a long time and significant amount of the sample, hence the protein purification was (and often still is) considered as the bottleneck of protein research. Affinity tags are proven to be tremendously effective tools for a wide variety of applications, primarily in shortened protein purification protocols, but also e.g. in immobilization of proteins for display on a surface, in detection and quantification of target proteins, in analysis of protein-ligand and protein-protein interactions[2]. Today, IMAC (Immobilized-metal affinity chromatography) is used routinely for purification of proteins for various purposes, and it is generally thought that small size and neutral charge of His-tag don't influence structure and function of the protein[3]. However, more and more reports are published stating negative effect of His-tag on protein's oligomerization state, structure, dynamics, function and activity[4].

We have constructed two variants of adenylosuccinate synthetase (AdSS) from *Helicobacter pylori* – with His_6-tag on N- or C-terminus. Both variants were purified (by IMAC and size-exclusion chromatography), characterized (protein stability, enzyme kinetics) and their properties compared together with wild-type variant of the enzyme. N-His-AdSS and C-His-AdSS were also crystallized and their 3D-structures determined. All our results combined indicate diminishing effect of His_6-tag only on N-terminus of *H. pylori* AdSS on the enzyme's properties.

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Keywords: adenylosuccinate synthetase; Helicobacter pylori; His-tag; enzyme kinetics; protein 3D-structure

Structure-based search for inhibitors of monoacylglycerol lipase of *Mycobacterium tuberculosis*

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Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis* (Mtb), poses a major health threat. The complexity of Mtb's life cycle presents a significant challenge, particularly its ability to enter a dormant phase characterized by low metabolic activity. This dormancy makes the bacterium elusive to traditional pharmaceutical interventions. One of the strategies used by Mtb during dormancy is to use host lipids as a primary source of energy. In this complex landscape of Mtb's biology, the enzyme monoacylglycerol lipase Rv0183 (mtbMGL) emerges as a critical player. The protein is involved in the degradation of host cell lipids, facilitating Mtb's survival during dormancy. Its potential as a drug target lies not only in its central role but also in its unique characteristic of being exported out of the cell. This export mechanism enhances its accessibility and druggability, making it an attractive target for therapeutic intervention. To exploit this opportunity, collaborative efforts with institutions like the European Lead Factory have been initiated. The aim is to identify specific inhibitors of mtbMGL that selectively target the bacterial enzyme without affecting its human orthologue. This specificity is crucial to minimize potential off-target effects and maximize therapeutic efficacy. We collaborate with the European Lead Factory to find specific inhibitors for mtbMGL that do not interact with the human orthologue. With a combination of activity assay-based screening and crystallographic data, we determined structural information about four inhibitors of mtbMGL. Furthermore, analysis of enzyme-inhibitor interactions has revealed similarities shared among these compounds, providing a foundation for rational drug design and optimization. By understanding the structural nuances of these interactions, we can tailor inhibitors to enhance potency, selectivity, and pharmacokinetic properties, ultimately advancing the development of novel anti-tuberculosis therapies. In summary, targeting mtbMGL with specific inhibitors represents a significant advancement in the fight against tuberculosis. Collaborative efforts and innovative research approaches are bringing us closer to developing effective treatments that can combat Mtb's resilience and overcome the challenges posed by its complex biology.

Keywords: Tuberculosis; Mycobacterium tuberculosis; lipid metabolism; structure-based; inhibitor

Characterising chromatin-binding domains in KAT6A histone acetyltransferase

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KAT6A is a multidomain histone H3 acetyltransferase and epigenetic reader that regulates gene expression in haematopoiesis and tissue/organ development. Chromosomal rearrangements fusing KAT6A N-terminal half, comprising the nuclear localisation domain (NEMM), double PHD finger (DPF) and MYST domain, to other gene regulators (such as CBP/p300 or TIF2) are associated with rare acute myeloid leukaemias (AML). Mutations within the KAT6A gene have been linked to paediatric global developmental delay disorders, affecting speech, intellectual ability, digestion, feeding and cardiac development. While the DPF, which binds histone H3, and the catalytic MYST domain have been structurally and functionally well characterised, the structure of the NEMM domain is predicted to form two Winged-Helices (WH1+2) and is vital for leukemogenicity of fusion proteins and KAT6A chromatin targeting. However, the precise structure and function of KAT6A WH1+2 domain remains unclear.

