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1. Introduction

The biological activity of most proteins is determined by their 3D structure. For instance, a substantial number of molecular diseases are caused by protein structural alterations, which are genetically encoded. Drugs operate by binding to proteins, inducing alteration of their functional structure and thereby affecting their biological activity. Hence the design and improvement of drugs is greatly facilitated by knowledge of the 3D structures of their macromolecular targets. In the light of these considerations, it is clear that elucidation of the 3D structure of proteins is of prime importance for understanding the underlying mechanisms of molecular diseases. It was initially believed that any protein that could be made soluble and could be purified would be relatively easy to crystallize. However, the results have indicated that solubility and purity of proteins, although being important factors, do not secure a yield of useful crystals. The crystallization behavior of proteins turns out to be very complex.

In an effort to identify the naturally occurring protein folds, large structural genomics consortia were set up. The somewhat disappointing outcome of these efforts is that only about 3% of all proteins that were targeted by these consortia yielded a crystal structure (http://targetdb.pdb.org/statistics/TargetStatistics.html), despite massive investments in high-throughput, automated protein production, purification and crystallization. It is clear that in order to improve the current situation, better strategies for protein crystallization are required, combined with techniques that allow the use of smaller nano-crystalline material.

2. Crystallization of bio-macromolecules

Biocrystallization involves the three classical steps of nucleation, growth, and cessation of growth, even though the protein crystals contain on average 50% of disordered solvent (Figure 1) (Matthews et al., 1968). However, crystal growth of biological molecules differs substantially from small molecule crystallogenesis. The reason is the much larger number of parameters involved in biocrystallization, as well as the specific physico-chemical properties of the biological compounds. The main difference from small molecule crystal growth is the conformational flexibility and chemical versatility of macromolecules and their greater sensitivity to external factors. An overview of different parameters affecting the crystallization of biomacromolecules is presented in table 1 (Bergfors T, 2009).
Fig. 1. Crystal packing in lysozyme crystals (pdb:1Lyz) shows large cavities. These cavities are filled with disordered solvent (not shown).

<table>
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<th>Intrinsic physico-chemical properties</th>
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Table 1. Overview of parameters affecting bio-macromolecular crystallization.
Another important prerequisite for successful crystallization is the quality of the macromolecular samples. Bio-macromolecules are extracted from living cells or synthesized *in vitro* and they are frequently difficult to prepare at a high degree of purity and homogeneity. Besides traces of impurities, the different treatments proteins are subjected to may decrease their stability and activity through different kinds of alterations. As a general rule, purity and homogeneity are regarded as conditions of prime importance. Accordingly, purification, stabilization, storage and handling of macromolecules are other essential steps prior to crystallization.

3. Purity of bio-macromolecular samples

The concept of purity has a special meaning when biological crystallogenesis is concerned. Molecular samples need to be not only chemically pure, but they must also be conformationally uniform (Giege et al., 1986). This concept is based on the fact that the best crystals are grown from solutions containing well-defined entities with identical physico-chemical properties. For X-ray crystallographic studies, the aim is to grow ‘single crystals’ diffracting to high resolution with a low mosaicity and prolonged stability in the X-ray beam. It is therefore understandable that contaminants may compete for sites on the growing crystals and generate lattice errors leading to internal disorder, dislocations, poor diffraction or early cessation of growth (Vekilov et al., 1996). Because of the high molecular weight of molecules in a single crystal (up to millions of daltons), and hence low molarity of their solutions even relatively small amounts of contaminant may induce formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (Skouri et al., 1996; McPherson et al., 1996). Successful crystallization of rare proteins and nucleic acids support the importance of purity and homogeneity (Wierenga et al., 1987; Thegesen et al., 1996; Aoyama et al., 1996; Douna et al.; 1993). Usually most of the contaminants are eliminated during the different purification steps, however traces of polysaccharides, lipids or proteases may still be present and hinder crystallization. Small molecules, like peptides, oligonucleotides, amino acids, as well as uncontrolled ions should also be considered as contaminants. Buffering molecules remaining from a purification step can be responsible for irreproducible crystallization. For instance, phosphate ions are relatively difficult to remove and may crystallize in the presence of divalent cations (Ca$^{2+}$, Mg$^{2+}$). Counterions play a critical role in the packing of biomolecules. Often macromolecules do not crystalize or yield different habits in the presence of various buffers adjusted at the same pH. Bio-molecular samples containing traces of contaminants can further be subjected to purification through recrystallization, column chromatography, ultra-centrifugation, fractionated precipitation, affinity purification and other techniques. Microheterogeneity in pure macromolecules can only be revealed by very sensitive methods. The most common causes for heterogeneity are uncontrolled fragmentation and post-synthetic modification.

