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Optimization of Protein Crystallization: The OptiCryst Project

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Supporting Information

ABSTRACT: Protein crystallization has gained a new strategic and commercial relevance in the postgenomic era due to its pivotal role in structural genomics. Producing high quality crystals has always been a bottleneck to efficient structure determination, and this problem is becoming increasingly acute. This is especially true for challenging, therapeutically important proteins that typically do not form suitable crystals. The OptiCryst consortium has focused on relieving this bottleneck by making a concerted effort to improve the crystallization techniques usually employed, designing new crystallization tools, and applying such developments to the optimization of target protein crystals. In particular, the focus has been on the novel application of dual polarization interferometry (DPI) to detect suitable nucleation; the application of *in situ* dynamic light scattering (DLS) to monitor and analyze the process of crystallization; the use of UV-fluorescence to differentiate protein crystals from salt; the design of novel nucleants and seeding technologies; and the development of kits for capillary counterdiffusion and crystal growth in gels. The consortium collectively handled 60 new target proteins that had not been crystallized previously. From these, we generated 39 crystals with improved diffraction properties. Fourteen of these 39 were only obtainable using OptiCryst methods. For the remaining 25, OptiCryst methods were used in combination with standard crystallization techniques. Eighteen structures have already been solved (30% success rate), with several more in the pipeline.

INTRODUCTION

Structural genomics, together with a myriad of postgenomic research activities, is being investigated worldwide to realize the enormous medical, social, and economic potential of the information coded by living organisms. In particular, the wealth of information obtained by structural genomics initiatives together with advances in computation has allowed protein-structure-based drug design to complement screening and combinatorial chemistry in providing the basis for more efficient drug development: ultimately, this approach will reduce the time of the synthetic cycle and the cost per drug.

Structural genomics has coincided with the era of the high-throughput culture, which has resulted in major advances in the

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automation of protein preparation and X-ray crystallographic analysis, as well as progress in automating and miniaturizing crystallization trials.[1−3] The number of such trials are indeed impressive, reaching thousands per day, yet for all current structural genomics projects, high throughput has not led to high output. This is especially problematic, since the production of suitable crystals is found to be a rate-limiting step even once an active protein target has been solubilized and purified.[4] As of September 2010 (Table S1), from 44,986 purified proteins, only 8,076 diffracting crystals were produced:[5] indeed, in the past decade, the proportion of purified proteins that has yielded suitable crystals within all major structural genomics projects worldwide has remained consistently and stubbornly at around 18%.

Although screening has been very valuable in finding initial conditions for crystallization, the conversion of those crystal "leads" into useful diffracting crystals has not always followed. This problem is becoming ever more acute as the supply of proteins referred to as the "low hanging fruit" is being exhausted and the more difficult ones of high therapeutic value remain unsolved. Essentially, then, large-scale screening has not been sufficient to deliver the desired numbers of useful crystals.

In this context, intensive research in the science of crystallogenesis can provide the tools to attain better control of the crystallization process, including the design of new and improved optimization methods to turn crystal leads into useful diffracting crystals.

Crystallization is a phase transition phenomenon in which crystals grow from an aqueous protein solution when the solution is brought into supersaturation by varying parameters such as protein concentration, pH, additives, etc.[6] The crystallization process can be illustrated by a phase diagram that indicates which state (liquid, crystalline, or amorphous precipitate) is stable when varying these crystallization parameters. In a typical crystallization phase diagram there are four representative zones indicating different degrees of supersaturation: (a) high supersaturation, where the protein will precipitate; (b) moderate supersaturation, where spontaneous nucleation will occur; (c) the metastable zone (just below the nucleation zone) of lower supersaturation, where crystals are stable and may grow but no further nucleation will take place—this region offers the best conditions for growth of well-ordered crystals; (d) undersaturation, where the protein is fully dissolved and will never crystallize. In an ideal experiment, once nuclei have formed, the concentration of protein in the solute will drop, thereby leading the system into the metastable zone where few single crystals will grow. In the majority of protein crystallization experiments, however, either no crystal forms at all or excess nucleation occurs, yielding numerous clusters of tiny crystals. Therefore, it is of the utmost importance to be able to control the crystallization process in order to drive the system into the appropriate area of the phase diagram.

The OptiCryst approach (running from December 2006 to August 2010) has been a concerted effort by seven SMEs (small and medium-sized enterprises) and four academic groups integrating complementary techniques.[7] The overall objective has been to address the critical postprotein production bottleneck in the field of structural genomics by creating a research platform focused on the development, implementation, and exploitation of new crystallization technologies that are based on understanding the science of crystallization rather than on trial and error. OptiCryst focuses only on techniques which have been shown to work on several model and target proteins and can be further applied in novel ways for use with challenging proteins. The consortium has handled 57 proteins to date, most of which have been selected on the basis that crystal hits could not be optimized by merely fine-tuning conditions. The project generated 39 crystals with improved diffraction. Fourteen of these 39 were only obtainable using OptiCryst methods. For the remaining 25, OptiCryst methods were used in combination with standard crystallization techniques. Overall, with the implementation of the OptiCryst approach, this 65% success rate in crystallization far exceeded that anticipated (Table S1). Eighteen structures have already been solved (a 30% success rate; Table S3), with more in the pipeline. Here we discuss some of the highlights of the project that have enabled us to achieve these improved success rates.

DEVELOPMENT OF TOOLS AND METHODOLOGIES

In this section we describe the techniques that have been developed within the consortium. The first section covers evaluation of potential hits, including dual polarization interferometry (DPI) to detect nucleation, automated *in situ* dynamic light scattering (DLS) to monitor and analyze crystal growth, and UV fluorescence to differentiate crystals from salts. In the second section, we describe novel ways to increase crystallization success rates, encompassing the development of screening kits for the counterdiffusion technique, the design and use of novel nucleants, the automation of seeding procedures, the novel use of seeding in counterdiffusion experiments, the utilization of clear drops, and the development of crystallization kits for growth in gels. For the full list of tools and methodologies developed within the consortium, please see Table S2 in the Supporting Information.

