Advanced methods in macromolecular crystallization VIII

(FEBS PC18-001)

Academic and University Center, Nove Hrady, June 10 - 16, 2018

Lectures - Sunday, June 10

L1

PRINCIPLES OF PROTEIN CRYSTALLIZATION I: THE NATURE OF PROTEIN CRYSTALS AND THE PHYSICAL CHEMISTRY OF THEIR FORMATION

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Protein crystallization is the self-assembly of protein molecules into an ordered, periodic structure, the protein crystal. Protein molecules however are large, complex, and flexible molecules and most proteins are therefore difficult to crystallize. To understand how to find conditions that allow crystal formation, we need to understand the physicochemical nature of proteins and how to modify their solubility and local surface property distribution. Once we understand what conditions mustbe fulfilled for crystallization to occur, the question is how to (a) obtain a protein that actually can crystallize, and (b) how to efficiently sample the multitude of possible reagent combinations that might provide the right conditions. The initial screening or sampling then informs us how to proceed further and how to optimize crystal growth, and often also indicates that further examination and modification of the protein itself may be necessary to achieve successful crystallization.

L2

CAPILLARY COUNTERDIFFUSION TECHNIQUE FOR PROTEIN CRYSTALLIZATION AND SCREENING

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Protein crystals are always grown from aqueous solutions and therefore actual crystallization experiments are affected by gravity. Typically, protein crystallization experiments display phenomena such as evaporation, sedimentation, and convective mixing, that alter the homogeneity of the volume of the solutions, and that sometimes provoke unwanted complex dynamics, which are difficult to control. Therefore, removing gravity driven phenomena, such as sedimentation and convection, is a way to control the space and time evolution of the experiments. Also, it also opens an alternative approach to design protein crystallization experiments by coupling diffusion mass transport and chemical precipitation.

In this talk, I will introduce the fundaments of the crystallization method named counterdiffusion, which is based on the coupling of the precipitation and the diffusion transport of the molecules of proteins and antisolvents used to reduce the solubility of the protein. The technique can be performed with different implementations, namely in gels, in capillary volumes and low gravity environments in space. I will introduce first the fundamental basis to understand the time evolution of the supersaturation and supersaturation rate along the crystallization reactor and how this can be used to design very efficient screening of the crystallization conditions. Then, I will explain why and how this technique can be utilized for the optimization of crystal size and crystal quality of proteins. The different implementations will be shown with the help of video demonstrations.

F. Otálora, J. A. Gavira, J. D. Ng. and J. M. García-Ruiz. Counterdiffusion methods applied to protein crystallization. Progress in Biophysics and Molecular Biology 101 (2009) 26-37.

J. M. Garcia-Ruiz and L. A. González-Ramírez, Capillary counterdiffusion experiments with prefilled Granada Crystallization Boxes. Protein Crystallization: Second Edition Terese Bergfors, Ed; IUL Biotechnology Series. International University line 2009; pp 395-400.

L3

CRYSTALLIZATION OF MEMBRANE PROTEINS IN LIPIDIC SYSTEMS

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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 currently 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 given at the *FEBS Lab Excercises* on Tuesday, June 28.

Useful 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., 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. J. Vis. Exp. 45: www.jove.com/index/details.stp?id=1712 (doi 10.3791/1712). www.jove.com/index/details.stp?id=1712

http://www.tcd.ie/Biochemistry/research/publications_mcaffrey.php

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L4

CONVENTIONAL CRYSTALLIZATION METHODS AND THEIR MODIFICATIONS

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Once the solubility of the protein has been optimized (Hofmeister series and DLS), typically simple hanging or sitting drop vapor diffusion experiments are used in order to obtain protein crystals suitable for single crystal X-ray diffraction experiments. The traditional type of experiment can be modified by several pre and post set-up techniques to overcome some of the shortcomings of the classical crystallization vapor diffusion technique:

A selection of pre set-up, vapour diffusion experiment alterations

1. one-for-all reservoir solution
2. use of dyes or fluorescent dye-labelled proteins
3. addition of proteases for *in situ,* limited proteolysis
4. microseed matrix seeding to outwit nucleation
5. insertion of an oil barrier that will slow down the equilibration rate
6. use of gels to, among other effects, slow down convection in the droplet
7. use of capillaries in vapor diffusion mode to minimize handling of crystals

A selection of post set-up, vapour diffusion experiment alterations

1. change reservoir precipitant concentration
2. change temperature
3. change pH
4. microseeding

Some of the shortcomings of conventional crystallization vapor diffusion set-ups will be discussed and the simplest but most effective modifications will be reviewed. Most important, the experimentation does not stop after the cover slide is placed over the reservoir! Actually, now the work starts.

Primers and Links

I. J. Drenth, Principles of Protein X-ray Crystallography (Third Edition, Chapter 16), Springer Science+Business Media LLC

II. T.M. Bergfors, *Protein crystallization strategies, techniques, and tips*, IUL Biotechnology series

III. A. Ducruix and R. Giegé, *Crystallization of nucleic acids and proteins*, Oxford University Press

IV. A. McPherson, *Crystallization of biological macromo lecules,* Cold Spring Harbor Laboratory Press

V. S. Iwata, *methods and results in crystallization of membrane proteins,* International University Line Biotechnology series

VI. N. Chayen, *Protein Crystallization Strategies for Structural Genomics*, IUL Biotechnology Series

VII. www.iobcr.org.

L5

Lecture by IUBMB speaker

MOLECULAR MOVIES WITH NANOCRYSTALS USING XFELS: SMALL IS BEAUTIFUL

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Biological processes are highly dynamic, while most of the structures of biomolecules determined by X-ray crystallography represent a static picture of the molecule. Serial Femtosecond crystallography (SFX) provides a novel concept for structure determination, where X-ray diffraction "snapshots" are collected from a fully hydrated stream of nanocrystals, using femtosecond pulses at the high energy X-ray free-electron laser, the Linac Coherent Light Source [1, 2]. As femtosecond pulses are briefer than the time-scale of most damage processes, femtosecond crystallography overcomes the problem of X-ray damage in crystallography [3]. The concept of fs crystallography extends to atomic resolution [4, 5] and has been applied to the study of light driven processes in Photosynthesis and important membrane protein drug targets crystallized in lipidic environments[6-16]. First experiments on the proof of principle for time resolved serial femtosecond crystallography [11-16] pave the way for the determination of molecular movies of the dynamics of proteins "at work". These new discoveries open new frontiers in structure-based drug development.

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4. Boutet S, Lomb L, Williams GJ, et al **Science 2012**, 337: 362-364.
5. Redecke L, Nass K, Deponte DP. et al **Science 2013,** 339, 227-30.
6. Liu W, Wacker D, Gati C et al **Science 2013,** 342: 1521-1524.
7. Weierstall, U, James, D, Wang, C et al. **Nature Communications 2014,** 5, 3309.
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9. Zhang, H., Unal, H., Gati, C et al. **2015**. **Cell** 161, 833-844.
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11. Aquila,A, Hunter,MS, Doak,RB, et al HN **Optics Express 2012,** 20 (3), 2706-16.
12. Kupitz, C, Basu, S, Grotjohann, I et al **Nature 2014,** 513, 261-5.
13. Tenboer, J., Basu, S., Zatsepin, N. et al **Science 2014,** 346, 1242-1246 .
14. Pande, K., Hutchison, C.D.M., Groenhof, G., **Science 2016**, 352(6286), 725-729.
15. Stagno, J.R., Liu, Y., Bhandari, Y.R., et al **Nature 2017**, 541(7636), 242-246.
16. Kupitz, C., Olmos, J.L., Jr., Holl, M. et al **Struct Dyn, 2017**, 4(4), 044003 .