Proteolysis normally takes place in many physiological processes and represents a major difficulty that needs to be overcome during protein extraction from the living cells that produce the desired protein (Achstetter et al., 1985; Barrett et al., 1986; Dalling et al., 1986; Bond et al., 1987; Arfin et al., 1988; Wandersman et al., 1989). The reason is that proteases are localized in various cellular compartments or excreted in the extracellular medium. Upon cell disruption, cellular compartments are mixed with extracellular proteases and control
over proteolysis is lost. Decrease of protein size and stability, modification of their charge or hydrophobicity, partial or total loss of activity are usually signs of proteolysis. Traces of protease may not be detectable even when overloading electrophoresis gels, but they can cause damage during concentration or storage of samples.

Co- or post-translational enzymatic modifications generate microheterogeneity in proteins when different groups, for instance oligosaccharide chains, occupy specific modification sites on the protein, or when correct modifications are unevenly distributed. Only certain modifications are reversible, for instance phosphorylation, but others like glycosylation or methylation are not. Microheterogeneity can also appear during storage, for instance by deamidation of asparagines or glutamine residues is a well-documented phenomenon.

Pure, chemically uniform macromolecules can be fully functional in a biochemical activity assay even though they are microheterogeneous. Conformational heterogeneity may have several origins: binding of ligands, intrinsic flexibility of molecular backbones, oxidation of cysteine residues or partial denaturation. In the first case, macromolecules should be prepared in both forms, the one deprived of and the other saturated with ligands. In the second case, controlled fragmentation may be helpful. In the last one, oxidation of a single cysteine residue leads to complex mixtures of molecule species for which the chances of growing good crystals are low (Van der Laan et al., 1989).

Although macromolecules may crystallize readily in an impure state (Holley et al., 1961), this is an exception and it is always preferable to achieve a high level of purity before starting crystallization experiments. In order to gain more information about the quality of the protein samples, different techniques can be used. For instance, spectrophotometry and fluorometry give information about the quality of samples if macromolecules or their contaminants have special absorbance or emission properties. SDS-PAGE indicates the size of protein contaminants, but not that of non-protein contaminants. Isoelectric focusing gives an estimate of the pI of protein components in a mixture and electrophoretic titration shows the mobility of individual proteins as a function of pH. The latter method can also suggest the type of chromatography suitable for further purification. Capillary electrophoresis is well adapted for purity analysis (Karger et al., 1996). Amino acid composition and sequencing of N- and C- termini verify in part the integrity of primary structure. Electrospay ionization and matrix-assisted laser desorption/ionisation mass spectrometry are also powerful tools in the analysis of recombinant protein chemistry. Nuclear magnetic resonance can detect small size contaminants and gives structural information on biomolecules (Wuthrich, 1995).

It is widely believed that the success of crystal trials is largely dependent on various, not very well identified, properties of the protein. For example, a positive correlation has been established between the degree of protein monodispersity in solution and the ability of the protein to crystallize. On the other hand, it’s thought to be a negative correlation between the degree of disorder in the protein and its ability to crystallize (Mikol et al., 1989).

