Advanced methods in macromolecular crystallization VI

The 1st joint FEBS-INSTRUCT crystallization course in the middle EU (FEBS PC14 005)

Academic and University Center, Nove Hrady, June 20 - 27, 2014

Lectures - Saturday, June 21

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 physico-chemical 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 MESOPHASES

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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 with reference to the latest update of the Membrane Protein Data Bank (www.mpdb.tcd.ie). 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 lipidic cubic phase or *in meso* method will be given at the *FEBS Lab Excercises* on Saturday, June 21.

Useful references

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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Lectures - Sunday, June 22

L3

NUCLEATION OF PROTEIN CRYSTALS: MECHANISMS AND SUGGESTED CONTROL STRATEGIES

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Crystallization of proteins starts with nucleation. This is a unique part of the process of crystal formation, in which a barrier, due to the excess free energy of the emerging solution-crystal interface, is overcome. Control of nucleation is crucial for the control of the number, size, perfection, polymorphism and other characteristics of the crystal population.

There have been significant recent advances in the understanding of the mechanisms of protein crystal nucleation. The foremost of these are the two-step mechanism of nucleation and the solution to crystal spinodal. The two step mechanism was initially proposed for protein crystals and it has been proven to apply to crystallization of small-molecule organic and ionic materials, colloids, polymers, and biominerals. According to it, crystalline nuclei appear inside pre-existing metastable clusters of size several hundred nanometers, which consist of dense protein liquid and are suspended in the solution. Thus, this mechanism affords a novel way to induce and control the protein crystal nucleation: by controlling the properties of the dense liquid clusters and the volume that they occupy. Recent studies have shown that the clusters are insensitive to electrostatic forces between the protein molecules. In contrast, both the cluster size and volume respond strongly to modifications of the hydrophobic interactions. These insights suggest that amphiphilic additives, broadly used in protein crystallization, such as glycerol, acetone, methyl pentane diol, and others, enhance crystal nucleation by fine-tuning the molecular hydrophobicity and in this way increasing the cluster volume.

At the high supersaturations typical of most crystallizing systems, the generation of crystal embryos occurs in the spinodal regime, where the nucleation barrier is negligible. The concept of solution-crystal spinodal helps to understand the role of heterogeneous substrates in nucleation and the selection of crystalline polymorphs: the substrates should have structural similarity to the expected crystal. This similarity allows them to guide the high concentration solution held in the clusters towards attaining crystalline order.

Vekilov, P. G. Nucleation. Crystal Growth & Design 10, 5007-5019, (2010).

Vekilov, P. G. & Vorontsova, M. A. Nucleation precursors in protein crystallization. Acta Crystallogr. F-Struct. Biol. Cryst. Commun. 70, 271-282, (2014).

L4

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 is critical information to contrast 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 in terms of 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. The final shape of protein crystals is controlled by the relative growth of different faces. This growth occurs by the addition of new building blocks to the crystal lattice. The three main growth mechanisms observed in protein crystal growth i.e. i) normal growth, ii) 2D nucleation mediated growth and iii) spiral growth, and its dependence on supersaturation, will be illustrated and their consequence on crystal shape and quality discussed.

L5

CONVENTIONAL CRYSTALLIZATION METHODS AND THEIR MODIFICATIONS

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With the phase diagram in mind, some of the shortcomings of conventional / common crystallization vapor diffusion set-ups will be discussed and alternative methods / modifications will be reviewed.

Classical vapour diffusion experiments can be modified by several pre and post set-up techniques:

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

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

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

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

Also the standard microbatch trials in Terasaki plates (droplets under paraffin oil) can be manipulated by choosing different oil (mixture of silicon and paraffin oil).

Most important, the experimentation does not stop after the cover slide is placed over the reservoir, so to speak.

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

L6

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.

L7

SEEDING STRATEGIES FOR "RANDOM" CRYSTAL SCREENING AND CRYSTAL OPTIMIZATION

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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]. One industrial group used the method to solve 38 out of 70 structures generated in a three year period, finding particular success with antibody complexes [2]. rMMS not only produces more hits, it also generates better-diffracting crystals - because crystals are more likely to grow in the metastable zone [3].