1. Evaluation of Potential Hits for Subsequent Optimization. Initial screening can give rise to a range of results encompassing precipitation, phase separation, and a variety of crystalline looking precipitates. Currently employed techniques to identify promising leads such as the "crush test" and the use of dyes are intrusive and often unreliable. However, each situation needs to be tackled in a different way. When evaluating possible hit conditions, one can be confronted with two difficult scenarios: the formation of indistinguishable amorphous precipitate or the formation of crystalline material, which could be either protein or salt. In the former case, DPI can be used to detect nucleation, leading to the formation of useful crystals, whilst *in situ* DLS allows characterizing, monitoring, and scoring the crystallization process of biological macromolecules. In the latter, UV-fluorescence, which is noninvasive, can be used to differentiate protein crystals from those of salt.

Use of Dual Polarization Interferometry To Detect Nucleation (Novel Imaging Method). Dual polarization interferometry (DPI)[8] can be used to differentiate between nucleation that leads to the formation of useful crystals and other solid state based transitions. The Opticryst consortium embarked on developing a DPI-based method to determine whether proteinaceous nucleation is likely to lead to useful crystals. DPI uses an alternating polarized laser beam to illuminate two slab waveguides within an optical multilayer structure, resulting in two independent interference fringe patterns at the output of the device. Changes in the refractive index (RI) of a protein solution in contact with the uppermost waveguide will manifest as a phase shift of propagating light in that waveguide and shift in position of the exiting fringes. In addition to the phase shift, the fringes are characterized by their contrast. This is a measure of the amount of light guided in the upper sensing waveguide compared with the lower reference guide. As a result, the contrast is affected by any losses that occur in the upper active (sensing) waveguide due to

absorption, scattering, or other physical phenomena. Because DPI is a waveguide technique, which probes very close to the surface along the whole waveguide length, it is sensitive to loss at a single point. This and the discrimination of loss from changes in RI at the surface, differentiates it from other typically used optical techniques.

A complete loss (or considerable decrease, in some cases) of the contrast had previously been observed when protein crystals grew on the surface of the sensor chip. Detailed analysis of the protein layer adsorbed to the surface, and comparative studies conducted simultaneously on the same solutions with polarized light microscopy showed that this drop in contrast corresponds to the early stages of crystal nucleation.[9] The usefulness of the technology was investigated with different protein crystallization methods, namely batch, microbatch, vapor diffusion, and dialysis. The dialysis approach was chosen, as it offers the possibility to actively control the crystallization processes.

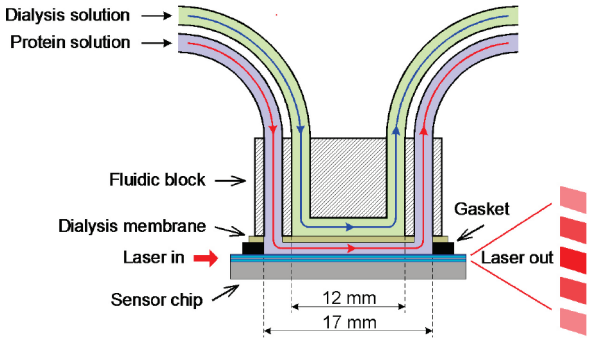

Figure 1. Diagram of the fluidic cell used to investigate the dialysis technique on protein crystallization using dual polarization interferometry. The polarized light propagates along the dual waveguide structure at the surface of the sensor chip and probes the interface with the protein solution above it. The dialysis solution is exchanged in a controlled way to perform dialysis on the protein solution across the dialysis membrane. Changes in the propagation of the light through the structure are measured as changes in the interference fringes generated when the light leaves the sensor chip.

Initial investigation by dialysis used a fluidic cell consisting of two parallel and independent sample channels, each separated from its own dialysate channel by a nanoporous ultrafiltration membrane. Figure 1 is a schematic representation of one of these cells.

Depending on the length of the exposed waveguide surface (5 or 15 mm) and the gasket depth (0.1 or 0.5 mm), the static protein volume probed was between 0.5 and 7.5 μL, with sample cell dimensions L = 17 mm, w = 1 mm, and h = 0.1−0.5 mm, and dialysate chambers L = 12 mm, w = 0.6 mm, and h = 1 mm. A typical experiment has protein in one channel and the buffer in which the protein has been dissolved in the second channel, which acts as a control. Once both channels are filled, they are isolated and flow is directed through the dialysis channels. In this way, the precipitant or an additive can be introduced with small steps in composition into the protein solution.

Figure 2 shows the dialysis of NaCl injected in small steps (1−13% w/v) at 40 μL/min into 30 mg/mL lysozyme (in 50 mM acetate buffer, pH 4.6, 20 °C) and buffer alone.

Overall, the dialysis method shows great potential for running real time crystallization screens against a wide range of precipitant conditions in a relatively short period of time for mapping crystallization phase space or screening additives. The technique is able to detect the onset of crystallization (nucleation) and has been successfully applied to differentiate crystallizing from noncrystallizing solutions of hen egg-white lysozyme, bovine liver catalase, and thaumatin from *Thaumatococcus danielii* (data not shown). Currently