A number of biophysical techniques and methods are employed to evaluate the quality and stability of protein solutions. Dynamic light scattering is a useful tool for non-invasive in situ monitoring of crystallization trials because it detects the formation of aggregates or nuclei before they become visible under a light microscope (Berne et al., 1976). Fluorescence and light scattering are helpful to rapidly identify stabilizing conditions compromising simple agents (salts, co-factors etc.). Emission fluorescence is used to measure changes if the protein unfolds or undergoes other conformational changes (Konev et al. 1967).
4. Solubility, supersaturation and phase transition

Biological macromolecules follow the same thermodynamic rules as inorganic or organic small molecules concerning supersaturation, nucleation and crystal growth. However, protein macromolecules are organized in tertiary and quaternary structures. The intramolecular interactions responsible for their tertiary structure, the intermolecular interactions involved in the crystal contacts, and the interactions necessary to solubilise them in a solvent are similar.

To crystallize a biological macromolecule, its solution must have reached supersaturation, which is the driving force for crystal growth. The under- and supersaturated states are defined by the solubility of the macromolecules. When the concentrations of the crystallization agent and the macromolecules correspond to the solubility condition, the saturated macromolecule solution is in equilibrium with the crystallized macromolecules. Below the solubility curve (fig. 2) the solution is under saturated and the system is thermodynamically stable. In this case, phase transition (crystallization) will not occur. Above the solubility curve, the concentration of the biological macromolecules is higher than the concentration at equilibrium. A supersaturated macromolecular solution contains an excess of macromolecules that will appear as a solid phase until the macromolecular concentration reaches the solubility value in the solution. The higher the supersaturation, the faster this solid phase appears. However, at very high supersaturation precipitation, not crystallization occurs, but insoluble macromolecules rapidly separate from the solution in an amorphous state.

![Solubility curve of a protein](image)

Fig. 2. Solubility curve of a protein, where the phase state of the protein is plotted against the concentration of both protein and precipitant. At the point (1), the protein may precipitate so fast that an amorphous precipitate or at best shower of microcrystals is formed. At (2) the conditions are just right for the protein to form a stable crystal nucleus, which will start to grow – passing (3) – into a stable protein at equilibrium with the mother liquor (4). At (5), the concentration of protein and precipitant are too low for crystal nucleation or growth, and the solution will remain clear. Note that the true solubility curve of any protein is highly multidimensional, with every parameter affecting protein solubility (cf. Table 1) representing a different independent axis.
5. Crystallization strategies

5.1 Crystallization screens
Finding crystallization conditions for a new protein target is largely based on a trial and error method. The first step is to set up screening trials, exposing the protein to a variety of agents in order to find useful “leads”, which can be crystals, crystalline precipitates and phase separation that point to conditions that are conductive to crystallization. The most popular screens to perform the initial screening step are called sparse-matrix screens. These screens rely on a compilation of conditions that had previously led to successful crystallization. Systematic screens sample the crystallization parameter space in a balanced, rational way using information on the protein properties. Systematic screens are usually used as second remedy or in order to optimize the crystallization conditions.

5.2 Choosing the crystallization method
There are different methods to crystallize biological macromolecules. However, all of them aim at bringing the solution of macromolecules to a supersaturation state (McPherson, 1985; Giege, 1987). It’s important to keep in mind that not only the various chemical and physical parameters influence protein nucleation and crystallization, but also the method of crystallization. Therefore, it’s wise to try different methods when searching for optimal crystallization conditions. As solubility is dependent on temperature (it could increase or decrease depending on the protein), it’s highly recommended to perform crystallization trials at constant temperature unless temperature variation is part of the experiment. Solubility of most chemicals is given in Merck Index. The chemical nature of the buffer is an important parameter for protein crystal growth. It must be kept in mind that the pH of buffers is often temperature dependent, this is particularly significant for Tris buffers. Buffers, which must be used within one unit from their pKa value, are well described in textbooks (Perin et al., 1974).