The theory and practice of the rMMS method will be described with case studies [e.g. 4], and studies of novel approaches to rMMS by Douglas Instruments [5] will also be presented, including working protein-protein and other complexes. Stefan will also talk about experimental design for optimization when you are using seeding [6].

1. D'Arcy, Allan, Frederic Villard, and May Marsh. "An automated microseed matrix-screening method for protein crystallization." Acta Crystallographica Section D: Biological Crystallography 63.4 (2007): 550-554.
2. Microseed Matrix Screening Crystallization of Antibody Fragments and Antibody-Antigen Complexes. RAMC, Strasbourg, France, 2011. Galina Obmolova, Biologics Research, Centocor R&D http://hamptonresearch.com/documents/ramc/RAMC2011_T11_Obmolova.pdf.
3. Further information on the theory and practice of the MMS method is available at the Douglas Instruments web-site, http://www.douglas.co.uk/mms.htm and http://www.douglas.co.uk/MMS_proc.htm.
4. Vera, L., Antoni, C., Devel, L., Czarny, B., Cassar-Lajeunesse, E., Rossello, A., & Stura, E. A. Screening using polymorphs for the crystallization of protein-ligand complexes. Crystal Growth & Design.
5. Shaw Stewart, Patrick D., et al. "Random microseeding: a theoretical and practical exploration of seed stability and seeding techniques for successful protein crystallization." Crystal Growth & Design 11.8 (2011): 3432-3441.
6. Shaw Stewart, Patrick D., and Peter FM Baldock. "Practical experimental design techniques for automatic and manual protein crystallization." Journal of crystal growth 196.2 (1999): 665-673.

Lectures - Monday, June 23

L8

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.

L9

CAPILLARY COUNTERDIFFUSION TECHNIQUE FOR PROTEIN CRYSTALLIZATION AND SCREENING

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Vapor diffusion and micro-bath under-oil are the most used techniques in Structural Biology laboratories for protein crystallization. In vapor diffusion technique the slow evaporation of a drop with a mixture of protein and precipitant brings the system towards the supersaturated region at certain rate while in batch methodologies protein and precipitating solutions are mixed to immediate reach a particular supersaturation value. Both techniques have inherent buoyancy driven convection and consequently crystals are grown in a heterogeneous environment compromising uniform crystal growth and quality. Chaotic mixing and convection can be reduced when the crystallization process proceed by diffusively mixing the protein and precipitant solutions. This effect can be achieve with liquid-liquid diffusion (free-interface diffusion) techniques in which protein and precipitant are allowed to diffuse one against each other in any media permitting diffusive mass transport (gels, capillaries, microfluidic devices or microgravity).

There are different ways to implement this technique. Among them, the most effective configuration proven to be useful for growing macromolecules crystals is the counter-diffusion (CD) technique. Unlike other techniques aimed at finding initial conditions close to equilibrium, counterdiffusion looks for initial high values of supersaturation thus provoking even the formation of amorphous precipitates at the earliest stages of the experiment. Then, by using a long protein chamber the technique exploits the simultaneous event of diffusion and crystallization giving rise to a supersaturation gradient along the length of the crystallization chamber.

In this talk we will discussed the effectiveness of counterdiffusion technique not only for improving crystal quality but also for the search of initial crystallization conditions when compared with traditional crystallization technique.

1. J.M. Garcia-Ruiz, Counterdiffusion methods for protein crystallization. *Methods in Enzymology*, **368** (2003) 130-154.
2. 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.

L10

"WHAT TO DO IF EVERYTHING HAS FAILED"

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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.

L11

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.

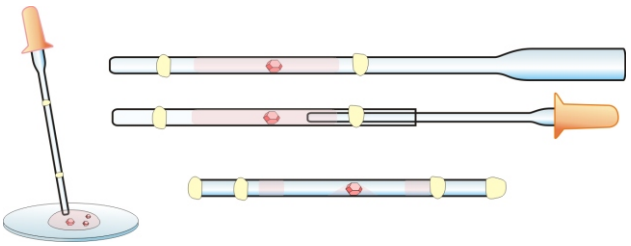

Figure 1. Schematic representation of how to prepare your crystal for room temperature X-ray diffraction test or data collection.

Lectures - Tuesday, June 24

L12

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.

L13

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.

