Advanced methods in macromolecular crystallization VII

The 2nd practical crystallization course in the middle EU cosponsored by INSTRUCT (FEBS PC16 003)

Academic and University Center, Nove Hrady, June 27 - July 2, 2016

Lectures - Monday, June 27

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

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.

Caffrey, M., Cherezov, V. 2009. Crystallizing Membrane Proteins In Lipidic Mesophases. Nature Protocols. 4:706-731. (PMID: 19390528).

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).

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

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L3

AN INTRODUCTION TO CRYSTAL MORPHOLOGY AND CRYSTAL GROWTH MECHANISMS

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The description of the shape of the crystals resulting from crystallization experiments contains critical information to check their reliability and reproducibility. Fortunately, crystallographers have developed since more than one hundred years ago a precise and unambiguous terminology based on crystal symmetry to define crystal shapes regarding morphology and habits, i.e. in term of combinations of crystal forms and their relative development.

The aim of this lecture is to introduce the main concepts, terms, and definitions used in the proper description of morphology of crystals and crystal aggregates obtained in protein crystallization experiments. I will also introduce the different growth mechanisms and growth phenomena that are involved in the morphogenesis of the crystals and how are they connected with the physicochemical crystallization conditions of the solutions from which they growth.

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

Lectures - Tuesday, June 28

L5

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.

L6

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.

L7

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.

L8

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 designing 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.

L9

SEEDING STRATEGIES FOR "RANDOM" CRYSTAL SCREENING AND CRYSTAL OPTIMIZATION

Stefan A. Kolek[1], Patrick D. Shaw Stewart[1], Bastian Bräuning[2]

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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 eight years since the method was published, theoretical understanding of the method has increased [2 - 4], and several important practical variations of the basic method have emerged [5, 6]. We will introduce the method and briefly describe its most important variations.

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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6. Abuhammad, A., Lowe, E. D., McDonough, M. A., Shaw Stewart, P. D., Kolek, S. A., Sim, E., & Garman, E. F. (2013). 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), 1433-1446.

L10

UV TO THE RESCUE!

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Anxiously inspecting thousands of drops in crystallization trial plates is a tedious, work intensive, but crucial step in the crystal structure determination process. Sliding the plate along, while checking each drop at a time through the lenses of a microscope (or pictures of drops taken by an automated imager), requires a detail oriented and patient eye. The excitement of discovering crystalline objects or even something that could be, or may become, or once was a crystal, diminishes often immediately because of one question: is it protein or is it salt? Usually, several options to find an answer to this question are at the scientist's disposal; however, the most time efficient, reliable, and gentle method is utilization of the intrinsic fluorescence of proteins which contain tryptophan residues. UV microscopes take advantage of this fluorescence and represent an in situ, rapid, yet non-destructive method in protein crystal hit discovery. Crystallization drop inspection will be discussed and commonly used methods for differentiating protein crystals from salt crystals will be presented.

L11

PUBLICATION OF SCIENTIFIC RESULTS WITH EMPHASIS ON CRYSTALLIZATION COMMUNICATIONS

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The crystallization communication (CC) is often the beginning crystallographer's first encounter with scientific publication. The fundamental principles of scientific publication will be presented with special emphasis on crystallization results either as a CC or as part of a 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. As of this year, all CC submissions to Acta F must now be made through ***publBio***.

Lectures - Wednesday, June 29

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

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].

1. McPherson A. (1999). Crystallization of Biological Macromolecules, Chapter 3. The Purification and Characterization of Biological Macromolecules, pp. 67 – 126, Cold Spring Harbor Laboratory Press, New York, USA.
2. Bergfors T.M. (1999). Protein Crystallization Techniques, Strategies and Tips, Chapter 3. Protein Samples, pp. 19 – 25 International University Line, La Jolla, CA, USA.