Protein samples often contain large amount of salts of unknown composition when first obtained. Thus it’s wise to dialyse a new batch of a macromolecule against a large volume of well-characterised buffer of given pH, to remove unwanted salts and to adjust the pH. Starting from known conditions helps to increase the reproducibility. Whatever the crystallization method used, it requires high concentration of biological macromolecules as compared to normal biochemistry conditions. Before starting a crystallization experiment, a concentration step is generally needed. It’s also important to
keep pH and ionic strength at desired value, since pH may vary when the concentration of macromolecules increases. Also, low ionic strength could lead to early precipitation. Many commercial devices are available based on Different concentration principles such as concentration under pressure, using centrifugation, or lyophilisation. The choice of method for concentration depends on the quantity and the stability of the macromolecules.

Before a crystallization experiment, solid particles such as dust, denatured proteins, and solids coming from purification columns or lyophilisation should be removed. This could be achieved by centrifugation or filtration, depending on the available quantity. The most common method to measure macromolecular concentration is to sample an aliquot, dilute it with buffer, and measure absorbance at 280 nm for proteins within the linear range of a spectrophotometer. Proper subtraction with the reference cell should be made especially when working with additives absorbing in the 260-300 nm wavelength range. When working with enzymes, an alternative method to measure the concentration of protein is to perform activity test, otherwise colorimetric methods can be performed.

5.2.1 Vapour diffusion

The most widely used method of crystallization is vapour diffusion. The protein solution is a hanging, sitting or sandwich drop that equilibrates against a reservoir containing crystallizing agents at either higher or lower concentration than in the drop. Equilibration proceeds by diffusion of the volatile species (water or organic solvent) until vapour pressure in the droplet equals the one of the reservoir. If equilibration occurs by water exchange from the drop to the reservoir, it leads to a droplet volume decrease. Consequently, the concentration of all constituents in the drop will increase. For species with a vapour pressure higher than water, the exchange occurs from the reservoir to the drop. In such a ‘reverse’ system, the drop volume will increase and the concentration of the drop constituents will decrease. The same principle applies for hanging drops, sitting drops and sandwich drops. Most people use a ratio of 1:1 between the concentration of the crystallizing agent in the reservoir and in the droplet. This is achieved by mixing a droplet of protein at twice the desired final concentration. When no crystal or precipitate is observed, either supersaturation is not reached or one has reached the metastable region. In the latter case changing the temperature by a few degrees is generally sufficient to initiate nucleation. Although unique in this respect, vapour diffusion permit easy variation of physical parameters during crystallization, and many successes were obtained by modifying supersaturation by temperature or pH changes. With ammonium sulphate as the crystallizing agent, it has been shown that the pH in the droplets is imposed by that of the reservoir. Consequently, varying the pH of the reservoir permits gentle adjustments of that in the droplets. From another point of view sitting drops are well suited for attempting epitaxial growth of macromolecule crystals on appropriate mineral matrices. In other words vapour diffusion provides a way to sample the crystallization space with the conditions continuously varying, as the equilibration proceeds. The kinetics of water evaporation determines the kinetics of supersaturating and accordingly affects nucleation rates. Evaporation rates from hanging drops have been determined experimentally in the presence of ammonium sulphate, PEG, MPD and NaCl as crystallizing agents. The main parameters that determine the rate of water equilibration are temperature, initial drop volume, water pressure of the reservoir, and the chemical nature of the crystallization agent. Theoretical modelling has shown in addition the
pivotal role of the drop to reservoir distance. It was shown that the effect of this parameter is negligible in classical set-ups and becomes only noticeable when drop to reservoir distance is more than 2 cm. From practical point of view, the time for water evaporation to reach 90% completion can vary from about 25 hours to more than 25 days. The fastest equilibration occurs in the presence of ammonium sulphate and the slowest in the presence of PEG. Equilibration rates are significantly slowed down by increasingly appropriately the distance between the drop and the reservoir. An alternative solution to decrease equilibration rates is to apply a layer of oil over the reservoir.

Fig. 4. Schematic drawing of sitting drop (left), hanging drop (middle), and batch crystallization (right). Well solution is blue, protein mixed with well solution is brown and oil is green.