Lectures - Monday, June 11

L6

PRINCIPLES OF PROTEIN CRYSTALLIZATION II: METHODS, EVALUATION, AND PROPERTIES OF 'REAL' CRYSTALS

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The second lecture covers aspects of the actual how-to of crystal screening and harvesting, including post-mortem analysis in case things do not turn out well. Practical aspects of protein crystallization include the use of robotics and prior information aiming to extract the most information from the least amount of precious material, or in other words, to maximize the efficency of the process. We discuss various screening setup techniques, some sampling theory and data mining results, as well as analysis and optimization of crystals. The crystals also need to be harvested and often cryo-protected before they can be exposed to X-rays. Real crystals have often defects or exhibit microscopic twinning. Finally, we introduce (there will be more lectures on this important subject) a few methods to rationalize reasons why no or no well diffracting crystals could be grown, with emphasis is on assessment of stability and conformational purity of the proteins.

L7

UNCONVENTIONAL CRYSTALLIZATION STRATEGIES AND TECHNIQUES FOR SCREENING AND OPTIMIZATION

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All prescription drugs on the market today, which account for billions of pounds in annual sales worldwide, directly or indirectly target proteins. Protein functions are determined by their three-dimensional structures, hence detailed understanding of protein structure is essential for rational design of therapeutic treatments. Examples include cancer, obesity, cardiovascular disorders, autoimmune diseases and a multitude of other ailments.

The most powerful method for determining the structure of proteins is X-ray crystallography which is totally reliant on the availability of high quality crystals, but producing useful crystals has always been, and still remains, the bottleneck to structure determination.

There is no 'magic bullet' that will guarantee the yield of good crystals, hence rational approaches leading to the development of new and improved technologies for obtaining high quality crystals is of crucial importance to progress.

This talk will present strategies for increasing the chances of success and highlight a variety of practical methods that resulted in successful crystallization in cases where standard procedures have failed. The methods involve active influence and control of the crystallization environment, in order to lead crystal growth to the desired result. Many of the techniques can be automated and adapted to high throughput mode and several have been patented and commercialised.

1. Chayen and Saridakis (2008) *Nature Methods* 5, 147-153.
2. Chayen, Helliwell and Snell *Macromolecular Crystallization and Crystal Perfection*, Oxford University Press, Oxford, UK (2010).
3. Saridakis *et al.* (2011) *Proc. Natl. Acad. Sci. U.S.A*. 108, 11081-11086.
4. Khurshid *et al.* (2014) *Nature Protocols* 9, Pages: 1621–1633.
5. Govada *et al.* (2016) *Scientific Reports - Nature* 6:20053 DOI: 10.1038/srep20053.
6. Nanev *et al.* (2017) *Scientific Reports* Nature Publishing Group 7:35821.
7. http://quicktech.imperialinnovations.co.uk/i/materials/CRMIP.html.

L8

INTERPRETATION OF THE CRYSTALLIZATION DROP RESULTS

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The crystallization drop is full of information for the person who knows what to look for. However, for the inexperienced observer, the interpretation of the phenomena in the crystallization drop is not always a straightforward process. While it is sometimes easy to recognize a crystal, what about all those other solid phases of proteins like oils, precipitates, spherulites, and gels? Are they worth optimizing, or should one continue screening for new conditions? What does it mean when the protein "oils out"? What does phase separation look like and how does it affect the outcome of the experiment? How can you recognize a promising precipitate from a "bad" one? What are the best types of crystals to use as seeds?

This lecture will give present pictorial examples of the most commonly encountered results in crystallization drops and discuss how to recognize the different phenomena, and what to do with them. It will also cover examples of UV-imaging, one of the methods for distinguishing salt from protein crystals. While highly useful, it still has some pitfalls and limitations. Examples of both false-negative and false-positive UV images will be discussed.

A pictorial library of crystallization drop phenomena can be accessed at: http://xray.bmc.uu.se/terese.

L9

NEW STRATEGIES TO IMPROVE PRODUCTIVITY - RMMS MICROSEEDING FOR CRYSTALLIZATION AND DLS FOR CRYOEM

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Random Microseed Matrix-Screening (rMMS), where seed crystals are added automatically to random crystallization screens, is a significant recent breakthrough in protein crystallization [1]. During the ten years since the method was published, understanding of the theoretical advantages of the method has increased [2 - 4], and several important practical variations on the basic method have emerged. Important variations that will be discussed include combining seeds from several hits [5], the best methods of selecting hits to optimize [2], and cross-seeding targets with crystals of homologous proteins [6]. We will also present an approach that allows the method to be applied to the crystallization of membrane proteins in LCP [7], and a novel approach to preparing samples for cryoEM using “in situ” dynamic light scattering [8]. This will include discussion of the composition of the ideal screen for cryoEM [9].

1. D’Arcy, A., Villard, F, and Marsh, M. “An automated microseed matrix-screening method for protein crystallization.” Acta Crystallographica Section D: Biological Crystallography 63.4 (2007): 550-554.
2. Shaw Stewart, P. D., Kolek, S. A., Briggs, R. A., Chayen, N. E., & Baldock, P. F. (2011). Random microseeding: a theoretical and practical exploration of seed stability and seeding techniques for successful protein crystallization.Crystal Growth & Design, 11(8), 3432-3441.
3. 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.
4. Shaw Stewart, P. D, & Mueller-Dieckmann, J. (2014). Automation in biological crystallization. Acta Crystallographica Section F: Structural Biology Communications, 70(6), 686-696.
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9. ttps://www.douglas.co.uk/cryoem.htm.

L10

TIPS AND TRICKS FOR PROTEIN CRYSTAL MANIPULATION AND HANDLING

José A. Gavira

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The possibility to solve any protein structure relies on the ability to obtain a crystal suitable for X-ray diffraction. Obtaining a crystal is just the starting point for a way that sometimes can be very tedious. The next steps will include; i) testing the crystal nature, ii) X-ray diffraction at room temperature, iii) cryo preservation prior iv) low temperature data collection and iv) derivatization. This manipulation can put at risk your crystal quality and therefore the quality of your structure. In this talk we will try to fill the gap between the microscopy and the X-ray "observation" of your crystals with some tips and tricks. We will also see how to include new additives, i.e. cryoprotectant, scatter atoms, etc., into your protein crystal avoiding or minimizing the lost of quality and finally how to perform *in situ* cryo-crystallography from crystals grown by the capillary counterdiffusion method.

1. Garman EF, Weik M. *J Synchrotron Radiat* 18, 2011, 313-7.
2. Deller MC, Rupp B. *Acta Cryst.* **F70**, 2014, 133-55.
3. Gavira JA, Toh D, Lopez-Jaramillo J, Garcia-Ruiz JM, Ng JD. *Acta Cryst.* **D58**, 2002, 1147-54.
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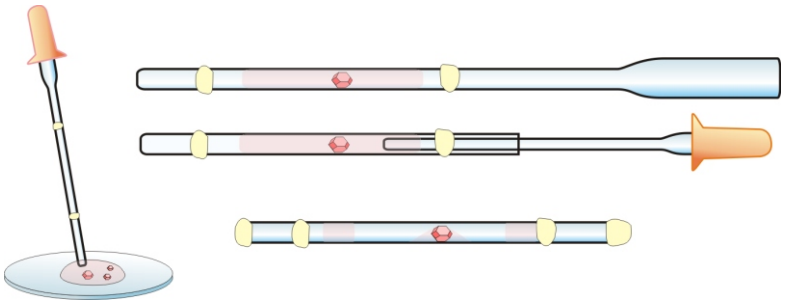

Figure 1. Schematic representation of how to prepare your crystal for room temperature X-ray diffraction test or data collection.