L14

PREPARATION OF MACROMOLECULAR COMPLEXES FOR CRYSTALLIZATION

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Many biological events involve the action of one macromolecule on another, thus triggering a series of recognition, signaling and modification events. 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 macro- molecules. 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]. 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. 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 [Fig.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 identification and/or obtaining target sequence; pattern recognition techniques; protein tertiary structure prediction, choice of expression vector and/or system; sequence verification and host transformation; expression and solubility analysis; protein characterization; binding properties and assays; complex formation; crystallization of complexes and struc-

ture determination [The 'crystallization circle' shown in Fig. 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 [2–5]. 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. Recently, there has been also renewed interest in seeking innovative biological solutions to reducing surface entropy, and some interesting work at the interface of traditional chemistry and biology is starting to show promise. 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: co-expression of the protein with the ligands of interest, the use of the ligands during protein purification, co-crystallization and soaks.

1. Hassell et al. Crystallization of protein-ligand complexes. Acta Cryst D (2006) 63:72–79.
2. Stura et al. Crystallization of macromolecular complexes: SVS. J Cryst Growth (2001) 232:580–590.
3. Griffin et al. Antibody fragments as tools in crystallography. Clin & Exp Immunol (2011) 165:285–291.
4. Derewenda. Use of recom. meth. & mol. engineering in prot. crystallization. Methods (2004) 34:354–63.
5. Hunte. Crystallization of memb. prot. mediated by antibody fragments. Curr Op Str Biol (2002); 12:503–8.