5.2.2 Batch crystallization methods
Another routinely used method for crystallization is the batch method. The biological macromolecule to be crystallized is mixed with the crystallizing agent at a concentration such that supersaturation is instantaneously reached. Crystallization trials are dispensed and incubated under low-density paraffin oil. The crystallization drops remain under oil, where they are protected from evaporation, contamination and shock. Since supersaturation is reached at the start of the experiment, nucleation tends to be higher, if compared to the vapour diffusion method. However, in some cases fairly large crystals can be obtained when working close to the metastable region. Although the microbatch method has not been compared in a statistically significant scale against hanging drop-vapour diffusion method, a comparison on a small scale has been performed (Baldok et al., 1999). The study demonstrated that the methods are not entirely identical, but are equally effective. The results suggest that vapour diffusion method and the microbatch technique will probably produce similar numbers of crystals, but may not produce crystals for the same conditions. Microbatch and vapour diffusion methods are both suitable for high throughput crystallization experiments where all the steps of dispensing, mixing and sealing are automated and performed by a robot. Other crystallization methods worth mentioning,
although with more limited success and use are crystallization in gel, dialysis, microfluidics, free interface diffusion. Microfluidic chips are also being used for high throughput crystallization screening.

5.2.3 Crystallization in gels
Special attention has been paid to crystallization in gels (Robert et al., 1987). The protein crystallization process consists of two main steps – the transport of growth units towards the surface of the crystals and second, the incorporation of the growth units into a crystal surface position of high bond strength. The whole growth process is dominated by the slower of these two steps and is either transport controlled or surface controlled. The ratio between transport to surface kinetics, which can be tuned by either enhancing or reducing transport processes in solution, was shown to control the amplitude of growth rate fluctuations. These are the reasons why gels if properly designed are expected to enhance the quality of crystals. It’s worth mentioning that crystals growing in gel do not sediment as they do in free solution. They develop at the nucleation site, sustained by the gel network. For small molecule crystals grown in silica gel, the gel often forms cusp-like cavities around the crystal and a thin liquid film that reduces contamination risk, separates the crystal from the gel. Such cavities have not been seen in macromolecular crystals. Recent studies have shown that silica gel can be incorporated in the crystal network almost without disturbing the crystal lattice. Such crystals that still diffract to a high resolution, are mechanically reinforced and are more resistant to dehydration, because the silica gel framework embedded in the crystal slows down water loss due to its hygroscopic properties. Although seeding can be used, it appears that most of the gel-grown crystals are obtained by spontaneous nucleation inside a macroscopically homogeneous gel. When the gel adheres to the walls of the container, no nucleation occurs on the cell walls, neither on dust. So, heterogeneous nucleation is strongly reduced, if not suppressed. Another type of nucleation, namely secondary nucleation, is due to attrition of a previous crystal by the solution flux. When nucleation occurs inside the gel, one observes that all the crystals appear at the same time and have about the same size. They are homogenously distributed in the whole volume.

5.2.4 Dialysis methods
Crystallization by dialysis methods allow for an easy variation of the different parameters that influence the crystallization of biological macromolecules. Different types of dialysis cells are used, but follow the same principle. The macromolecule is separated from a large volume of solvent by a semi-permeable membrane that gives small molecules free passage, but prevents macromolecules from circulating. The kinetics of equilibration will depend on the membrane cut-off, the ratio of the concentration, the temperature and the geometry of the cell.

The method of crystallization by interface diffusion was developed (Salemme, 1972) and used to crystallize several proteins. In the liquid/liquid diffusion method, equilibration occurs by diffusion of the crystallization agent into the biological macromolecule volume. To avoid rapid mixing, the less dense solution is poured gently on the most dense (salt in general) solution. Sometimes, the crystallizing agent is frozen and the protein layered above to avoid rapid mixing.
5.3 The role of heterogeneous substrates in the process of protein nucleation and crystallization