L11

EVALUATION OF CRYSTALLIZATION TRIALS WITH THE UVEX MICROSCOPE

James Gordon

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The utilisation of the instrinsic fluorescence properties of Tryptophan can be a powerful tool in any protein crystallography experiment. By exposing a protein crystal to UV light this fluorescence can been imaged using the UVEX imaging system. In this demo we will be using the UVEX to accurately differentiate between Protein and salt crystals. Moreover we will be using UV to look for crystal hits that can sometimes be obscured when using a bright-field microscope.

Lectures - Tuesday, June 12

L12

FROM PROTEIN EXPRESSION AND PURIFICATION TO ITS CRYSTALLIZATION

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X-ray crystallography is used to generate atomic resolution structures of protein molecules. These structures provide information about biological function, mechanism and interaction of a protein with substrates or effectors including DNA, RNA, proteins, cofactors or other small molecules and ions. This technique, however, requires preparation of pure and highly concentrated protein samples. High purity (? 95%), homogeneity and stability of the protein sample are critical factors for successful crystallization experiments. Recombinant protein production using *Escherichia coli* is the method of choice when large quantities of protein are required. Alternatively, eukaryotic organisms such as *Saccharomyces cerevisiae* (yeast), insect and mammalian cell lines can be used, especially when post-translation modifications are required. After the protein expression, the protein of interest must be purified from the cells. The purification method of choice is fast protein liquid chromatography, as there are a vast number of chromatography media including metal-affinity, size-exclusion, hydrophobic interaction, and ion-exchange, readily available for use in purification. Many proteins are expressed with a variety of N- or C-terminus tags that are highly specific to a particular kind of medium, thereby facilitating the purification and detection of recombinant proteins. Once a protein has been expressed, purified, and concentrated, it must maintain its structural integrity for the duration of the experiments. The presentation will cover cloning of genes and overexpression of proteins in bacterial and eukaryotic systems, protein purification with and without tags, and assessment of protein purity and stability.

L13

PROTEIN AS THE MAIN VARIABLE IN CRYSTALLIZATION

Ľubica Urbániková

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Preparation of high quality protein crystals is essential for the structure determination using X-ray techniques. Statistics from the projects of structural genomics shows that the success rate of high-throughput crystallization is only 10-30 % and thus preparation of protein crystals becomes the rate-limiting step.

Crystallization is influenced by many parameters, from which the most important one is the protein itself, its purity, homogeneity and specific properties, namely its propensity to form crystals. Protein crystallizability may be enhanced by the methods of molecular biology. This may involve the preparation of proteins with various kinds of fusion partners or tags, removal of their most flexible parts (N- and C-termini or flexible loops), increasing the homogeneity by modifications of free cysteines or potential sites of glycosylation, replacement of unfavourable amino-acid residues at the surface of the molecule, etc.

Requirement of protein purity and homogeneity will be discussed and stressed. The influence of protein modifications on its crystallizability and/or crystal packing and quality will be documented on results obtained in our laboratory and examples from literature. Some rational approaches and strategies oriented on enhancing the protein crystallizability as well as the possibility of its computational prediction will be presented.

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L14

"WHAT TO DO IF EVERYTHING HAS FAILED"

Terese Bergfors

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Protein crystallization projects usually have two stages. The initial one involves screening parameters to find promising lead conditions. Useable crystals may already appear at this stage, but the most typical scenario is that a second round of experiments is required to optimize the potential leads. This lecture will present some of the major considerations in choosing particular strategies or "routes" for screening and optimization. However, since the pathway in a crystallization project often contains dead-ends, the protein crystallizer also needs to be equipped with a plan for dealing with the "detours". When it seems that everything has failed, what are the options left to try?

To address this problem, the following questions will be discussed:

- Can pre-screening the protein buffer improve the protein behavior in the crystallization drops?
- How many conditions should the initial screen contain: 150 or 1500?
- So many crystallization kits! Which one to choose?
- Which is more critical: the choice of precipitant or the kinetic pathway?
- How does one recognize the kind of leads that are worth optimizing? For example, should one try optimizing drops with phase separation or keep screening for new conditions?
- What kind of tools exist for predicting if a protein is going to crystallize? How reliable are they?

This lecture will answer these questions from the perspective of an academic laboratory with little automation and which works on a limited, but very focused, group of targets from *Mycobacterium tuberculosis.*

Bergfors, T. M., ed. Protein Crystallization, 2nd Edition, 2009, International University Press, La Jolla California. *Now available as an e-book.*

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L15

CRYSTALLIZATION AND CRYSTALLOGRAPHIC ANALYSIS IN MICROFLUIDIC CHIPS

Claude Sauter

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A decade ago microfluidic technologies opened new possibilities for the crystallization of biological macromolecules. Indeed, microfluidic systems offer a lot of advantages for crystal growth: they enable an easy handling of nano-volumes of solutions as well as an extreme miniaturization and parallelization of crystallization assays. In addition they provide a convection-less environment a priori favorable to the growth of high quality crystals. Pioneer examples implementing free interface diffusion [1] and nano-batch [2] crystallization in microfluidic chips demonstrated the value of this technology, especially for high throughput screening applications in structural genomics.

Examples of microfluidic devices available on the market or in development will be described to illustrate how different steps of a structural study can be carried out 'on chip' from the crystallization to the observation of crystals and their characterization using synchrotron radiation [3,4]. The perspective of using inexpensive microfluidic chips for screening best crystallization agents and for automated crystal diffraction analysis and their complementarity with conventional crystallization setups will be discussed.

1. Hansen CL, Skordalakes E, Berger JM, Quake SR. A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion. *PNAS* **99**, 16531-6.
2. Zheng B, Tice JD, Roach LS, Ismagilov RF. A droplet-based, composite PDMS/glass capillary microfluidic system for evaluating protein crystallization conditions by microbatch and vapor-diffusion methods with on-chip X-ray diffraction. *Angew Chem Int Ed Engl.* **43**, 2508-11.
3. Sauter *et al.* (2007). From macrofluidics to microfluidics in the crystallization of biological macromolecules. *Crystal Growth Design* **7**, 2247-50.
4. Pinker et al. (2013). ChipX: a novel microfluidic chip for counter-diffusion crystallization of biomolecules and in situ crystal analysis at room temperature. *Crystal Growth & Design* **13**, 3333–40.

L16

PUBLICATION OF SCIENTIFIC RESULTS WITH EMPHASIS ON CRYSTALLIZATION COMMUNICATIONS

Howard Einspahr

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The fundamental principles of scientific publication will be presented with special emphasis on crystallographic results either as a crystallization communication (CC) or as part of a crystallographic structure report. Included will be an introduction to ***publBio***, a collection of novel web-based tools for authors developed by IUCr Journals to facilitate drafting of crystallographic publications and speed editorial processing after submission. Note that all CC submissions to Acta Cryst F must now be made through ***publBio***.