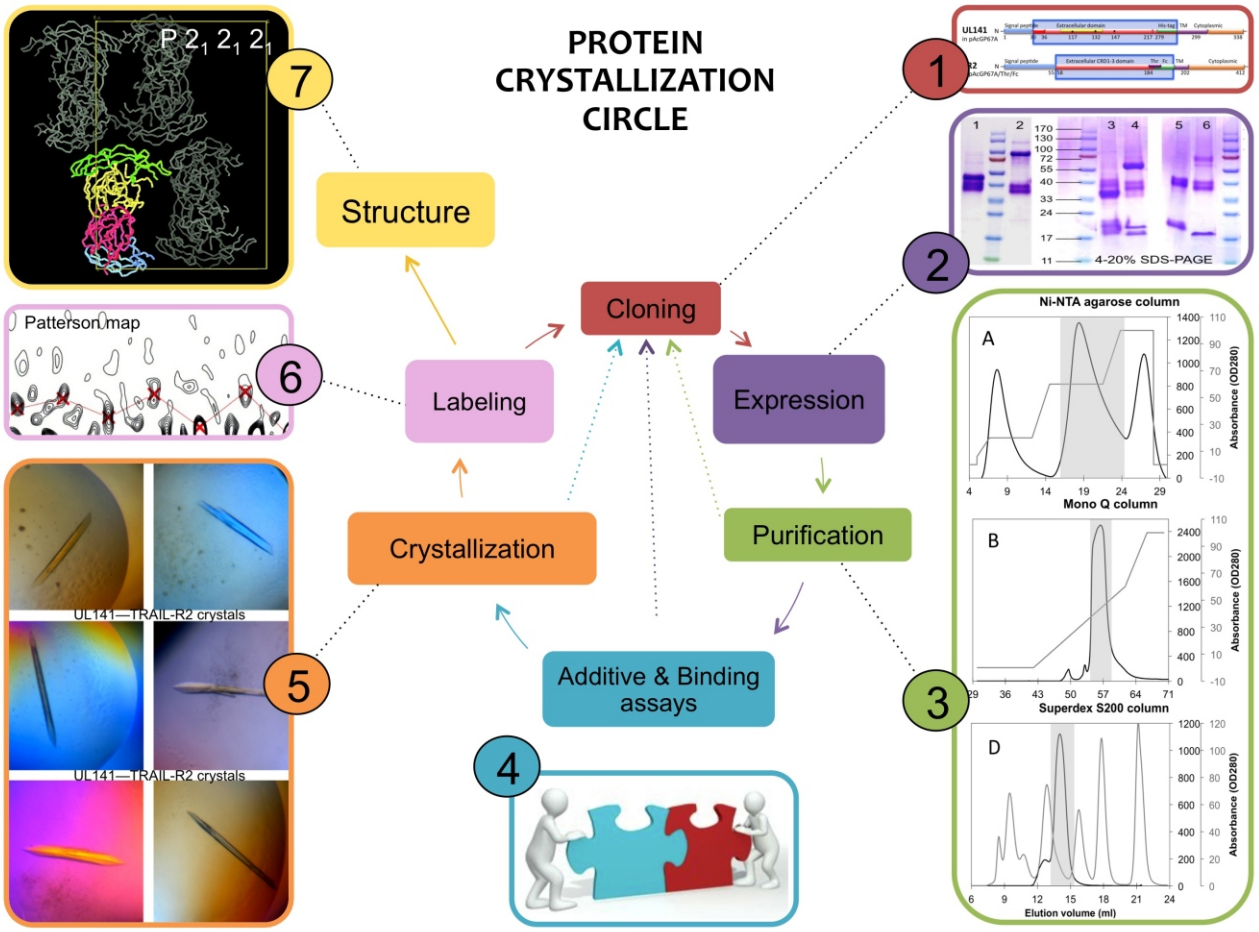

Figure 1. Protein crystallization circle: A number of ways to influence protein crystallization.

L15

PROTEIN AS THE MAIN VARIABLE IN CRYSTALLIZATION

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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.

1. Dale G.E., Oefner C., D'Arcy A. (2003) *J. Struct. Biol.* 142, 88-97.
2. Derewenda Z.S. (2004) *Methods* 34, 354-363.
3. Derewenda ZS and Vekilov PG (2006) *Acta Cryst.* D62, 116-124.
4. Goldschmidt L., Cooper D., Derewenda Z., Eisenberg D. (2007) *Protein Science.* 16:1569-1576.
5. Smialowski P., Schmidt T., Cox J., Kirschner A., Frishman D. (2006) *Proteins: Struct. Funct. Bioinf.* 62, 343–355.

L16

"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.*

Bergfors, T.M. *Screening and optimization methods for nonautomated crystallization laboratories.* 2007, Methods in Molecular Biology, vol. 363, 131-152.

L17

A HISTORICAL PERSPECTIVE ON PROTEIN CRYSTALLIZATION FROM 1840 TO THE PRESENT DAY

Richard Giegé

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Protein crystallization has been known since 1840 and proved to be straightforward in many cases, but it constituted often a real bottleneck, especially for structural biology projects. This stimulated the birth of the biocrystallogenesis field with both 'practical' and 'basic' science aims. In the early years of biochemistry, crystallization was a tool for the preparation of biological substances. Today, biocrystallogenesis aims to provide efficient methods for crystal fabrication and, amongst others, a means to optimize crystal quality for X-ray crystallography [1–6].

The historical development of crystallization methods for structural biology occurred first in conjunction with that of biochemical and genetic methods for macromolecule production, then with the development of structure determination methodologies and, recently, with routine access to synchrotron X-ray sources. Previously, the identification of conditions that sustain crystal growth occurred mostly empirically but, in recent decades, this has moved progressively towards more rationality as a result of a deeper understanding of the physical chemistry of protein crystal growth and the use of idea-driven screening and high-throughput procedures [7]. Protein and nucleic acid engineering procedures to facilitate crystallization, as well as crystallization methods in gelled-media or by counter-diffusion, represent recent important achievements, although the underlying concepts are old [7–9]. Clever screening and seeding procedures and the new nanotechnologies have brought significant improvements in the practice of protein crystallization [7, 10–13]. Finally, the old topic of protein crystallization in living cells became recently rejuvenated with the advent of X-ray free-electron lasers that opened the new field of *in vivo* X-ray crystallography [14, 15].

Today, the increasing number of crystal structures deposited in Protein Data Bank could mean that crystallization is no longer a bottleneck. This is not the case, however, because structural biology projects always become more challenging and thereby require adapted methods to enable the growth of the appropriate crystals, notably macromolecular assemblages.

Selected references:

1. A McPherson (1990) *Eur J Biochem* **189**, 1-23.
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6. I Russo Krauss, A Merlino, A Vergara & F Sica (2013) *Int J Mol Sci* **14**, 11643–91.
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12. J Newman et al (2005) *Acta Cryst* **D 61**, 1426–31.
13. A McPherson & B Cudney (2006) *J Struct Biol* **156**, 387–406.
14 JD Ng, RC Stevens & P Kuhn (2008) *Methods Mol Biol* **426**, 363–76.
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Lectures - Wednesday, June 30

L18

CRYSTALLIZATION AND CRYSTALLOGRAPHIC ANALYSIS IN MICROFLUIDIC CHIPS

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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.