In general, additives play an important role in protein crystallization. Heterogeneous substrates are usually regarded as additives when they are purposefully added to the solution in order to obtain a desired effect (inhibition of nucleation, habit change of crystals). However, impurities of foreign substances may also exist in the solution originating from other sources (the solvent, crystallization agent, etc.). Heterogeneous crystallization which is induced by a properly chosen additive may allow better control of nucleation and growth. The first report of a nucleant inducing nucleation of macromolecules was the epitaxial growth of protein crystals on minerals (McPherson et al., 1988). Other candidate nucleants followed like zeolites, silicates, charged surfaces, porous materials etc. and have been tested for multiple proteins (Sugahara et al., 2008, Takehara et al., 2008). Previous results showed that horsehair and dried seaweed showed increased hits when added to sparse-matrix crystallization trials. The increase in crystallization was 35% when horsehair was added to 10 test proteins (Thakur et al., 2008). The underlying mechanism is explained with epitaxial nucleation in the case of minerals, electrostatic interactions if the nucleants contain charged surfaces, nucleation through specific favourable protein-protein interactions or physical entrapment in the caves of porous materials.

Seeding techniques can be advantageous in both screening of crystallization conditions to obtain crystals as well in the later optimisation steps. The streak seeding technique may provide a fast and effective way to facilitate the optimization of growth conditions without the uncertainty that is intrinsic in the process of spontaneous nucleation (Bergfors, 2003). A probe for analytical seeding is easily made with an animal whisker mounted with wax to the end of a pipette tip. The end of the fibre is then used to touch an existing crystal and dislodge seeds from it. Gentle friction against the crystal is normally sufficient. The probe is then used to introduce seeds into pre-equilibrated drop by rapidly running the fibre in a straight line across the middle of the drop containing protein and precipitant. Sitting drop set-ups are preferable since hanging drops tend to evaporate more quickly.

![Fig. 5. Lysozyme needle crystals growing on sliced human hair as a nucleant, the black bar in the left picture represents 200 micrometer.](image)

6. Combining heterogeneous crystallization and high throughput methods

A method for the introduction of heterogeneous nucleants in high throughput crystallization experiments has recently been developed (Nederlof et al., 2011). The method includes preparing of crystallization plates that are locally coated with fragments of human
hair, allowing automated, high throughput crystallization trials in a fashion entirely compatible with standard vapour diffusion crystallization techniques. The effect of the nucleants was assessed on the crystallization of 11 different proteins in more than 4000 trials. Additional crystallization conditions were found for 10 out of 11 proteins when using the standard JCSG+ screen. In total, 34 additional conditions could be identified. The increase in crystallization conditions ranged between 33.3% (two additional conditions were identified for myoglobin on top of four homogeneous crystallizations) to 1.2% (we identified a single additional condition for insulin, which crystallized in 85 out of 96 conditions); the median increase in crystallization hits was 14%. The method is straightforward, inexpensive and uses materials available in every crystallization lab.

7. Lab automation

In recent years, setting up protein crystallisation trials and analysis of the results has become largely automated. More and more of the crystallisation methods mentioned in section 5 have been made amenable to automation, with the sitting drop method still the most popular experiment type in this respect. Lab automation includes the use of dispensing robots, imaging robots, in situ crystal analysis as well as automated diffraction analysis (Stevens et al., 2000, Berry et al., 2006).

7.1 Automation in dispensing

Dispensing robots that are used routinely are either specialized for dispensing well recipes (e.g. Formulator, MatrixMaker) or drop-setting (NT8, Phoenix, Mosquito), but there are also more generic robots that can do both (Hamilton Star, Tecan Evo). In general, the experimenter will start crystallisation trials with a set of pre-defined conditions, contained in one or more screens. Over 150 of these screens can be bought from commercial vendors in a wide range of formats. A number of these are designed on the basis of statistical analysis of results obtained at structural genomics initiatives. When initial hits are found with screens like these, secondary optimisation experiments need to be performed to produce diffracting crystals. In this stage, interaction with a Lab Information Management System (LIMS), where experiment design can be coupled to experiment preparation and analysis, greatly enhances the potential throughput in a lab and thereby the success rate. There are a number of these software packages that can be used to create grid experiments around an initial hit condition, as well as randomized sparse-matrix screens based on initial successes.