L17

ANALYTICAL ULTRACENTRIFUGATION: NEW MULTIWAVELENGTH SEDIMENTATION ANALYSIS OF PROTEINS IN SOLUTION

Martin Máša

Product Support Agencourt, Centrifugation and AUC Beckman Coulter Česká republika s.r.o., Murmanská 1475/4, CZ 100 00 Praha

Analytical Ultracentrifugation allows the characterization of molecules while they float free and unbound, letting you characterize them in their native state. While the sedimentation process of course did and will not change, new developments in 2 areas allow to unravel more and more of the information that for long time had been hidden in the sedimentation process. In this presentation the NEW in Analytical Ultracentrifugation like e.g. 2 dimensional spectrum analysis for the characterization on size and shape of heterogenous samples and Multiwavelength Sedimentation Analysis will be explained and recent application examples presented.

Lectures - Wednesday, June 13

L18

USING FLUORESCENCE TO FIND YOUR CRYSTALS

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A wide variety of crystallization solutions are screened to establish conditions that promote the growth of a diffraction-quality crystal. Screening these conditions require the assessment of many crystallization plates for the presence of crystals. A range of optical techniques and automated systems for screening are available. One disadvantage of some automated imaging systems is the need for certain characteristics, such as the presence of tryptophan, for crystal detection. Another disadvantage is the cost of the instrumentation, which is typically greater than $50,000.

We have developed a visible fluorescence approach that can give unambiguous macromolecule crystal detection and have now coupled it to a smartphone-based imaging method [1] that can be implemented for as low as $25-$35. Since the method uses trace fluorescent labeling (TFL) [2] with visible wavelength fluorescent probes, one can use different colors for the imaging of complexes as the presence of each color in the crystal being verification that those molecules are present. The TFL method involves the covalent attachment of a fluorescent dye to ~0.1 to 0.5 % of the protein molecules and has been shown to not affect crystal nucleation or diffraction quality at these levels. [3] The images give a direct indication of what the protein is doing in response to each screening condition as only the macromolecule has fluorescent probe attached to it.

There are several advantages of the TFL technique for detecting crystals. The ability to distinguish salt crystals from macromolecule crystals, facilitating the analysis of screening results, and the low-cost of imaging. This approach can be used for the imaging of protein complexes, which can save beam time.

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L19

CRYSTALLIZATION RESULTS ANALYSIS AND OPTIMIZATION USING IONIC LIQUIDS

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The default outcome of most crystallization screening experiments is a lack of crystalline results. If there are no obvious crystals formed, then the typical response is to either try still more screening solutions or to modify the protein, either at the genetic level, by chemical or by enzymatic means. Screening can be carried out using incomplete factorial methods, but this requires the design and then the subsequent analysis of a statistically balanced screen. This requires additional effort on the part of the experimenter, as commercial vendors have not provided such screens, or the software for their analysis. We are addressing this problem with the development of software that can use the scored results from commercial or in-house developed screens for the analysis process. Two analytical approaches have been developed to date, the associative experimental design [1] (AED) and, more recently, genetic analysis (GA), a method first proposed by Saridakis [2]. Both methods use scored crystallization screening results, which process is greatly facilitated by the use of trace fluorescent labeling (TFL). While we use a 10-level scoring scale, the methods have been successfully tested using a more limited 5-level scale; the important consideration being that the scores numerically track the desirability of the results. In use, the scored screening results, attached to the subject conditions, are read in and the programs output a listing of the screen components evaluated to be most effective in obtaining the highest scores and a listing of their combinations to give new screening conditions for use in preparing new screening blocks that do not match any of those used for input. Comparative testing of the two methods is currently in progress. To date, neither is shown to be "better", although they usually give different outputs.

While the above analytical methods generally yield more crystallization hits in a single 96 condition screen than were found in the (typically) 4 screens used for input, a number of non-crystalline outcomes are still found. Those conditions are being used for subsequent experiments into the use of ionic liquids (ILs) as crystallization additives. Ionic liquids are salts that are typically liquid at temperatures 100 °C. We are testing 23 commercially available ILs, along with dH2O as a control, with 12 of the screening conditions for each protein. Most of the screening conditions selected were those that gave precipitated protein, and we have been able to derive crystals from ~30 % of those apparently "failed" outcomes. The advantages of ILs is that their solution properties can be modified by changes in their cation or anion structures. The use of TFL is critical to these experiments as the ILs frequently crystallize. Sufficient data has been acquired that analysis of IL structure vs. efficacy can now be carried out.

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L20

ADVANCED AND NON-CONVENTIONAL METHODS FOR CONTROLLING THE SIZE AND THE SHAPE OF PROTEIN CRYSTALS

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Proteins, nucleic acids, polysaccharides and lipids are considered the most important molecules of life. The function of these molecules in sustaining life depends on their three-dimensional structure and on their highly specific mutual interactions, dictated by their structure and bonding properties. Structural knowledge of such molecules and their macromolecular complexes (combination of all of them) is therefore continuously increasing our understanding of the processes of life and the mechanisms of biological processes and suggests novel ways to treat a wide range of diseases, ranging from congenital anomalies through bacterial and viral infection to autoimmunity and many kinds of cancers. On the other hand, X-ray crystallography is ubiquitous in this search, as it is the most powerful technique for structure elucidation of macromolecules, reaching quasi-atomic resolution in the most favorable cases, and without a priori limitations on the size and complexity of the molecules studied. X-ray crystallography requires the growth of large and well-diffracting crystals (to perform conventional crystallography) or nanocrystals in size (for free electron lasers technology research, usually called XFEL), the production of such crystals being the most intractable stage in the process of structure determination.

This talk provides a review of different advanced methods that help to increase the success rate of a crystallization project, by producing larger and higher quality single crystals for determination of macromolecular structures by crystallographic methods. For this purpose, the present talk is divided into three parts. The first part deals with the fundamentals for understanding the crystallization process through different strategies based on physical and chemical approaches. The second part presents new approaches involved in more sophisticated methods not only for growing protein crystals, but also for controlling the size and orientation of crystals through utilization of electromagnetic fields and other advanced techniques. The last section deals with three different aspects: The importance of microgravity, the use of ligands to stabilize proteins, and the use of microfluidics to obtain protein crystals suitable for high-resolution X-ray, neutron crystallography, and the new trends in crystallography by XFEL techniques.

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L21

DLS MEASUREMENTS PRIOR TO CRYSTALLIZATION EXPERIMENTS

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Dynamic Laser Light Scattering (DLS) is for many years well established and this days increasingly used to score and optimise protein solutions prior to crystallisation experiments, as today serial crystallography (SX) data collection is coming more and more in routine use at high intensive microfocus beamlines [1]. For SFX, as well as SFX many high quality micro- or nano-cyrstals are required and the preparation of such crystal suspensions requires more efforts than those for the conventional single crystal (MX) approach. DLS is used to analyse the dispersity and homogeneity of protein, or other biomolecule suspensions. Automated methods to crystallize macromolecules are widely used and can easily generate thousands of crystallization droplets. Nevertheless, evaluation of crystallization experiments to find optimal growth conditions, or to find ideal conditions to upscale crystallization conditions from single crystals to suspensions of nano-crystals remains a bottleneck. Methods to analyze protein solutions in cuvettes, in capillaries, in flow mode as well as in droplets applying DLS [2-6] will be presented. And a further, today also commonly applied approach is the use of a combined white/UV illumination for microscopic determination of whether crystal-like objects are biomolecular or salt. This system will be explained and examples will be presented as well. [7]. As mentioned before, DLS is a most appropriate method to determine the size and mode of aggregation of proteins and other biomolecules in solution, but its use has so far been limited because the need to make measurements in cuvettes required rather large sample volumes. Protein crystallisation experiments

are usually carried out in multi-well plates with droplet volumes in the range of 0.5 to 10 µl. Within the lecture an advanced and most suitable method to image, measure and analyse the particle size directly in drops and in particular to investigate the stage of nucleation and the progress of crystal growth by *in-situ* DLS, i.e. directly in the droplets will be shown. This methods has several advantages: no additional pipetting is necessary to perform measurements; the crystallisation process can be monitored online *in situ*, without interruption; measurements can be taken from even small volumes. This particular DLS technique has been adapted to an automated CCD-camera-based plate-screening system and will be applied also in the tutorials of the workshop (Spectro-Imager, Xtal Concepts Germany), allowing monitoring and evaluation of the entire process of crystallisation in an automated way. The data obtained provide information to understand in detail the process of crystal growth and allow to optimize growth conditions. Images taken from various droplets/set ups will be presented along with corresponding DLS measurements.