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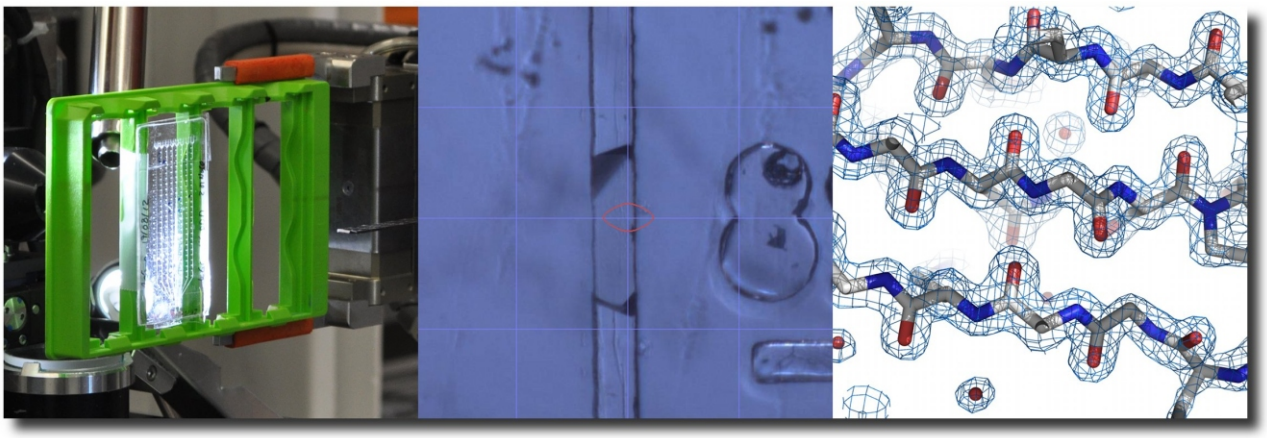

L19

CRYSTAALLIZATION SCREENING - NEW APPROACHES

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We use trace fluorescent labeling (TFL) as a means of rapidly identifying crystals in the screening plate. The method involves the covalent labeling of between 0.1 to 0.2 % of the protein molecules with a fluorescent probe. Our standard labeling process uses the amine reactive dye 5(6)-carboxyrhodamine 6G succinimidyl ester (Invitrogen, C-6157), with reaction conditions (pH) adjusted to label random side chain amines. Previous results had shown that labeling below 1% does not affect the nucleation rate or diffraction data quality. Identification of crystalline outcomes is based on intensity; for TFL protein packing density is highest in the crystalline form which will fluoresce more brightly than other precipitated forms. We are finding that there are many outcomes where the fluorescent images have regions of high intensity, but no corresponding crystalline structures are apparent using white light transmission microscopy. Under the governing paradigm, that intensity = structure, we hypothesized that these are likely lead conditions and testing that hypothesis with optimization screening. We are only using one screen (Hampton Research High Throughput), with 6 plates set up for each protein (three with and three without TFL) to test if there are any effects of the label on the crystallization process.

To date the number of TFL+ hits in the initial screen data is slightly higher than for the TFL- hits. However, the additional leads can only come from the TFL+ screening plates. Our standard optimization method uses capillary counter diffusion (CCD). Overall success rates for optimization of the TFL-derived leads are ~40 % from CCD experiments. However, we are now exploring the use of ionic liquids (IL's) as crystallization optimization additives. Seven commercial off the shelf IL's are being tested in this first round of experiments, with the IL's used at 0.1 M final concentration. The proteins employed in this study are not the usual models, but rather part of several ongoing research projects in this laboratory. Based upon the results to date the IL-based optimizations are at least equivalent, with the added benefit that the IL optimizations can be set up more quickly. While the results vary, they clearly show that there is an IL structure dependence for the outcome, suggesting that the IL structures can be modified to further improve their effectiveness. Independent of the approach employed, even proteins that did not give crystals in the initial screen have given crystals after optimization of the TFL-derived leads.

L20

TIPS AND TRICKS FOR PROTEIN CRYSTAL MANIPULATION AND HANDLING

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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.