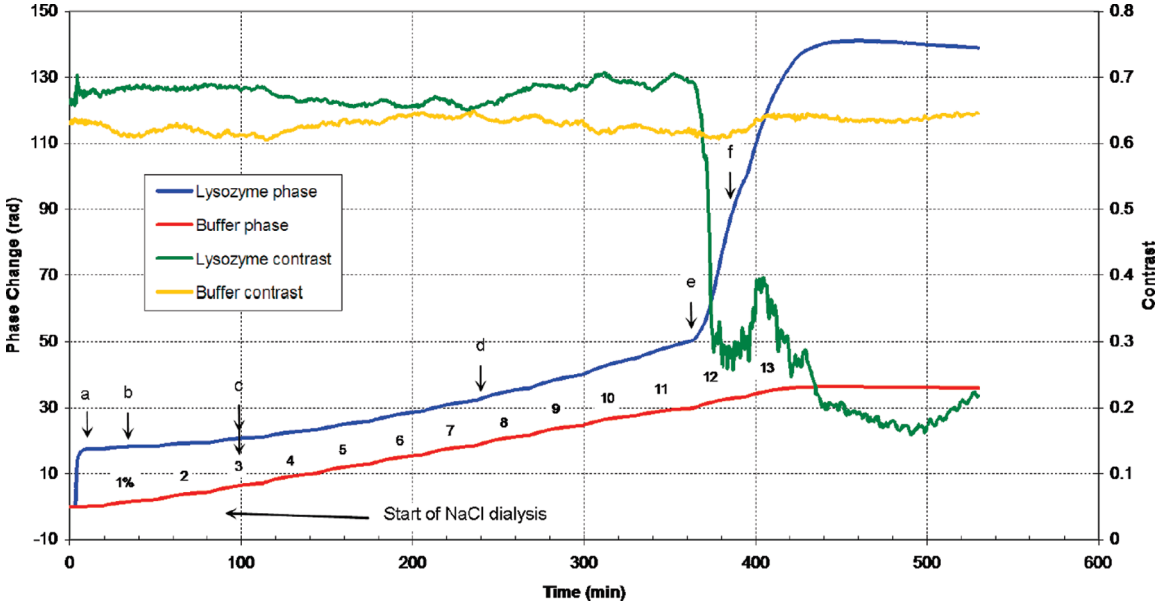

Figure 2. Dialysis experiment using dual polarization interferometry. The graph illustrates phase and contrast changes (shown for one polarization only—transverse magnetic mode) of 50 mM sodium acetate at pH 4.6 buffer (control) and 30 mg/mL lysozyme (sample) dissolved in the same buffer in response to NaCl (1−13% w/v) dialysis. The letters and arrows correspond to different stages of the dialysis as explained in the text. The following sequence of events was observed during the experiment: (a) On introduction of the protein sample, there was an increase in phase due to the RI of the bulk protein solution and the adsorption of a protein layer to the surface of the chip. (b) After onset of dialysis, desorption of protein from the surface occurred due to electrostatic shielding (salting in). (c) An increase in bulk RI was observed due to increasing salt concentration (continuous increase in phase). (d) Protein association occurred at the surface (slope of the phase in protein channel increases above that due to just salt). (e) The loss of fringe contrast suggested crystal nucleation on the surface. (f) Protein crystals and precipitation (large and rapid increase in phase) were observed.

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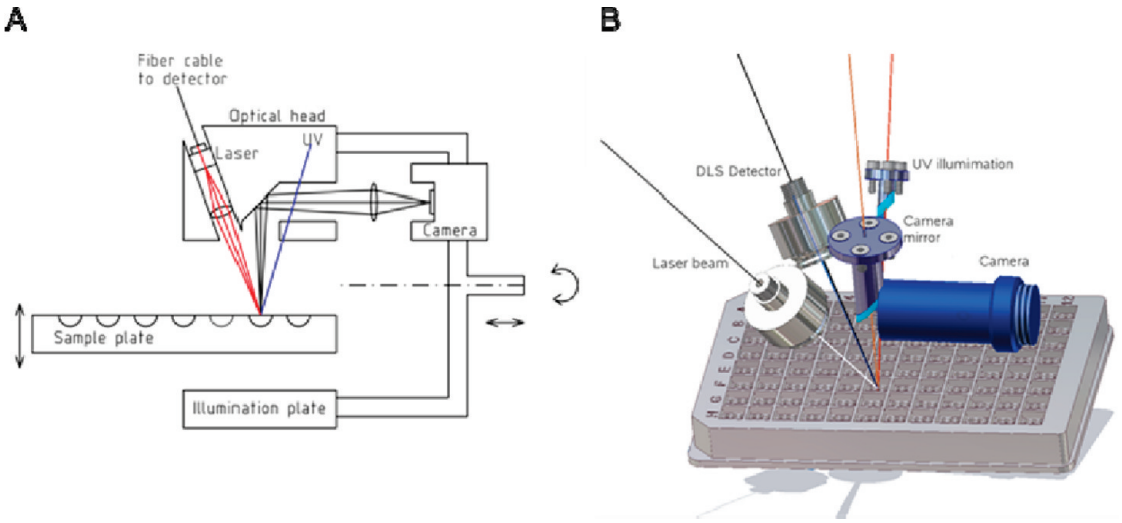


Figure 3. Scheme showing the *in situ* DLS imaging system named SpectroLight 500 and a close-up of the optics to analyze crystallization droplets in multiwell plates and other crystallization compartments. The hardware is manufactured by Nabitec GmbH and marketed as the Spectrolight 500 series by Molecular Dimensions Ltd.

the method is being developed further to include increased automation and further reduction in cell volumes.

Automated in Situ Dynamic Light Scattering To Monitor and Influence Crystal Growth. Crystal nucleation requires a higher degree of supersaturation than crystal growth. The aim of an ideal crystallization experiment is therefore to initiate crystallization at conditions that induce nucleation and subsequently "back off" in order to lead the system to metastable conditions before an excessive number of nuclei have had time to form. Therefore, the key to a successful crystallization experiment is to know the appropriate time at which to intervene. This is usually done by trial-and-error screening, but a more systematic approach is to use *in situ* DLS, which enables the analysis and scoring of crystallization experiments as well as their optimization to obtain crystals suitable for X-ray analysis. DLS is mainly used prior to crystallization to analyze the homogeneity and monodispersity of a protein solution.[10] It has also been known for many years that the aggregation behavior of proteins and other biomolecules can be investigated by DLS.[11] Recently, the technique was extended to screen and identify ideal buffers and additives in which a protein is most stable.[12]

Early crystallization studies demonstrated that good DLS data for a purified protein predicted successful crystal formation,[13] while the latest developments in the field have allowed the investigation of the submicroscopic processes taking place during crystallogenesis. Initial studies of this type, using DLS for the prediction of crystallization conditions, were done already 25 years ago in standard DLS cuvettes.[14] However, these measurements required huge quantities of sample solution (30 μL), and thus, this rational approach could not compete with the emerging high throughput techniques. More recently, the use of DLS as feedback tool for the separation of nucleation and growth during batch crystallization experiments was investigated.[15] Prior to OptiCryst, Wessel and Ricka measured DLS in small droplets—as used in modern high throughput vapor diffusion experiments.[16] This approach provides a possibility to combine the empirical screening with rational feedback. In terms of the OptiCryst project, a more advanced DLS technique was established, allowing measurements to be made directly in crystallization droplets in a range of commercially available formats, e.g. 96- and 24-multiwell plates (Figure 3).[17]

For DLS experiments in hanging and sitting droplets, various commercially available multiwell plates, sealing sheets, and foils were tested. An example is shown in Figure 4, which illustrates the nucleation process in microbatch setups and capillaries.