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L22

CRYSTALLIZATION OF PROTEIN-NUCLEIC ACID COMPLEXES

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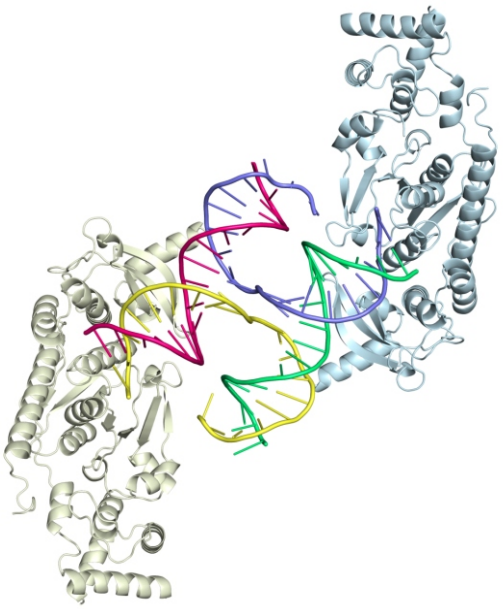
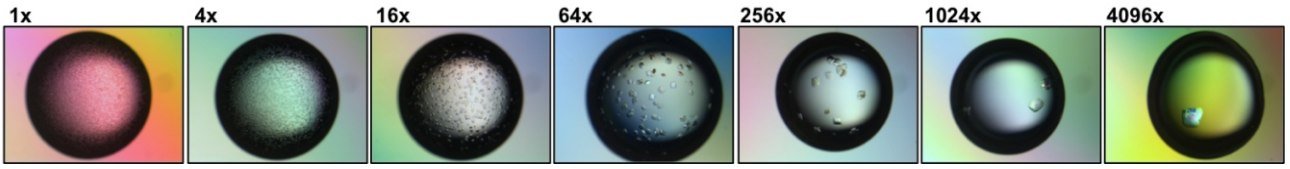
The general workflow for crystallizing protein-nucleic acid complexes for crystallographic studies is analogous to the work with only proteins and obtaining diffraction quality crystals is still a bottleneck. However, technological advances in sample preparation as well as high-throughput screening have helped to accelerate the workflow and made it feasible to approach more difficult projects. Moreover, the structural analysis of protein-nucleic acid complexes provides a wealth of information about their function that can only partially be deduced from nucleic-acid binding proteins alone. The crystallization of protein-nucleic complexes poses additional challenges but also certain benefits compared to the work with proteins alone. The lecture and demonstration will give a general overview for the work with protein-nucleic acid complexes. Several topics from experimental design, sample preparation, stabilizing complexes, optimizing poorly diffracting crystals to phasing techniques will be covered. Designing suitable nucleic acid substrates for promoting complex crystallization is critical. It is even possible to engineer crystal contacts and to promote complex crystallization in different functional states of a biological process. Therefore, a special focus will lie on strategies for design-





ing nucleic acid substrates. Furthermore, practical considerations for the biochemical and biophysical charac terization of nucleic acid complexes in solution as well as in crystals will be discussed.

The optional exercise will focus on design strategies for nucleic acid substrates that promote complex formation and crystallization. In addition, we will discuss methods to characterize protein-nucleic acid complexes and their crystals.

L23

Evening lecture

STRUCTURAL IMPLICATIONS OF THE HELIOBACTERIAL PHOTOSYSTEM ON EVOLUTION OF PHOTOSYNTHESIS

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The origin of photosynthesis is related to the first reaction centers which could convert the energy of sun light. As much our knowledge about photosystems and reaction centers has increased with structures of these membrane proteins since the mid-1980s the trajectory of this important part of evolution is by far not understood.

With an estimated age of 4.6 billion years the planet earth had life with primitive organisms at least since 3.7 billion years ago. In these early times the earth atmosphere was strict anaerobic or oxygen free. Photosynthesis evolved from ancestral photosynthetic bacteria which had an early branching to heliobacteria these gram-positive bacteria (firmicutes) are still to be found in muddy volcanic waters or even in rice paddies. The structure of the photosystem from Heliobacterium modesticaldum at 2.2 Ĺ resolution gives the first insight how an ancestral photosystem has been organized [1].

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Lectures - Thursday, June 14

L24

PREPARATION OF PROTEIN SAMPLES FOR CRYSTALLIZATION EXPERIMENTS

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Protein crystallization process is influenced by a large number of various factors and one of the most important is the property of the protein sample to be crystallized. Preparation and characterization of the protein sample plays a crucial role in protein crystallization.

In the lecture, the most widely used techniques to judge evaluate protein sample purity and quality before crystallization experiments will be reviewed and discussed. For crystallization trials highly pure and homogeneous protein sample is usually recommended, however, if larger amount of protein is available 'impure' protein sample can be also screened. Guidelines and tips for protein handling before crystallization trials will be addressed.

Common methods to analyze protein purity and stability will be described: (a) SDS polyacrylamide gel electrophoresis (PAGE), (b) native PAGE, (c) isoelectric focusing, (d) size exclusion chromatography (gel filtration), (e) mass spectrometry, (f) dynamic light scattering (DLS), and (g) differential scanning fluorimetry (DSF).

Recommended protein concentration for initial crystallization screening is in range of 5 - 20 mg. The higher protein concentration provides more opportunity for crystal nucleation to occur but, on the other hand, also can cause protein aggregation. The best concentration is usually tuned as one of the variables during optimization procedure. All components of the protein buffer should be carefully considered since they might influence crystallization. Storage conditions have to be checked experimentally for each protein, but most protein can be stored at -70 C or 4°C. Lyofilization should be avoided and if inevitable, extensive dialysis before crystallization is recommended.

For more general reading further references are recommended [1, 2].

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L25

PREPARATION OF MICRO- AND NANO-CRYSTALS FOR FREE-ELECTRON-LASER AND SYNCHROTRON RADIATION SOURCES

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[2]Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Str.4, 72076 Tübingen
[3]Center for Free-Electron Laser Science (CFEL), Notkestr. 85, 22607 Hamburg, Germany
[4]Institute of Biochemistry, University of Lübeck, Germany
[5]XtalConcepts, Malowring 19, 22525 Hamburg, Germany
[6]67 Green Avenue, Lawrenceville, NJ 08648, USA

During the last years serial crystallography (SX), applying suspensions of micro- and nano-crystals for serial data collection was established at most high brilliant synchrotron radiation beamlines, as well as at XFEL radiation sources (SFX) [1]. Before macromolecular structures were primarily determined applying the single crystal method (MX) for diffraction data collection. The new method of Serial Crystallography was in principle developed for X-ray Free Electron Laser Sources, however became more and more in routine use at microfocus beam lines, as this approach also offer new possibilities to analyse proteins that do not form crystals suitable for conventional X-ray diffraction. However, growth and preparation of high quality micro-crystals and suspensions optimal for data collection experiments at this modern micro-beam insertion-device synchrotron (SR) beamlines and growth of nano-crystals required for data collection at Free-Electron-Laser (FEL) beamlines is a new challenging task. X-ray free-electron laser sources use extremely intense pulses of X-rays with more than 10^{12} photons in 10 to 100 fs duration. When fo-

cused to micron dimensions these pulses vaporize the sample but the diffraction pattern is collected before that radiation damage sets in. The SFX method [2-4] can thereby achieve high resolution diffraction on sub-micron macromolecular crystals without the need for cryogenic cooling.