Our work has uncovered that KAT6A WH1+2 possesses DNA-binding activity and can bind various DNA sequences, with a selective preference for unmethylated CpG motifs. Electrophoretic mobility shift assays (EMSAs) and mutagenesis pinpoint WH1 as a CpG-specific binding domain through a conserved positively charged motif, whilst WH2 contributes non-sequence specific DNA binding. Ongoing crystallisation trials with protein-DNA complexes have yielded fragile needle-shaped crystals, but further optimisation is needed to achieve diffraction to high resolution (≤2A). In parallel, we have begun crystallisation trials to provide an updated characterisation of other KAT6A chromatin-binding regions. Future work will use cryo-EM and biochemical methods to elucidate how KAT6A WH1+2 and neighbouring chromatin-binding domains interact with nucleosomes. This comprehensive characterisation not only improves our understanding of KAT6A in health and disease but also holds potential for future therapeutic development.

Keywords: Acetyltransferase; Protein-DNA complex; Structural biology

Protein allostery – a programmatic approach

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X-ray crystallography gives us the best insight into the structure of proteins, giving us a picture of what these large molecules look like at the atomic level. However, the main drawback of this method is that it only gives us one of the many possible conformations that the molecule takes in time. To study allostery, as a fundamentally dynamic phenomenon, it is necessary to use molecular dynamic simulations (MD) based on crystal structures as starting points. The results of MD simulations are trajectories that contain the positions of thousands of atoms in millions of steps and therefore represent a very complex data set. The methodology and programs for the visualization of MD trajectories as a series of time-dependent angular diagrams will be presented, as well as the monitoring of correlations between the movements of individual protein parts as a basis for the study of allostery[1].

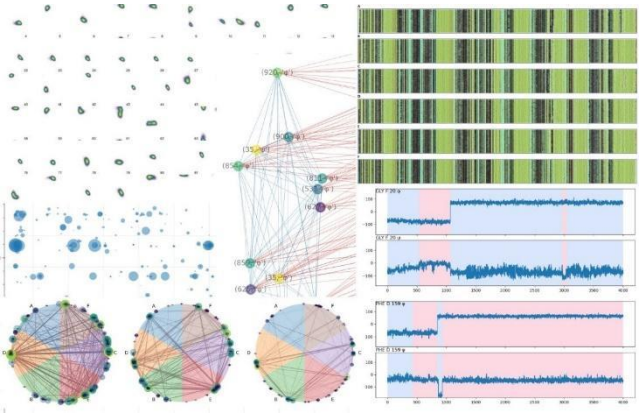

Figure 1. Various visual representations of long MD simulations of the oligomeric protein obtained by programmatic means.

[1] This research is a part of the ALOKOMP project and is financed by Croatian Science Foundation (project grant IP-2019-04-6764).

Keywords: Protein allostery, Purine nucleoside phosphorylase, MD simulations, Python

Unveiling Hub Protein Properties in Bacteria

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Hub proteins play a pivotal role in cellular regulation by interacting with numerous other proteins. Understanding the unique properties that enable these proteins to engage with multiple binding partners is crucial for elucidating their functional roles. This study investigates whether hub proteins share common features that predispose them to have a large number of interactants. To accomplish this, I analyzed datasets of hub and non-hub proteins extracted from three interactome databases, encompassing a diverse range of evolutionary distant bacterial species, including E. coli, B. subtilis, M. genitalium, H. pylori, P. aeruginosa, M. tuberculosis, Synechocystis sp., and S. coelicolor. Proteins were classified as hubs if they had 10 or more confirmed interaction partners in at least two databases, whereas non-hubs were defined as proteins without any known interaction partners in these databases. Significant differences were observed in gene ontology terms between a hub and non-hub datasets. However, no specific domains were consistently enriched across both groups, indicating that hub properties might be attributed to other intrinsic features of the proteins. Subsequent analysis focused on different protein features, such as intrinsic disorder and amino acid composition. The results show that the presence of intrinsically disordered regions is a key element in predicting whether proteins function as hubs. Across all studied proteomes, hub proteins exhibited a markedly higher prevalence and count of disordered regions compared to non-hub proteins. In solved crystal structures, these regions often correspond to interactive loops or extended N- and C-terminal unstructured domains. The amino acid analysis within these disordered regions highlighted a significant enrichment of lysine and arginine residues in hub proteins that probably contribute to their ability to interact with multiple binding partners and regulate various biological processes. In conclusion, this study highlights the distinct characteristics of hub proteins, particularly the dominance of intrinsically disordered regions and specific amino acids, in their capacity to engage with multiple partners and regulate diverse biological processes.