L14

UNCONVENTIONAL CRYSTALLIZATION TECHNIQUES FOR SCREENING AND OPTIMISATION

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The past decade has seen momentous progress in the miniaturisation, automation and analysis of crystallisation experiments. However, production of high quality crystals still presents a major barrier 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 have 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. Saridakis, E. and Chayen. N.E (2013) Trends in Biotechnology 31, 515-520.
2. Saridakis et al. (2011) Proc. Natl. Acad. Sci. U. S. A. 108, 11081-11086.
3. Macromolecular Crystallization and Crystal Perfection. N.E. Chayen, J.R. Helliwell and E.H. Snell. Oxford University Press, Oxford, UK 2010.
4. Saridakis, E. and Chayen, N.E. (2009) Trends in Biotechnology 27, 99-106.
5. Chayen, N.E. and Saridakis, E. (2008) Nature Methods 5, 147-153.
6. Chayen, N.E. (2004) Curr. Opin. Struct. Biol. 14, 577-583.

L15

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

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Protein crystallization has been known since 1840 and can prove to be straightforward but, in most cases, it constitutes a real bottleneck. 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 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].

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.
2. A Ducruix & R Giegé (eds) (1999) Crystallization of nucleic acids & proteins: A practical approach, IRL Press, Oxford, (2nd ed).
3. A McPherson (1999) Crystallization of Biological Macromolecules. Cold Spring Harbor Lab. Press.
4. CE Kundrot (2004) *Cell Mol Life Sci* **61**, 525–36.
5. NE Chayen, JR Helliwell & EH Snell (2010) Macromolecular Crystallization and Crystal Perfection. Oxford University Press.
6. I Russo Krauss, A Merlino, A Vergara & F Sica (2013) *Int J Mol Sci* **14**, 11643–91.
7. M Jaskolski, Z Dauter & A Wlodawer (2014) *FEBS J* in press.
8. R Giegé (2013) *FEBS J* **280**, 6456–97.
9. BW Low & FM Richards (1954) *JACS* **76**, 2511-8.
10. J-M Garcia-Ruiz & A Moreno (1994) *Acta Cryst* **D50**, 484-90.
11. T Bergfors (2003) *J Struct Biol* **142**, 66–76.
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.

Lectures - Wednesday, June 25

L16

CRYSTALLIZATION AND CRYSTALLOGRAPHIC ANALYSIS IN MICROFLUIDIC CHIPS

Claude Sauter

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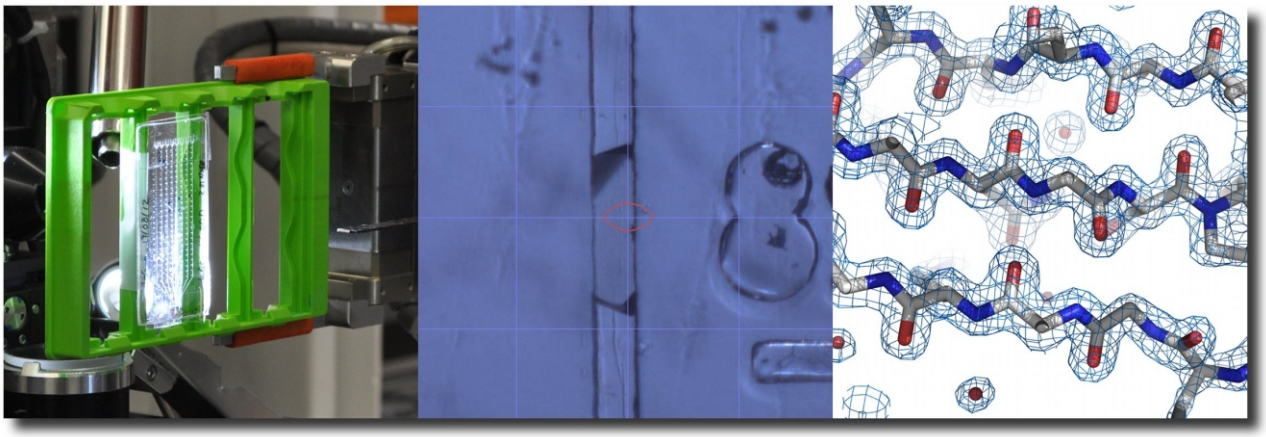
A decade ago microfluidic technologies opened new possibilities for the crystallization of biological macromolecules. Indeed, microfluidic systems offer a lot of advantages for crystal growth: they enable an easy handling of nano-volumes of solutions as well as an extreme miniaturization and parallelization 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.L7



L17

OPTIMIZATION OF CRYPTIC LEADS DERIVED FROM TRACE FLUORESCENT LABELING SCREENING

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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.