However, it requires a large number of crystals, and crystals all with same dimensions that are flowed across the beam, or transported on tape drives, or delivered controlled with special chips to cross the X-ray beam, and snapshot diffraction patterns are rapidly acquired. Today in the field of conventionell protein crystallogenesis several fully automated instruments are available and the search for crystallization conditions of macromolecules can easily carried out. Nevertheless, to identify optimal growth conditions to obtain high quality, nano-sized X-ray suitable crystals is a remaining and growing bottleneck in most cases [5, 6]. To take advantage of the new established and upcoming high brilliant SR- and FEL- radiation sources, which open new routes in structural biology [7] and allowing to collect diffraction data from micro- or nano- crystals [1] advanced crystallization and crystal scoring techniques need to be established or need to be developed further. To meet future crystal requirements for SX and SFX we investigated and optimized two approaches to produce high quality nano- and micro- crystals. In one approach we optimized the growth of nano sized crystals *in vivo,* in cells [8] and in a second approach we developed a advanced hardware combination allowing the controlled optimization of a single drop vapour diffusion experiment for production of nano- and micro- crystals [9]. Details and examples will be presented.

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L26

MEMBRANE PROTEIN STRUCTURES USING TYPE I AND TYPE II CRYSTALS, BICELLES, NANODISCS AND CRYO EM

Hartmut Luecke

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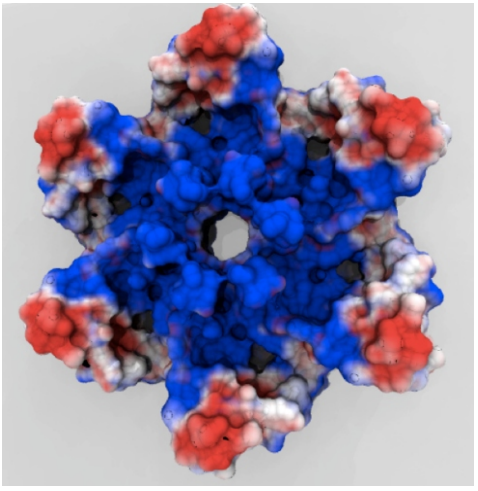
The Lipidic Cubic Phase (LCP) method for membrane protein crystallization has been refined to allow large-scale screening of membrane proteins. The various parameters (LCP lipid, water content, bilayer lipid additive, pH, ionic strength, precipitating agent etc.) can be varied. Several distinct seven-transmembrane proteins where crystallized and their high-resolution structures determined. In cases where the LCP method fails, the bicelle method or detergent-based methods were employed to crystallize other membrane proteins.

Bacteriorhodopsin (BR): High-resolution maps from X-ray diffraction of bacteriorhodopsin crystal obtained in LCP and some of its photointermediates have yielded insights to how the isomerization of the bound retinal drives ion transport. Although some important mechanistic details are still undecided, the events of the photochemical cycle are now understood to reflect changes in specific hydrogen bonds of protein groups and bound water molecules in response to motions of the retinal chain. A nearly complete lipid bilayer is also present in the x-ray-derived atomic model.

Human health: Half the world's population is chronically infected with *Helicobacter pylori*, causing gastritis, gastric ulcers and an increased incidence of gastric adenocarcinoma. Its proton-gated inner-membrane urea channel,



*Hp*UreI, and a urease are essential for pathogen survival in the acidic environment of the stomach. The channel is closed at neutral pH and opens at acidic pH to allow the rapid access of urea to cytoplasmic urease. Urease produces NH_3 and CO_2, neutralizing entering protons and thus buffering the periplasm to a pH of roughly 6.1 even in gastric juice at a pH below 2. The structure of urea channel reveals six protomers assembled in a hexameric ring surrounding a central bilayer plug of ordered lipids. Each

protomer encloses a channel formed by a twisted bundle of six transmembrane helices. The bundle defines a previously unobserved fold comprising a two-helix hairpin motif repeated three times around the central axis of the channel, without the inverted repeat of mammalian-type urea transporters. Both the channel and the protomer interface contain residues conserved in the AmiS/UreI superfamily, suggesting the preservation of channel architecture and oligomeric state in this superfamily. Predominantly aromatic or aliphatic side chains line the entire channel and define two consecutive constriction sites in the middle of the channel. The novel hexameric channel structure provides a new paradigm for the permeation of urea and other small amide solutes in prokaryotes and archaea. Our high-throughput screens have identified sub-micromolar inhibitors of *H. pylori*'s acid acclimation system. Follow-up microsecond-scale unrestrained molecular dynamics studies provide a detailed mechanism of urea and water transport by *Hp*UreI. In parallel we have determined the structure of the 1.1 MDa cytoplasmic urease complex by cryo EM to 3.1 Å resolution.

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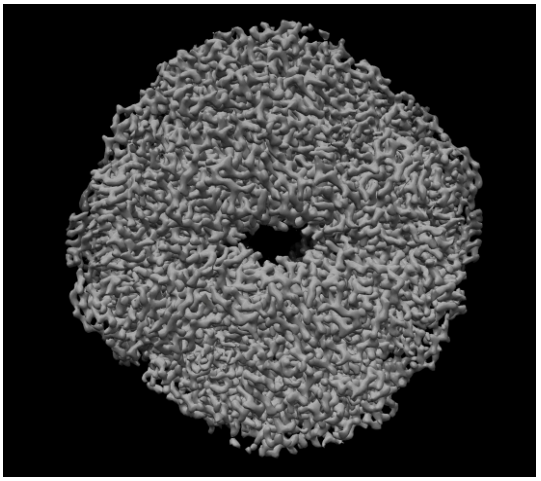

Luecke, H. & Sachs, G. "*Helicobacter pylori's* Achilles' Heel" (2013) *Immuno-Gastroenterology* **2**, 76.

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L14

CRYSTALLIZATION OF MACROMOLECULAR COMPLEXES WHILE STUDYING HOST–VIRUS SYSTEMS

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Majority of biological events involve the action of one macromolecule on another, thus triggering a series of recognition, signaling and modification events. Compared to the relatively well-conserved processes found in cellular organisms, viruses demonstrate huge variations in terms of genomic composition, patterns of evolution, and protein function. While studying protein–protein interactions in virus–host systems, these variations on the pathogen side must be considered. The details of such macromolecular interactions are critical to our understanding of biological function and bestow greater knowledge than the three-dimensional structures of single macromolecules. Although substantial progress has been made in macromolecular docking, it still remains difficult to predict the mode of interaction between macromolecules even when the structures of the interacting partners are known [1-2, 4]. Given the large size of such complexes, crystallography remains the method of choice to determine their structure, and therefore crystals for such complexes need to be grown.