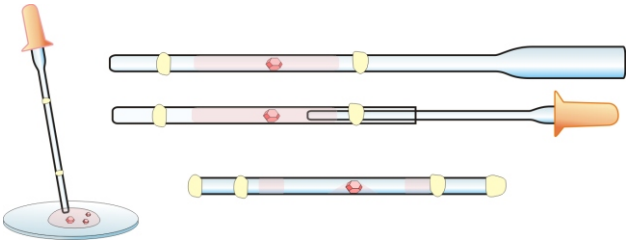

Figure 1. Schematic representation of how to prepare your crystal for room temperature X-ray diffraction test or data collection.

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L21

DLS MEASUREMENTS PRIOR TO CRYSTALLIZATION EXPERIMENTS

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Automated methods to crystallize macromolecules are Dynamic Laser Light Scattering(DLS) is today a well established method to optimise protein solutions prior to crystallisation experiments by analysing the dispersity of the protein suspension. 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 remains a bottleneck. We have investigated and improved methods allowing to analyze the process of evaluating crystallization experiments and identifying crystal growth conditions. Besides imaging drops, two methods have emerged as most promising. One is dynamic light scattering (DLS), which has already many applications, but which we today is most useful for detection of aggregation and nucleation in droplets as well as in counter diffusion capillaries [1-6]. The other is the use of a combined white/ UV illumination for microscopic determination of whether crystal-like objects are biomolecular and identification of crystals in crystallisation set ups [7]. DLS is today a more common used 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. We will describe a advanced and most suitable method to image, measure and analyse the protein 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. This has several advantages: no additional pipetting is necessary to perform measurements; the crystallisations process can be monitored online in situ, without interruption; measurements can be taken from even small volumes. This new DLS technique has been adapted to an automated CCD-camera-based plate-screening systems 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. We will also describe a method to support the identification of protein crystals, exploiting the fact that most proteins and other biomolecules fluorescence when illuminated with UV light. The imaging and scoring systems mentioned before incorporate all of the techniques described above in one device. Images taken from various droplets/set ups will be presented along with corresponding DLS measurements. Details will be presented.

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L22

SOME METHODS TO MAXIMIZE THE PROBABILITY OF PROTEIN CRYSTALLIZATION

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Protein crystallization occurs at suitable supersaturation conditions. In this lecture, several methods that can adjust the supersaturation conditions so as to achieve better crystallization probability will be introduced. Specifically, application of methods like desiccation [1-3], cross-diffusion microbatch [4-5], cycling-temperature strategy [6], heterogeneous nucleation [7-9], and some special physical environments [10-11], can be helpful to find more new crystallization conditions during crystallization screening process.

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L23

ASSESING 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, July 1

L24

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.

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L25

LARGE VOLUME CRYSTAL GROWTH IN RESTRICTED GEOMETRY FOR NEUTRON CRYSTALLOGRAPHY

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Neutron Macromolecular Crystallography (NMC) is the prevalent method that accurately determines the positions of hydrogen atoms in macromolecules. There are two major limiting factors in determining protein structure with neutron diffraction; one is obtaining large crystal volumes and the other is the extended experimental duration. This lecture will demonstrate that crystallizing hyperthermophilic proteins by counter-diffusion crystallization can produce suitable protein crystals for NMC. Counter-diffusion crystallization in restricted geometry, such as in capillary tubes, allows diffusion to govern mass transport processes so that a spatial-temporal gradient of supersaturation is formed along its length. Consequently, proteins can crystallize in an optimized supersaturated condition where a single crystal can fill up the diameter of the capillary. We have employed the same process in capillary vessels having diameters exceeding 1mm while minimizing the ratio between buoyant and viscous forces. As a result, protein crystals suitable for neutron crystallography can be grown in this configuration with volumes greater than 1mm^3 while eliminating invasive crystal manipulation. Using crystals grown by this method, we have undergone neutron diffraction analysis of recombinant proteins from a hyperthermophilic archaeon. These proteins are excellent crystallization targets because of their thermal and mechanical stability and high propensity to crystallize. As an initial proof of principle, a novel inorganic pyrophosphatase (IPPase) was among the first of these proteins to be grown in large diameter capillaries resulting in a 9mm^3 volume crystal. The next generation neutron beamlines at the

Spallation Neutron Source (SNS) along with an effective counter-diffusion crystallization procedure can improve structures of a diverse range of proteins of interest to the crystallographic community. Thus the crystallization of macromolecules can grow to volumes never before achieved, potentially leading to enormous increases in the number of neutron crystallographic protein structures.