L18

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***.

L19

DLS MEASUREMENTS PRIOR TO CRYSTALLIZATION EXPERIMENTS

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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 have found useful for detection of aggregation and nucleation in droplets as well as in counter diffusion capillaries [1-4]. 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 [5]. DLS is a widely accepted 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. DLS is also a well established method to optimise protein solution quality prior to crystallisation experiments by analysing the dispersity. 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 relative new 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 two automated CCD-camera-based plate-screening systems (Spectro-Imager 501, and Spectrolight 600, Molecular Dimensions, UK) 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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Lectures - Thursday, June 26

L20

RECEPTOR–LIGAND INTERACTIONS CAN PROMOTE CRYSTALLIZATION

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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 (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 crytallize multiprotein complexes. The presentation is organized under several subtitles like identification/obtaining target sequence; pattern recognition techniques; protein tertiary structure prediction, choice of expression vector/system; sequence verification and host transformation; expression and solubility analysis; protein characterization; binding properties and assays; complex

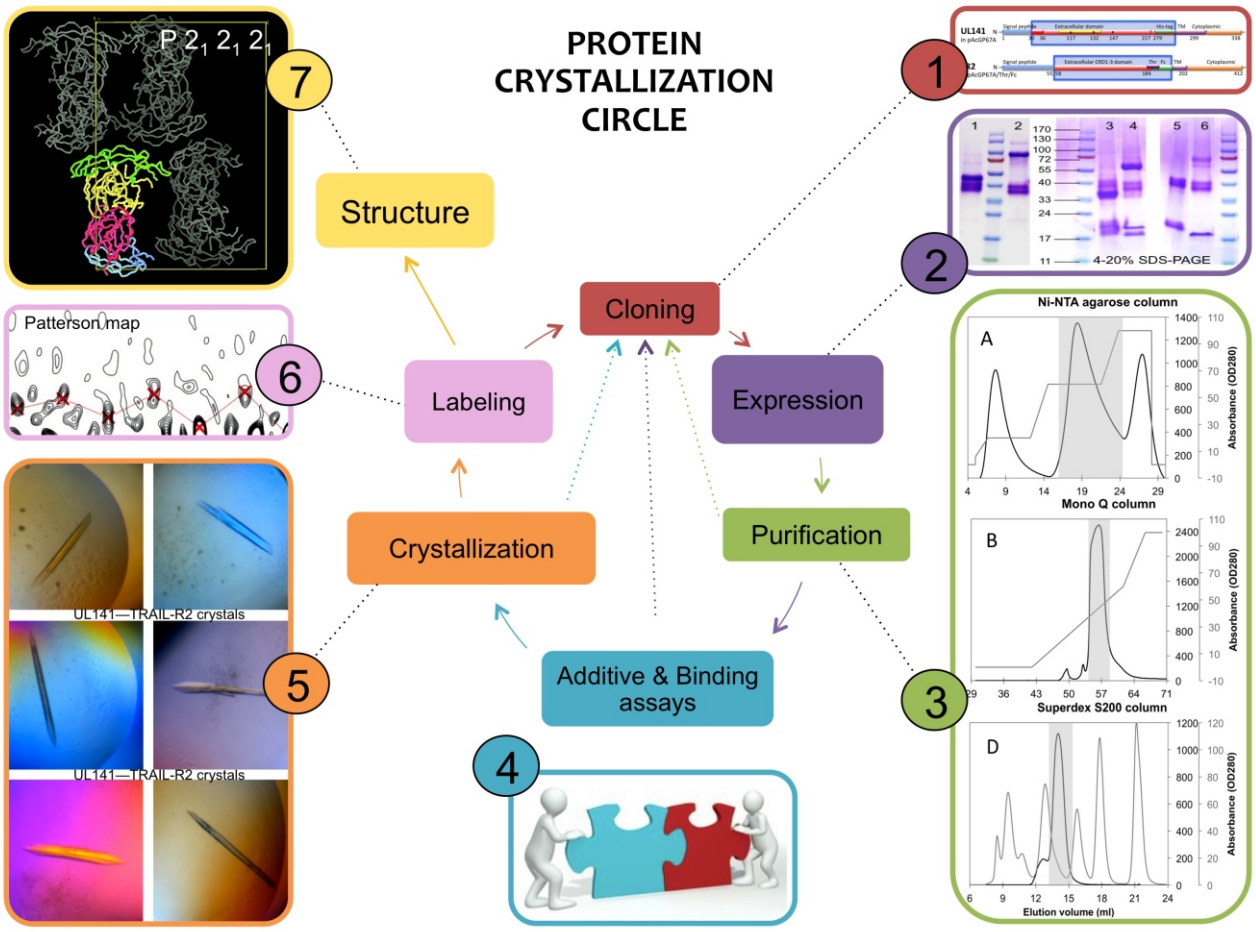

Figure 1. Protein crystallization circle: A number of ways to influence protein crystallization.

formation; crystallization of complexes and structure determination.

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 [1–4]. 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.

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L21

OPTIMISATION OF CRYSTAL GROWTH FOR NEUTRON CRYSTALLOGRAPHY

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Neutron macromolecular crystallography (NMX) is a key technique for the unambiguous identification of hydrogen atoms in macromolecular crystal structures. As the available neutron sources are weak, the crystal volume required for a neutron data set is most often the limiting factor for the more widespread use of this technique. If normal hydrogenated proteins are used, a minimum crystal size of at least 1 mm3 is necessary in order to achieve significant signal-to-noise ratios with the latest neutron sources [1]. If perdeuterated proteins are used the minimum crystal size can be as "little" as approximately 0.15 mm^3 [1], but this is still (assuming an isometric crystal) 0.53 x 0.53 x 0.53 mm, i.e. of a size that most X-ray crystallographers no longer try to produce. However, even with these large crystals, a single neutron diffraction dataset can take several days or weeks to collect. These and other technical difficulties partly explain why only 63 neutron crystal structures (0.07 % of the total number) have been deposited in the Protein Data Bank (http://www.rcsb.org/pdb) to date. Thus if neutron crystallography is to become a more routine technique for the structural biology community then the ability to control crystal size must become more accessible and routine for scientists working in the field. This is particularly important in order to push the limits of neutron crystallography towards more challenging targets such as membrane proteins.