These advances mean that DLS can be used for the analysis, scoring, and optimization of the crystallization processes, as well as exploiting phase diagrams on much smaller scales than are typical. This is possible, since high-quality DLS data can be obtained in volumes as small as 500 nL and in capillaries with an inner diameter of just 0.1 mm. Since the integrated mechanics allow adjustment in the *x*, *y*, and *z*-directions in steps as small as 10 μm, application of *in situ* DLS to even smaller formats is being investigated.

Since DLS can detect changes of interaction between molecules in solution, nucleation during a crystallization experiment can be monitored.[17−19] Figure 5 shows early nuclei and aggregates measured by *in situ* DLS. It is possible to distinguish nuclei from aggregates because during aggregation usually the monomer disappears rapidly while nucleation shows coexistence of nuclei and monomers. For most proteins, there is a gap in particle size distribution between the protein's monomeric state and the nuclei that are formed during nucleation, as described in phase transition and nucleation theory.[20,21]

In the case of lysozyme, the hydrodynamic radius of the monomers is 1.5−6 nm (depending—if pH and buffer and protein concentration are constant—on the precipitant concentration) while the minimum size of nuclei is 80−100 nm. Nucleation is assumed to be a highly dynamic process. If the size of an ordered aggregate exceeds a critical radius, the probability increases that nucleation will occur: such a nucleus is the precursor to a crystal.

UV Fluorescence To Differentiate Protein from Salt Crystals. UV-fluorescence is a promising technique to distinguish salt from protein crystals *in situ*.[22] This method has been efficiently incorporated into the imaging and scoring hardware, SpectroLight500 (Figure 6), developed by the Hamburg-based research partner and commercialized by Molecular Dimensions Ltd. The SpectroLight, which is able to analyze multiwell plates in a high-throughput mode, contains an advanced, combined white/UV light source

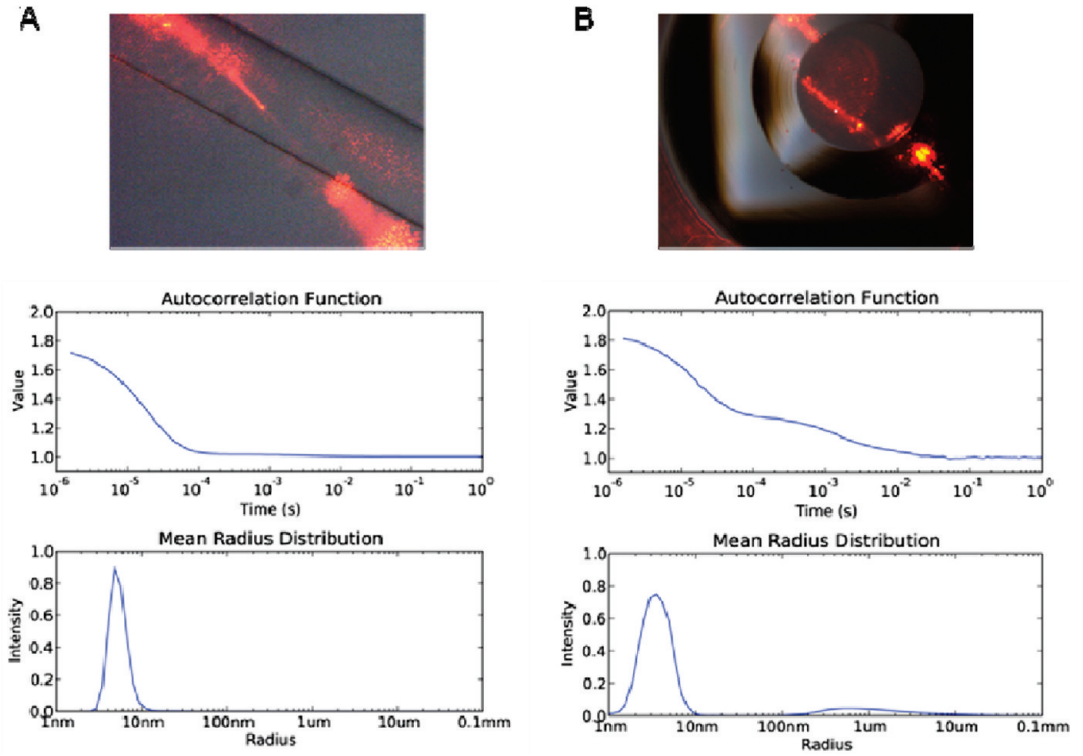


Figure 4. (a) DLS *in situ* measurement in a capillary counterdiffusion crystallization experiment with glucose isomerase. Below is the corresponding autocorrelation function and radius distribution, and on the right (b) an *in situ* DLS measurement of a microbatch lysozyme crystallization experiment is shown.