L26

OPTIMISATION OF CRYSTAL GROWTH FOR NEUTRON CRYSTALLOGRAPHY

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In neutron macromolecular crystallography where the available neutron sources are weak, compared to X-ray sources, the crystal volume required for a neutron data set is most often the factor limiting the more widespread use of this technique. If normal hydrogenated proteins are used, a minimum crystal size of at least 1mm^3 is necessary. If perdeuterated proteins are used the minimum crystal size can be as 'little' as approximately 0.15 mm^3 [1]. However, even with these large crystals, a single neutron diffraction data set can take several days or weeks to collect. For neutron crystallography to become more accessible to the structural biology community, the ability to control crystal size must become routine. This is particularly important in order to push the limits of neutron crystallography towards more challenging targets such as membrane proteins. Despite the knowledge gained in crystallization of model systems [2, 3], the principles are often difficult to implement in practice. Many proteins are not readily available in large quantities, which has driven crystallization methods towards small sample volumes often using vapour diffusion. One disadvantage of these methods is that the level of supersaturation is difficult to control effectively, especially as a function of time. Novel devices [4-8] have attempted to address this issue with somewhat different strategies, such as controlling the vapour pressure externally or using (micro)fluidics, dual polarization interferometry or fluorescence-based approaches.

I will introduce the crystallisation devices that combine precise temperature control with real-time observation through a microscope-mounted video camera [9, 10]. Latest instrument consists in the crystal growth bench that, in addition to accurate temperature control, also allows composition of the crystallisation solution (*e.g.* precipitant concentration, pH, additive) to be controlled and changed in an automated manner [10]. Our approaches allow the rational optimisation of large crystal growth based on a multidimensional phase diagram.

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L27

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

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Until recently macromolecular structures were primarily determined applying the single crystal method of X-ray crystallography. The new method of Serial Crystallography (SX&SFX), developed for X-ray Free Electron Laser Sources (FEL) however comes also more and more in routine use at high brilliant microfocus synchrotron 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 today 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 focused to micron dimensions these pulses vaporize the sample but the diffraction pattern is collected before that radiation damage sets in. The SFX method [1-3] can thereby achieve high resolution diffraction on sub-micron macromolecular crystals usually too small for conventional analysis and without the need for cryogenic cooling. However, it requires a large number of size-controlled crystals that are flowed across the beam as snapshot diffraction patterns are rapidly acquired. Today in the field of conventionell protein crystallization 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 X-ray suitable crystals still remains a bottleneck in most cases [4,5]. To také advantage of the new established and upcoming high brilliant SR- and FEL- radiation sources, which open new routes in structural biology [6] and allowing to collect diffraction data from micro- or nano- crystals via Serial Femtosecond Crystallography (SFX) [1-3] advanced crystallization and crystal scoring techniques need to be established. To meet future crystal requirements at FELs we followed two approaches to produce high quality nano- and micro-crystals during the last years. 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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L28

A RATIONAL APPROACH TO CRYSTALLISING PROTEINS IN THE PHARMACEUTICAL INDUSTRY, THE IMPACT OF MICRO SEED MATRIX SEEDING

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Crystallization is often referred to as a bottleneck in protein structure determination. However applying rational, knowledge based strategies and attention to detail, has proved to be very successful in obtaining crystallization systems for studying protein/inhibitor complexes in drug discovery.

This seminar will describe the rationale and the methods that have been successfully used to crystallize many medically relevant proteins [1] in four different pharmaceutical companies (Hoffmann-la Roche, Morphochem, Novartis and Actelion). The first part will deal with protein characterization prior to initiating crystallization trials [2], methods and strategies of screening and examples of protein modification for to improve crystallization.

The second part will focus on seeding and describe general seeding methods as an introduction to the most important subject of the talk, Microseed Matrix Seeding (MMS) [3-4]. One of the most powerful methods introduced into protein crystallization in the past 10 years in particular for establishing suitable co-crystallisation or soaking systems for obtaining X-ray structures of inhibitors complexes.

Examples will be given of increased hit rates, elimination of twinning, improved diffraction and promoting different space groups.

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