Keywords: hub proteins, bacteria, interactions, intrinsically disordered regions

In Depth Crystallographic Study of Pancake Bond Between Radical Cations in Crystals – Novel TMPD+ Chloride Salt Co-crystal With 2,5-Dichlorohydroquinone

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Pancake bond is an interesting type of π–interaction that forms between conjugated π–systems such as planar organic radicals. Such interactions can have non-negligible covalent character and they can also contribute to properties of the material, like electric conductivity and magnetism.[1,2] Pancake bonds are described as two-electron multicentric bonds as they involve multiple centers. Since crystals of planar organic radicals represent a great playground for studying pancake bond, here we present a crystallographic study on a novel compound, TMPD chloride salt cocrystal with 2,5-dichlorohydroquinone (1) with pancake bonded TMPD cation dimers. N,N,N′,N′-Tetramethyl-p-phenylenediamine (TMPD or Wurster's blue) has been known as an electron donor since 19th century and as such it can be a strong basis of compounds with interesting properties.[3] In this work, mentioned phenomenon is studied in detail by different crystallographic methods, by variable-temperature crystallography (80 – 400 K) and X-ray charge density.[4] These methods show changes in the structure caused by cooling or heating the crystal and reveal fine details of electron density between the stacked radicals. This work represents the first study of charge density between pancake-bonded radical cations.

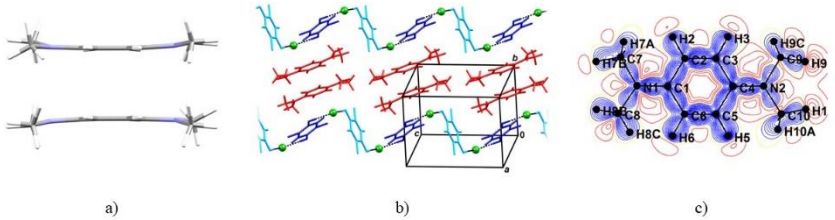

Figure 1. a) Dimer of TMPD+ radical cations; **b)** crystal packing of compound 1; **c)** deformation density map of TMPD+ radical cation in compound 1 after multipole refinement.

Keywords: crystallography; pancake bond; radical cations; charge density

Influence of N7G modification on RNA

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The research presents new focus on biocrystallographic studies of RNA duplexes containing in the sequence either guanosine or its regioisomer N7G, N7-2'-deoxy-guanosine, in order to investigate the influence of this modification on RNA structure.

As our co-workers found out during thermodynamic studies, introducing this modification in place of guanosine causes the increase in duplex stability for certain specific cases. This said cases are duplexes that contain non-canonical (non-Watson-Crick) base pairing of N7G with the nucleotide of complementary strand (namely G-A and G-G "mismatches").

The aim of the research is to obtain crystallographic data to examine structures of all of those previously studied duplexes, characterize differences and similarities between them, and combine this new biocrystallographic studies with previously obtained results from thermodynamic studies. We would like to find out the structural differences between helices with N7G modification and normal guanosine, following that with the differences between the helices containing the G-A and G-G base pairing and lastly, to confirm if there is any sequence specificity. The results would hopefully shed some light on why the increase in stability occured in certain cases

Current progress of the work includes synthesizing 8 oligonucleotides with different nucleotide modifications, obtaining 8 different, comparable RNA duplexes and succeding in obtaining crystals of two of them. There are ongoing efforts to optimize crystallizations and obtain crystalline forms of other duplexes.

To summarize, studied modifications of RNAs include N7G regioisomer of guanine, "mismatches" like G-A and G-G, other modified nucleosides, and combined two or three different modifications in one structure.

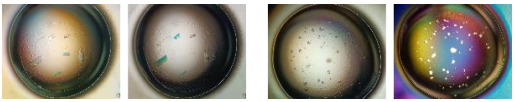

Figure 1. Photos of crystals obtained for two duplexes with N7G modifications; left: MG1-3 duplex with N7G-A base pairing; right: MG1-4 duplex with N7G-G base pairing.