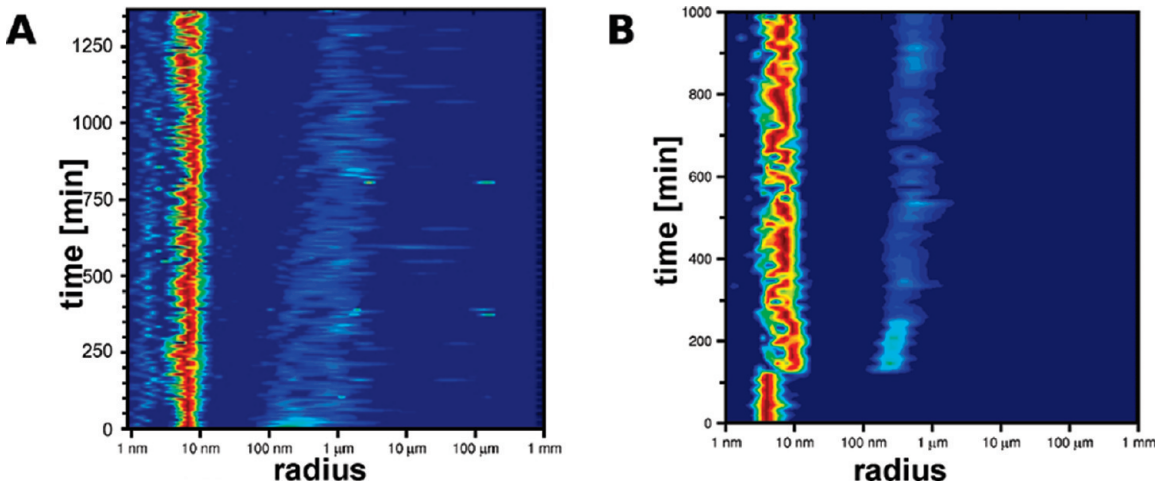


Figure 5. (a) Series of DLS measurements of glutathione-*S*-transferase during crystallization, nucleation, and further crystal growth. Smaller particles of 6.5 nm coexist with larger particles, and with time an increasing hydrodynamic radius of the larger particles can be observed. (b) Solution of lysozyme measured by *in situ* DLS for 100 min prior to adding precipitant. After adding the precipitant, immediately a shift in the monomer radius and the appearance of larger particles clearly separated from the monomers can be detected. As in part a, the hydrodynamic radius of the larger particles is increasing with time.

that excites efficiently tryptophan fluorescence. The camera optics with polar filter and zoom option allow the observation of crystal birefringence.

Protein crystallization can also be imaged *in situ* by attenuated total reflection−FT-IR spectroscopic imaging (ATR-FT-IR), which enables the examination of many different samples under a range of conditions in order to identify protein crystals.[23] The technique has been used to test crystals in arrays of <1 μL crystallization drops and successfully scored crystals or precipitates that contained protein, saving the time and effort of optimizing around inappropriate conditions. In one example, a membrane protein crystal that seemed promising by the eye, was shown to be salt.[23]

2. Increasing the Success Rate of Crystallization. In this section we discuss the key strategies explored in the project to enhance the likelihood of achieving crystals. Minimizing the amount of protein required in crystallization screens was prioritized by developing new,

smaller crystallization kits. Obtaining crystals from disregarded clear drops was also investigated as were the development of novel nucleants and the automation of seeding procedures to increase the success rate of nucleation.

Screening Kits for the Counterdiffusion Technique. Counterdiffusion methods have different kinetics from those of the more typically used batch and vapor diffusion. In practice, this means that it is possible to obtain sequentially amorphous precipitation, microcrystals, and crystals of the highest quality in a single capillary.[24] Counterdiffusion-based methods scan a large area of a phase diagram, thereby self-selecting optimal crystallization conditions (Figure 7b). This has been exploited to produce protein crystals in the presence of cryogenic reagents and anomalous scatterer atoms for *in situ* data collection and *ab initio* structure determination while preserving crystal integrity and quality.[25,26]

In order to understand the coupling between mass transport and crystallization, we have studied the most relevant parameters of the counterdiffusion technique (length and diameter of the capillary) and determined that for screening purposes it is sufficient to use capillaries of 0.1 mm × 30 mm; whereas optimization of crystal quality can be enhanced with either 0.2 mm × 30 mm or 0.3 mm × 30 mm using low concentration of agarose. With these dimensions in mind, a new counterdiffusion device, the Granada Crystallization Box (GCB-Domino, Figure 7a), was developed by Triana Science and Technology. Its smaller dimensions, compared to previous designs, allows the same number of experiments to be performed, but in a smaller capillary volume of 0.24 μL (L = 30 mm, i.d. = 0.1 mm). Despite these very small dimensions, the capillaries are long enough to scan a wide area of the full phase diagram. Furthermore, the GCB-domino can be incorporated into currently available robotic systems, both pipetting and imaging, due to its simplicity and adaptability.

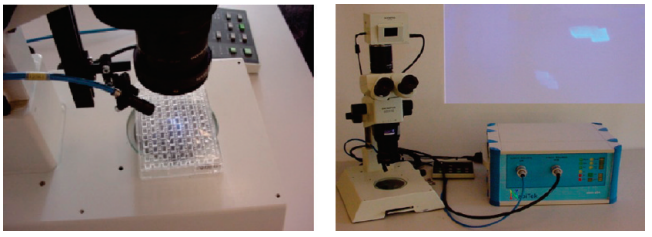


Figure 6. X-ta*Light* 100, which offers a UV-fluorescence source and is shown attached to a microscope.

Using counterdiffusion, several precipitants can be tested in one single experiment, thus making optimization faster. However, the precipitants must mix in solution at much higher concentration compared to mixing in vapor diffusion or batch. For this purpose, Triana has developed a 24 condition kit that is based on the solutions suggested by Kimber and colleagues[27] for vapor diffusion but adapted for counterdiffusion experiments. The full screen of 24 conditions can be implemented with fewer than 6 μL of protein solution in capillaries of 0.1 mm inner diameter or fewer than 12 μL if the experiment is set up at two temperatures: 4 and 20 °C.[28] This has been successfully applied to the crystallization of the N114A mutant of the SH3 domain of Abl tyrosine kinase complexed with a high-affinity peptide ligand[29] and to the crystallization of the oxy and cyano forms of the HbII-III complexes from *Lucina pectinata* hemoglobins.[28,30]

Apart from setting up counterdiffusion in specific devices such as capillaries, free interface diffusion experiments can be implemented in plates such as Laminex (Figure 8), which is developed and commercialized by Molecular Dimensions Ltd. Laminex offers considerable advantages for viewing and imaging crystal growth experiments, since the experiment is sandwiched between planar surfaces and the optical path creates no aberrations even when using viscous lipidic cubic phases.