The conditions under which a given protein crystallises cannot be inferred or predicted. Protein crystallisation is a very delicate process that depends on a large number of environmental variables (e.g. precipitant and protein concentration, equilibration rate, temperature) that are difficult to control precisely in the setups typically used. The difficulty of obtaining diffracting crystals is often the bottleneck also in X-ray crystallography, but with the current synchrotron instrumentation there is little incentive to increase the crystal size much beyond 200 m. The optimisation of crystallisation conditions for X-ray work has therefore concentrated in massively parallel, automated methods striving to use a minimal amount of protein per setup. In growing crystals for neutron crystallography the challenges are different, as increasing the crystal volume while maintaining the diffraction quality is the primary driver. While the theoretical background of the crystallisation process is well established and studied with model systems [2, 3], the principles are often difficult to implement in practice, as the level of supersaturation cannot be controlled effectively in crystallisation setups using small enough amounts of precious protein. Novel devices [4, 5] attempt to address this issue with somewhat different strategies.

I will introduce the crystallisation devices that combine precise temperature control with real-time observation through a microscope-mounted video camera [6, 7]. 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 [7]. Our approaches allow the rational optimisation of large crystal growth based on a multidimensional phase diagram.

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L22

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

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Growth and preparation of high quality micro-crystals optimal for data collection experiments at modern micro-beam insertion-device synchrotron (SR) beamlines and growth of nano-crystals required for data collection at future 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 focused to micron dimensions these pulses vaporize the sample but the diffraction pattern is collected before that radiation damage sets in. The SFX method [1,2] 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 [3,4]. Considering the tremendous advantages of the new and upcoming high brilliant SR- and FEL- radiation sources, allowing to collect diffraction data from micro- or nano- crystals via the new method of Serial Femtosecond Crystallography (SFX) [1,2] new crystallization and crystal scoring techniques need to be established. SFX offers new possibilities to analyse proteins that do not form crystals suitable for conventional X-ray diffraction in vitro and will open new routes in structural biology [5]. 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 [6] 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 [7]. Details and examples will be presented.