In an era that has been dominated by structural biology for the last 30-40 years, a dramatic change of focus towards sequence analysis has spurred the advent of the genome projects and the resultant diverging sequence/structure deficit [3-4]. The central challenge of computational structural biology is therefore to rationalize the mass of sequence information into biochemical and biophysical knowledge and to decipher the structural, functional and evolutionary clues encoded in the language of biological sequences. However, many other efforts have been performed to understand the relationship between the structure of proteins and their biological function. In addition, a number of protein candidates generated by genomics programs, has increased the interest in all the aspects of gene design, protein expression, purification and crystallization [Figure 1].

In this lecture, we attempt to provide a critical assessment of what one may experience during protein crystallography and to identify major issues yet to be resolved in attempt to crystallize multiprotein complexes. The presentation is organized under several subtitles like definition of ligand, receptor and their affinity; methods to determine binding properties; strategy for expression, purification and crystallization of macromolecular complexes and structure determination, choice of expression vector and/or system; expression and solubility analysis; protein characterization; binding properties and assays; complex

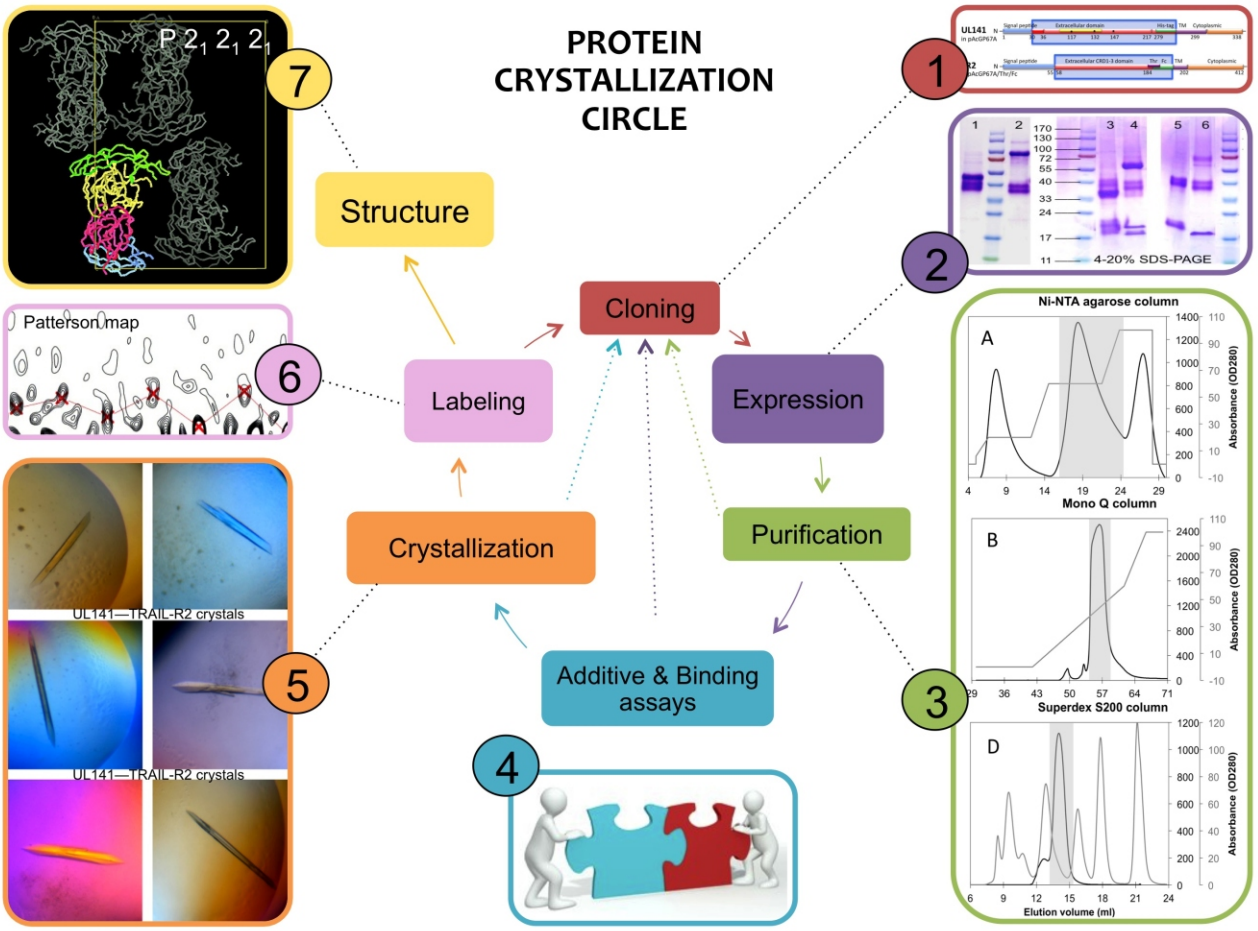

Figure 1. Protein crystallization circle: A number of ways to influence protein crystallization.

formation.. all included in the 'crystallization circle' shown in Figure 1.

We will also discuss a number of ways to stabilize proteins for crystallography that we have been experiencing, including genetic engineering, co-complexing with natural ligands and binding of antibody fragments or alternative scaffolds [2–6]. It is important to remember that in a three-dimensional crystal four or more (in a two-dimensional case we need at least three) different molecular contacts are needed to be able to form a lattice with a non-symmetrical object, and that the lattice interactions will always outnumber the specific contacts that give rise to the physiological complex. However, obtaining diffraction-quality crystals has long been a bottleneck in solving the three-dimensional structures of proteins. Often proteins may be stabilized when they are complexed with a substrate, nucleic acid, cofactor or small molecule. These ligands, on the other hand, have the potential to induce significant conformational changes to the protein and *ab initio* screening may be required to find a new crystal form.

This lecture presents an overview of strategies in the following areas for obtaining crystals of protein–ligand complexes: (1) co-expression of the protein with the ligands of interest, (2) the use of the ligands during protein purification, (3) co-crystallization and (4) soaks.

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L28

ASSESSING THE DIFFRACTION QUALITY OF CRYSTALS

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What is a good crystal? Once you have obtained a crystal of suitable size, with nice edges and good morphology, there is more to be done in order to prepare for the collection of a good quality dataset.

This talk will provide an introduction to the X-ray screening of protein crystals in order to select those with the best chance of producing good quality datasets. The discussion will include the visual analysis of images to estimate diffraction quality and identify features in the diffraction pattern, crystal indexing, and the treatment of twinned crystals. An overview of the procedure for finding the best cryo-protection conditions is included. Finally we will provide an overview of the considerations involved in setting up a good data collection experiment, based on crystal information obtained, the instrument and the kind of experiment that is to be performed.

Lectures - Friday, June 15

L29

INTRODUCTION TO SINGLE PARTICLE CRYO-EM

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We will cover the recently revolutionized technique of Cryo-EM whose leading developers were awarded the Nobel prize in Chemistry in 2017. The recent "resolution revolution" in cryo-EM, driven by developments in instrumentation such as direct detectors and the Volta phase plate, coupled with major improvements in data analysis, has put Cryo-EM at the forefront of structural biology, a novel method for attaining high-resolution models of difficult targets (low yield, moderately flexible). Since 2015, several records have been achieved with the highest resolution structure reported so far for glutamate dehydrogenase (soluble protein, 1.8 Å), anthrax toxin (membrane protein, 2.9 Å) and hemoglobin (only 64 kDa, 3Å). Another advantage is the possibility of studying glycosylated proteins as well as the option of reconstituting membrane proteins into nanodiscs, providing a protein-enclosed lipid bilayer and thus a more native-like environment as opposed to detergents.

We will cover several of the important topics, from sample requirements, to quality control and data processing, highlighting the many variables that need to be controlled to achieve a high-quality structure.