Utilization of Clear Drops. The clear drops that frequently arise during screening are considered to be a dead end and are widely disregarded. In 2004, the utilization of specially designed plates known as EasyXtal Tools (made by Qiagen) that subjected hanging drops to controlled evaporation was reported.[31] The key feature of these tools is the replacement of conventional coverslips with screw caps that can be loosened and tightened to different extents and at defined intervals. When loosened, an immeasurable gap is formed which facilitates controlled evaporation of the crystallization droplets with the aim of driving them to supersaturation. The evaporation is then arrested by tightening the screw caps before nucleation becomes excessive. During the OptiCryst project, this technique has been refined and appropriated to both screening and optimization. It has been colloquially named the "twist" method and has been demonstrated to facilitate the detection of leads that would not have been found by standard screening procedures, to enable the use of significantly less protein than typically required and also to shorten the time scale required for crystal growth.[32] Furthermore, once a lead was obtained, the

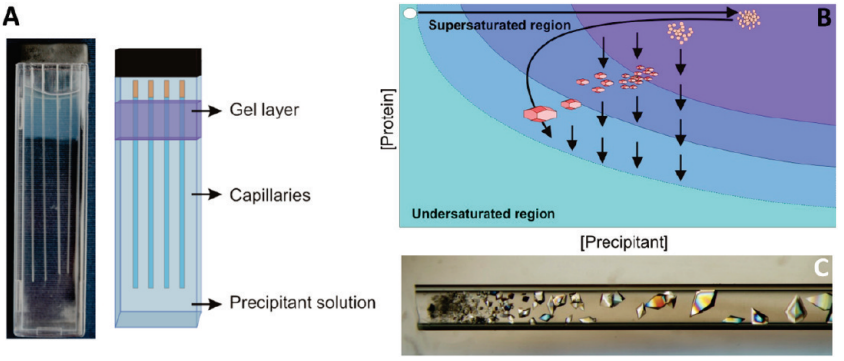


Figure 7. (a) Typical GCB-domino experiment. The box is filled with precipitant solution topped with a layer of agarose together with four 0.1 mm capillaries filled with protein solution. (b) In a counterdiffusion experiment, the precipitant diffuses into the protein solution inside the capillary. The interplay between precipitation and mass transport generates a supersaturation wave traveling along the capillary with time. (c) Formation of well-shaped crystals of thaumatin inside a capillary as a result of the evolution of supersaturation from high nucleation density (right) to discrete nucleation (left).

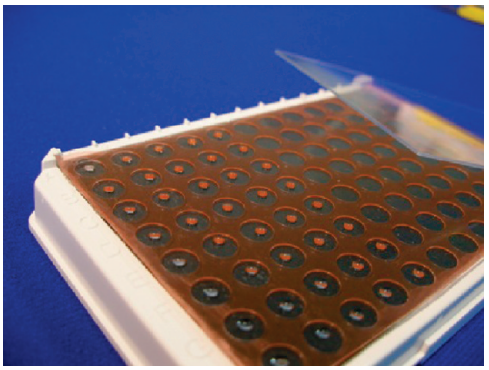
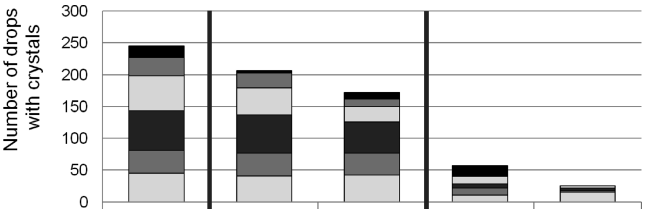


Figure 8. Laminex is a plate for crystal growth experiments, which occur in the narrow space between two plastic sheets or films. Laminex can be exploited with free interface diffusion, vapor diffusion, and microbatch.



	MMS in hit solution	MMS from Crystal Former	MMS from Triana capillaries	Mixed crystals, 15 unrelated proteins	Mixed precips. (same prot.)
Gluc. Isom.	18	3	10	17	0
Hemoglob.	29	24	12	0	0
Thaumatin	55	42	24	12	3
Thermolys.	62	60	49	6	5
Trypsin	36	36	35	11	2
Xylanase	45	41	42	11	15

Figure 9. Activity of seed stocks harvested from unconventional sources in microseeding (MMS) experiments. A total of 261 wells were set up using 6 test proteins in "pregnant" conditions that seldom or never gave crystals without seeding but generally gave crystals when seeds were present. Seed stocks made from crystals harvested from the Crystal Former microfluidic device by Microlytic (column 2) were nearly as effective as seed stocks from regular sitting drop experiments ("Hit Solution", column 1). Similarly, seed stocks from capillaries supplied by Triana (column 3) worked well. A seed stock made from crushed crystals of 15 unrelated proteins that were combined and suspended in 100% PEG 600 was less effective (column 4) but still gave crystals of 5 out of 6 test proteins. Mixtures of precipitates collected from screening experiments gave crystals of 4 out of 6 test proteins (column 5). The last two results are significant because these seed stocks can be used before any crystals have been obtained in regular screening experiments.

method has been used for optimization to yield highly diffracting crystals. The improvement of myosin binding protein crystals both visibly and in terms of diffraction is testament to this.[33,34] In order to render this technique high throughput, Molecular Dimensions has designed a small SBS format plate with screw caps which can be used with all robots (Figure S1).