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L23

ADDITIVES IN MACROMOLECULAR CRYSTALLIZATION

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Macromolecular crystallization is a multi-parameter process which depends, amongst others, on exact composition of the crystallization solution. While the major components of the mixture, such as water, precipitant, protein and/or other macromolecules and buffer compounds determine the trajectory of the crystallization process as for its speed and the basic outcome, minor components, which can be usually present for some in sub-mM to mM and for other in units to tens of volume % concentrations can vary dramatically as for their effect on a given crystallization condition. Chemical or macromolecular impurities can also effectively play the role of additives in crystallization but let us limit the term "additive" only to compounds deliberately added to the crystallization mixture to change the outcome of a crystallization experiment. A particular additive action can affect protein itself, its solubility, dissociation or association, kinetics of crystallization experiment, properties of solvent, environment for electrostatic interactions (permitivity), and introduction of "missing" or withdrawal of unwanted compounds. They can be divided according to their chemical nature, which often determines their action: metals or ions, salts, organics, amphiphiles, chaotropes, chelators, carbohydrates, co-factors, linkers, reducing agents, solubilizers, and other. When applied to a particular crystallization experiment, the chemical nature of additives should be considered. A rational approach to additives screening should include application of several selected representatives from each major class of additives to test the overall effect, which can be further tuned if a useful result is observed.

The most critical point in additives application is addition to a defined low concentration in a manner that can be easily and reliably reproduced. Effects of additives can appear and disappear with their varied concentration. Both manual and robotic introduction of additives is possible, each providing its pros and cons. Given the small volumes of droplets in robotic crystallization, the ways of achieving exact low final concentrations without much disturbance to the original crystallization condition are limited. The classical manual addition of chemicals is usually performed from a ~ 10-20x higher concentration drop near the target crystallization drop by transferring a fraction of the target drop volume, which enables immediate optimization in the case of unwanted effects (precipitation, phase separation, etc.). Examples of additive effects include: reduction of nucleation rate, change of crystal morphology, change from cluster growth towards single crystals, or change of protein behaviour. Several examples will illustrate the use of additives.

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L24

SCREENING THE DIFFRACTION QUALITY OF PROTEIN 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.

L25

ALTERNATIVE CRYSTALLIZATION TECHNIQUE

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In general, the crystallization of proteins is a very complex process. Experiences of many scientists point out that majority of proteins is difficult to crystallize and even if a protein tends to crystallize relatively easily there are many parameters that must be taken into account. There are multiple reasons that point out the difficulty of protein crystal growth. Therefore, finding of successful crystallization conditions for a particular protein remains a highly empirical process. During optimization a variable set of parameters is screened to determine appropriate conditions for nucleation and growth of single crystals suitable for X-ray diffraction analysis. In parallel to modern high-throughput approaches used in the protein crystallization, in recent years we performed basic research on physico-chemical properties and molecular interactions influencing crystal growth. Empirically, we have explored another tool useful for optimization strategy that was first described by Tomčová and Kutá Smatanová (2007). A new crystallization procedure modifying protein crystal morphology, internal packing and influencing crystal growth was tested particularly. For the first time the metal ion salts were added simultaneously to the protein drop and even to neighboring drops to allow a cross-influence effect of additives during crystallization experiment. The presence of metal ions significantly influences the crystal growth, as the modification of crystal morphology and internal packing were observed. This newly discovered cross-crystallization method (Tomčová & Kutá Smatanová, 2007; Tomčová et al., 2006) was called Cross-Influence Procedure (CIP).

Ivana Nemcovicova and Ivana Kuta Smatanova: Chapter 11: Alternative Crystallization Technique: Cross Influence Procedure (CIP). In the *Crystallization and Materials Science of Modern Artificial and Natural Crystals*, Pages 249-276, Edited by: Elena Borisenko, ISBN 978-953-307-608-9, Publisher: InTech (2012).

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