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Keywords: RNA; RNA modification; N7G; biocrystallography

Development of Structural Analysis Services of Protein-ligand Complexes in 'Fast track' mode

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According to modern knowledge, proteins are involved in cellular processes and their abnormalities are responsible for most known diseases making them promising molecular targets for drug design. Understanding the essential molecular interactions that define life processes is dependent on the ability to capture the finest details at the molecular level. The capacity to characterize the three-dimensional structures of proteins is one of the most important achievements of modern science accelerating the discovery of new drugs and their subsequent improvement.

Crystallographic studies of the protein-ligand complexes facilitate a rational design of drug molecules, which are potent and more specific allowing to reduce side effects and frequency of use. Moreover, such research allows more effective discovery of innovative drugs and therapies for previously incurable diseases.

However, the development and optimization of production and crystallization of a particular protein target is a highly time-consuming procedure and often the bottleneck step in the drug discovery process.

To overcome this limiting step, meet Clients' expectations and support the growing interest in SBDD programs, Selvita developed the 'Fast track' analysis. The developed platform involved the creation of a library of proteins and their structures for which all necessary materials and processes have been prepared, developed, optimized, and tested allowing to deliver the final result of the analysis in a relatively short time (depending on the selected protein from one month up to three months).

The created library includes proteins with already known and unknown structures. The selected proteins are strategic therapeutic targets for drug development available on the market (e.g., estrogen receptor) or are highly attractive for therapeutic purposes due to scientific reports (e.g., maternal embryonic leucine zipper kinase). An extensive approach of crystallization screening resulted in a comprehensive characterization of previously unpublished Type I iodothyronine deiodinase structure. Presented results display the design of the platform, solutions for protein production and crystallization bottlenecks, and altogether optimization of all processes that lead to establishing a fast pipeline that accelerates the SBDD research for our Clients.

Keywords: protein crystallography, structure-based drug design, fast track analysis, protein-ligand complexes, protein library

Development of Novel X-ray Crystallography Services at Selvita

Bebel A., Szubert F., Zrubek K., Mrugala B., Siuda M., Biterova E., Dawidowski D., Kumar M., Stach N., **Zieba B.**, Cukier C., and Lupa B.

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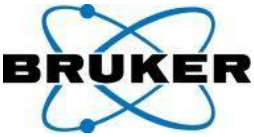
Macromolecular X-ray crystallography has been an enabling method in drug discovery in terms of both mechanistic understanding of protein-small molecule interactions and the development of new interacting compounds. As a drug discovery-oriented CRO, Selvita has developed a state-of the-art X-ray Crystallography Laboratory to serve the needs of complex integrated drug discovery projects as well as stand-alone protein analysis services. As part of the constant growth, we developed two novel service platforms: the 'Fast track' protein-ligand structural analysis platform and the Fragment-Based Drug Discovery (FBDD) platform. To facilitate a rational design of drug molecules, high-resolution structural information is essential. However, optimization of protein production and crystallization is highly time-consuming and often presents a bottleneck in the drug discovery process. To overcome this limiting step, we have developed the 'Fast track' structural analysis platform that includes a library of therapeutically relevant proteins and their structures together with tools that allow us to quickly deliver new structures with bound ligands of choice. The library includes proteins of both previously known and unknown structures, with targets strategic for drug development (such as estrogen receptor) or highly attractive for therapeutic purposes (e.g., MELK). Here, we present the design of the platform, our solutions to protein production and crystallization bottlenecks, and exemplary results obtained for the targets with previously known and unknown structures. Another crucial step in drug development is the identification of novel small molecules that modulate protein activity. FBDD has emerged as an effective method to sample low molecular weight, chemically diverse scaffolds, and led to the development of multiple drugs currently on the market or in clinical trials. Macromolecular X-ray crystallography (MX) has achieved substantial success as a primary screening method in multiple FBDD campaigns. Accordingly, at Selvita we have developed a high-throughput MX-based FBDD platform, additionally supported by our proprietary diverse fragment library and biophysical validation tools. Here, we present the details of MX capabilities within the FBDD platform, as well as the results of two MX-based FBDD case studies focused on searching for novel fragment binders of Endothiapepsin from E. *parasitica* and human VBC complex.

Keywords: FBDD; X-ray crystallography; drug discovery



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