Design and Use of Novel Nucleants. To date, nucleation has been facilitated mainly by seeding, epitaxy, charged surfaces, or mechanical methods.[35] Some of these approaches have been useful for individual proteins, but none has yet turned out to be of general use. The consortium has focused on the application of mesoporous materials containing pore sizes on the order of magnitude of protein molecules (5−10 nm) that trap protein molecules and create a local supersaturation maximum that facilitates nucleation. One example is a carbon-nanotube-based material, known as "buckypaper", which has been successfully applied for the crystallization of nonstructural protein 9 of the Transmissible Gastroenteritis Virus (Nsp9).[36] Over a dozen different porous materials were tested during the Opticryst project, out of which the most effective is thus far a bioglass.[37] This material has been commercialized in 2009 by Molecular Dimensions under the name of "Naomi's Nucleant" and can be used for both screening and optimization. In the case of screening, a grain of bioglass is placed in each screen drop. For optimization, the grains are inserted into drops at conditions of supersaturation which are slightly lower than nucleation conditions. The presence of Naomi's Nucleant can give rise to large single crystals that are often attached to the nucleant.[35]

Automated Seeding. Microseeding in random screens was introduced by D'Arcy et al.,[38] and it has been shown by D'Arcy and others that the technique, referred to as Microseed Matrix-Screening (MMS), gives a helpful improvement in crystallization in about 75% of cases where at least one crystal hit can be found.[39,40] The technique often gives (1) more hits and (2) better-formed crystals,[41] probably because crystallization is more likely to take place in the metastable zone of the crystallization phase diagram. D'Arcy et al.[38] suspended crushed seed crystals in the reservoir solution taken from the well where the crystals grew. In our study, variations of this technique were investigated, including suspending seed crystals in various solutions.

Microseeding experiments were carried out with six proteins: glucose isomerase, thaumatin, thermolysin, trypsin, and xylanase. All experiments were carried out in sitting drop plates with the Oryx8 crystallization robot by Douglas Instruments (this robot works well for microseeding because it uses contact dispensing). In order to quantify the effectiveness of different seed stocks, "pregnant" conditions were identified for the six test proteins. These were defined as conditions that seldom or never give crystals when seeds are not added, but which generally give crystals when crushed seed crystals are added. Protein crystals were identified using intrinsic UV fluorescence with the UV Pen-280 by Douglas Instruments.

Figure 9 shows that seed stocks can be harvested from capillaries and microfluidic devices. The three devices used all carry out crystallization by free interface diffusion. The Crystal Former HT 96-channel device, by Microlytic North America Inc. (Woburn, MA) (column 2) is a plate where crystallization takes place in specially formed conduits (around 150 μm width by 10 mm long). Crystals were harvested by removing the sealing film on the back of the plate under a microscope, crushing the crystals in the conduit with a probe, flushing the crushed crystals with 10 μL of the screening solution used, and transferring to a test tube on ice. Crystals were also grown in the counterdiffusion screening kit (24 conditions with 0.2 mm i.d. capillaries), by Triana Science and Technology (Granada, Spain). We placed a 10 μL drop of the Hit Solution onto a glass slide and pushed the crystals out of the capillary with a fine wire into the drop. We then crushed the crystals with a glass probe and transferred the suspended crystals to a test tube on ice.

The seed stocks from the Crystal Former (column 2) gave almost as many crystals as the conventional approach of harvesting seed stock from a sitting drop (column 1), while the seed stocks from capillaries (by Triana) were also very effective (column 3). Both approaches seem to be very useful, especially since it is often very difficult to translate crystallization conditions found in microfluidic

devices to sitting drop or microbatch-under-oil conditions. In addition to the examples shown, seed crystals of glucose isomerase were also successfully harvested from the Topaz system by Fluidigm and used in microseeding experiments.

Column 4 of Figure 9 shows the effectiveness of a seed stock made from crystals of 15 unrelated proteins (suspended in 100% PEG 600). This seed stock gave fewer crystals (column 4), but these results are promising because any approach that gives crystals when no seed crystals are available is particularly valuable. Crystals of five of the six test proteins were obtained using this stock. We also tested the method of Habel and Hung,[42] who made seed stocks by collecting protein precipitates from the wells of a screening experiment (we naturally excluded wells that contained visible crystals). Column 5 shows that the seed stock made from mixed precipitates also has potential, giving a small number of crystals of four of the six proteins tested.

Coupling of Seeding with the Counterdiffusion Technique. The seeding technique, which is based on the decoupling of nucleation and growth,[43−45] has traditionally been used with the batch and vapor diffusion methods because it eliminates the nucleation energetic barrier. However, for the success of the seeding technique, the protein system has to be in the metastable region of the phase diagram to avoid undesirable nucleation events. In theory, this would make seeding unnecessary with counterdiffusion because the latter self-screens for the best crystallization condition starting at high supersaturations.[24]

Nonetheless, we found that microseeding was helpful for the crystallization of several test proteins using the counterdiffusion technique. Seed crystals grown in, for example, vapor diffusion were crushed with a glass probe and mixed with protein stock prior to loading into the capillaries. The introduction of seeds increased the number of capillaries that contained crystals in random capillary screening experiments for 8 of the 9 proteins studied, and in five cases the number of conditions found at least doubled. The approach is particularly useful if protein samples are not available at high concentrations (either because they are insoluble at higher concentrations or because protein samples were prepared at a concentration that was appropriate for a sitting drop etc.).

Use of Gels in Crystallization Experiments. Crystals can be grown in small volumes of gel inside capillaries, thus combining the advantages of growth in gel with those of the counterdiffusion method.[46] Since nucleation can be promoted or inhibited depending on the gel chosen, the formation of gels at different pH values, in the presence of precipitant agents at different concentrations and in the presence of detergents, has been studied.[47] We have found that the use of gels in crystallization experiments provides a 2-fold advantage versus gel-free solutions: (1) it reduces convection; and (2) it allows controlling the nucleation. These two advantages result in an improvement of the crystal quality (i.e., resolution limit), especially with agarose and silica gels. Moreover, agarose gel increases the density of nucleation, whereas TMOS gels inhibits it, depending on the concentration of gel employed. The improved crystal quality achievable using the counterdiffusion technique has been tested with recombinant SmelDhp.[48] This protein was grown using both vapor-diffusion and counterdiffusion to obtain well-faceted crystals (0.6 mm × 0.2 mm × 0.15 mm).