L16

OPTIMISATION OF CRYSTAL GROWTH FOR NEUTRON CRYSTALLOGRAPHY

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While synchrotron X-ray methods dominate the field of macromolecular structure determination, neutron diffraction remains an extremely valuable complementary technique. The power of the neutron technique resides in the ability to determine hydrogen positions, without radiation damage issues, allowing room temperature structure determinations. Further, new advances in instrumentation, exemplified by the LADI-III diffractometer at the ILL, exploit efficient Laue techniques that allow the use of much smaller crystals than previously possible and provide rapid and accurate structural information. Sub-mm^3 crystals are now regularly being used for data collection, structures have been determined to atomic resolution for a few small proteins, and much larger unit-cell systems are being successfully studied [1-2]. This trend is expected to continue with further improvements to existing instrumentation,

such as the construction of entirely new instruments, such as the TOF Laue diffractometer "NMX" at the European Spallation Source (ESS). However, despite all the advances in the field, relative to X-rays, significantly larger crystals will always be required for neutron diffraction studies, particularly with the tendency to study ever-larger macromolecules or complexes. Therefore further development of instrumentation and methods for large crystal growth are required.

In my lecture I will introduce the crystallization devices, based on the detailed knowledge of the phase diagram, we have developed especially with the focus on X-ray and Neutron Macromolecular Crystallography. Knowledge of the phase diagram has key importance when designing and controlling a crystallization process for a substance. The 1st generation instrument combines the use of temperature control and seeding and allows for grow of large crystals in crystallization batch [3]. A crystallization batch in the metastable zone is seeded with small protein crystals. The seeds are maintained inside this region of the phase diagram for as long as possible by doing a temperature step each time the crystal solution equilibrium is achieved. The temperature steps are repeated until crystals of suitable size for diffraction measurement are obtained. The 2nd generation instrument, adds new functionality to the first instrument thanks to a fluidic cell enabling to perform a temperature controlled dialysis crystallization experiment [4,5]. The new crystal growth apparatus combines accurate temperature control with control of the chemical composition of the crystallization solution and therefore it allows very sophisticated experiments to be performed. Systematic phase diagrams in multi-dimensional space can be investigated using far less protein material than previously. We have demonstrated that it can be beneficial to provide sufficient scattering volumes for neutron studies that require large-volume well-ordered single crystals.

The recently developed fluidic device, once adapted, is intended to be useful in monitoring and controlling the crystallization processes of challenging biological macromolecules, such as membrane proteins.

1. M. Blakeley, *Crystallography Reviews* **15**, 157 (2009).
2. M. Blakeley *et al*., *IUCrJ.* **2**, 464 (2015).
3. M. Budayova-Spano *et al*., *Acta Crystallogr.* **D63**, 339 (2007).
4. M. Budayova-Spano, *Patent FR10/57354, UJF*, (extension: EP117730945, US13821053, JP2013528746), (2010).
5. N. Junius *et al*., *J. Applied Crystallogr*. **49**, 806 (2016).

L31

STATE-OF-ART BIOLOGICAL SMALL-ANGLE-SCATTERING AND NEW POSSIBILITIES ON FREE ELECTRON LASERS

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Biological Small Angle X-ray Scattering (SAXS) became one of the standard techniques in structural biology. As a solution scattering method SAXS does not rely on high quality protein crystals and is not limited in protein size and folding state. On the other side, SAXS cannot provide high resolution protein structures, however modern scattering data evaluation permits model building of macromolecular assemblies in the range down to 15 Angström. High quality SAXS data can be recorded at standard lab based SAXS devices, but the full advantage of solution scattering can be obtained at dedicated SAXS synchrotron beamlines. Using SAXS, environmental parameters can be altered and parameters such as temperature, pH-value and salinity adjusted to the protein activity. The most fascinating and challenging approach is the investigation of kinetics and dynamics of protein reaction. Starting from simply fast mixing experiments using protein and ligand solutions for investigation of protein kinetics, modern synchrotron beamlines allow as well pumped-probed experiments for exploring the dynamic of protein-protein interactions and protein domain motions. Following this idea of investigation on biological systems "at work" new possibilities are provided by Free Electron Lasers. These upcoming high brilliance X-ray sources are able to record X-ray diffraction data in the fs time range, which is suitable to record protein dynamics in detail.

In this lecture the basics of biological SAXS will be explained and high end application of the SAXS method introduced. Some technical details of Free Electron Lasers will be given and future prospective applications of this method discussed.

L32

STRUCTURE/FUNCTION STUDIES OF PAPS SYNTHASE ISOFORMS AND ITS MUTANTS: CONTRIBUTIONS FROM CRYSTALLOGRAPHY AND COMPUTATIONAL MODELING

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3'-Phosphoadenosine 5'-Phosphosulfate (PAPS) synthase (PAPSS) catalyze the formation PAPS in two steps. First inorganic sulfate reacts with alpha-phosphoryl part of ATP to form adenosine 5'-phosphosulfate (APS) and pyrophosphate (PPi) catalyzed by ATP-sulfurylase (ATPS) domain activity of PAPSS. APS released from the ATPS domain is instantaneously bound by APS kinase (APSK) domain of the dimeric PAPSS. The 3'-oxyanion of the APS attacks the gamma-phosphoryl of the new ATP that is now bound to APSK domain. With this nucleophilic attack ATP is cleaved between beta-gamma position to form ADP and PAPS. ATPS being an alpha-beta phosphoryl splitter it has the characteristic HXGH motif and APSK being a beta-gamma splitter has the typical P-loop GXXGXXK motif. There are two isoforms of PAPSS. PAPSS1 is predominantly expressed in tissues such as skin and brain and its structure has been partially resolved. PAPSS2(a/b) is heavily expressed in the liver. PAPSS2 defects has been reported to cause skeletal deformity. Thus, it is imperative to study the structure/functions of PAPSS1 and PAPSS2a/b owing to its clinical relevance in bone metabolism and orthopedic medicine. PAPSS1 and PAPSS2a/b are about 73% identical in amino acid sequences, nevertheless the kinetics of PAPSS formation between these isoforms are distinct. Structural comparisons of PAPSS1 and PAPSS2b would allow to identify the specific features of PAPSS2b. This would then allow to explain the kinetic differences between PAPSS 1 and 2a/b. For the sulfurylase half-reaction, previous molecular simulation studies predict that in addition to the characteristic $H_{425}NGH_{428}$ motif, at least one arginine residue plays a key role in the catalytic reaction. The second oxyanion negative charge is balanced by the positive charge of the arginine nitrogen making it unreactive. Thus, the reactive free oxyanion of the sulfate is facilitated closer to the alpha-phosphoryl for the actual ATPS catalytic reaction to occur. Similarly, with APSK the proposed D_1xD_2T motif (Venkatachalam et.al. unpublished) and the corresponding molecular details will be resolved. Data from this will allow to elucidate whether the beta-carboxylate anion of D_1 or the neighboring D_2 would remove the proton from the 3'-OH ribose of APS making it an oxyanion nucleophile which would then react with gamma-phosphate of ATP to form PAPS and ADP. In addition, mutants of $H_{425}NGH_{428}$ motif are being studied by X-ray crystallography to understand the rationale behind increased activity of N_{426}-K and null backward activity of G_{427}-A. In summary, this talk will illustrate how an interplay between X-ray crystallography, molecular dynamics simulations and hybrid quantum/MM calculations of reaction coordinates can be utilized to describe the structure/function relation of a dimeric multi-functional, multi-domain enzyme.