Whereas sephadex and polyacrylamide gels cannot be incorporated easily in automatized systems and, therefore, are not suitable for the screening of proteins, automation is feasible using capillaries with low concentration of agarose gel (0.1%), that is, below the critical gelling concentration. This is because gels can be manipulated as low viscous solution, making the robotic handling of capillaries feasible.

Another way of using gels is to set up gelled trials in microbatch, which has enabled, for the first time, the automatic dispensing of 0.3−2 μL gelled drops in high-throughput mode using TMOS at low concentration.[49]

■ IMPROVEMENT OF CRYSTAL QUALITY OF TARGET PROTEINS

The objective of the OptiCryst project was to address the fact that, in the pipeline from clone to structure, there is a persistent bottleneck on going from purified protein to diffracting crystal. As shown in Table S1, the proportion of proteins that have yielded suitable crystals within all major structural genomic projects worldwide has remained consistently and stubbornly at around 18%. The OptiCryst project has for the first time cracked this bottleneck by developing and commercializing new technologies (Table S2).

The consortium collectively handled 60 new target proteins that had not been crystallized previously. The project generated 39 crystals with better diffraction. For 14 of these 39, only OptiCryst methods could yield crystals. For the other 25, OptiCryst methods were used in combination with standard crystallization techniques. Overall, with the implementation of the OptiCryst approach, the success rate in crystallization rose to 65%. Moreover, 18 structures have already been solved (a 30% success rate; Table S3), with more in the pipeline.

Examples of structures solved by the consortium include the immunoglobulin-like C1 domain of MyBP-C (Table S3, entry 25) obtained to a resolution of 1.55 Å[33] and the SARS-unique domain (SUD), a domain encoded in the genome of the SARS coronavirus, which is lacking in all other coronaviruses and, therefore, suspected to be involved in the extraordinary human pathogenicity of the SARS virus. Crystals of this domain became useful for structure determination only after major optimization efforts.[50]

The most recent success using capillary counterdiffusion has been the determination of the structures of dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114 (Table S3, entry 15)[48] and of the third PDZ domain of the neural postsynaptic density-95 protein (PSD95-PDZ domain, Table S3, entry 17).[51] More recently, a different polymorph of the R217W Xylanase mutant (Table S3, entry 11) was crystallized to a resolution of 1.8 Å by the oils barrier and counterdiffusion methods, where other techniques had failed.

The search for optimal crystallization conditions for a gluthathione-*S*-transferase from *Wucheria bancrofti* (*Wb*GST, Table S3, entry 42) was facilitated by the use of *in situ* DLS during initial crystallization trials. This led to rapid optimization of crystallization conditions from which crystals suitable for X-ray analysis could be obtained (data to be published).

McpS (methyl accepting chemotaxis protein) is a recently identified chemoreceptor which functions in mediating chemotaxis by recognizing most of the tricarboxylic acids cycle (TCA) intermediates in the soil bacterium *Pseudomonas putida* KT2440. We have obtained crystals of the full length protein together with two of its cofactors (i.e.: malic and succinic acids) to a resolution of 1.8 Å (Table S3, entry 32). The analysis of its 3D-structure has been crucial in addressing how McpS can recognize both substrates.

PtxS, which binds to a highly conserved promoter, is a transcriptional regulator involved in glucose metabolism in *Pseudomonas putida*. We have obtained crystals of native PtxS bound to DNA. Crystals were analyzed directly from the capillary where they grew in a microfocus beamline to a resolution of 2 Å (Table S3, entry 31).

TodT is a response regulator of the TodS/TodT two-component system which controls expression of the toluene dioxygenase (TOD) pathway for the metabolism of toluene in *Pseudomonas putida* DOT-T1E. Crystals of TodT bound to its DNA recognition sequence could only be obtained by the counterdiffusion method (Table S3, entry 33).

Another protein from Wucheria Bancrofti, a thioredoxin (*Wb*TRX), was recently crystallized after its instability was discovered by DLS. It has been shown by DLS monitoring that WbTRX was unstable at 10 °C as well as at 20 °C. Once purification and crystallization processes were adapted to ultrafast throughput, X-ray suitable crystals could be obtained from screening after one week. Optimization of the crystallization conditions led to crystals that diffracted up to 1.9 Å (structural data to be published, Table S3, entry 41).

In the case of CD81 (Table S3, entry 44), a human membrane protein that plays a key role in the infection of human hepatocytes by hepatitis C virus (HCV), we applied *in situ* DLS within 96 well plates in order to screen for optimum buffer and detergent conditions. It was observed that a small change in detergent concentration resulted in a clear shift from a polydisperse to a nearly monodisperse solution. The subsequent crystallization experiments applying the new detergent conditions yielded protein crystals (up to 6 Å), which were detected by combined UV/vis-imaging.

CONCLUSIONS

The Opticryst consortium has developed new crystallization technologies and tools and applied them to the crystallization of a number of proteins. In order to increase the efficiency in evaluating crystal leads, DPI has been used to detect useful nucleation, *in situ* DLS to monitor crystal growth, and UV-fluorescence to differentiate protein crystals from salt. In order to increase crystallization success rates, new crystallization screening kits for the counterdiffusion technique that minimize protein consumption have been developed along with technologies to utilize clear drops, the design of novel nucleants, improvements in the automation of seeding, coupling seeding with the counterdiffusion technique, and new kits to be used with gels.

By using these advances in combination with a thorough understanding of each protein target, rational approaches to crystallization are now within our reach.

ASSOCIATED CONTENT

Supporting Information. Tables of scores for all major strutural genomics projects, major technological developments during Opticryst, and details of the crystallization strategies used in the OptiCryst project; and photograph of the screw cap plate developed by Molecular Dimensions Ltd to allow an easy control of evaporation compared to standard grease-based setups. This material is available free of charge via the Internet at http://pubs.acs.org.

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