7.2 Automated experiment imaging

Automated experiment analysis is an essential part of the lab setup. Due to the increase in throughput obtained by using dispensing robots it is impossible to routinely scan the results manually under a microscope. The dynamic nature of these experiments can cause the crystallographer to miss events, even crystals. Imaging robots vary from semi-automated microscopes with a moving plate stage and camera to fully automated incubators that are capable of following all lab experiments from start to finish without human intervention. Ideally, images are displayed to the user in the context of the experiment design, so that the results are easily interpreted. If this functionality is integrated with the experiment design and dispensing the optimisation circle is complete. Such LIMS systems (Bard et al., 2004) can be further expanded to follow up on harvested crystals, to assess their diffraction quality and finally the structural data derived. (see 7.4)
7.3 In situ crystal analysis

When crystals are found an assessment needs to be made whether the crystals are indeed protein crystals or just salt crystals. And the quality of the crystal needs to be established as well as their usefulness for collecting diffraction data. It has always been difficult to distinguish protein crystals from salt crystals without actually collecting diffraction data. Historically, destructive methods have been used like the “crunch” method and protein dyes, the idea being that crystals similar to the ones destroyed will have the same properties. These methods have not always been conclusive and often the true nature of the crystal was only revealed on the X-ray beam. In recent years, three new techniques have been developed in this field; in situ diffraction analysis, UV detection and second harmonic microscopy.

7.3.1 In situ diffraction analysis

A number of years ago, Oxford Diffraction has come up with a device for X-ray diffraction analysis of crystals in the plate where they were grown (Skarzynski 2009, le Maire, et al., 2011). The idea is fairly simple, you center a crystal in the X-ray beam using a visual alignment tool and you subsequently take a single or a small number of X-ray diffraction images to assess whether a crystal is indeed protein, and to get some idea about the diffraction quality (mosaicity, resolution). An advantage is that the method is non-invasive (bearing in mind potential radiation damage) and fast. The method is not suitable for complete diffraction analysis, as the sample can only be rotated by 6°. It is also possible to automatically screen a complete crystallisation plate for potential diffraction. When suitably diffracting crystals are found they will still needed to be harvested and frozen for complete diffraction analysis.

7.3.2 UV detection of protein crystals

An increasing number of imaging devices (see 7.2) make use of a secondary light path in the UV range to detect protein crystals. These imagers make use of the fluorescence in UV by proteins, mostly caused by tryptophan (Judge et al., 2005). Since the protein concentration in the crystal will be much larger than in solution, any protein crystal will light up under UV, provided that the protein contains tryptophan. This is a relatively fast and non-invasive method, UV illumination can cause some ionisation in the drop, but this effect is much less than with X-ray illumination. In order to maximize its use, the experiment media (plates, seals) have to be chosen with care, some plastics are not sufficiently translucent in UV, or fluoresce themselves, adding noise to the image. One also has to bear in mind that some non-protein crystals (ATP, other co-factors added), might also fluoresce in UV. Having visible light and UV cameras integrated in a single imaging device greatly enhances its usefulness to distinguish protein from salt crystals.

7.3.3 Second harmonic microscopy

Fairly recently, a new development in the field of in situ crystal analysis has been reported. The technology makes use of a phenomenon called second harmonic generation (SHG), more often referred to as “frequency doubling” (Wampler et al., 2008). When an intense laser pulse travels through a highly polarizing, non-centrosymmetric material, light emerges with exactly half of the wavelength of the incident beam. The explanation is that two photons of the incident beam merge, creating a single photon with twice the energy. If the incident beam is in the near-infrared, the emerging beam, will be in the visible range. As mentioned, the technology requires intense laser light, delivered in femtosecond pulses. Most chiral crystal classes, with the exception of octahedral and icosahedral crystals, allow
for SHG, thus encompassing over 99% of all protein crystals grown so far. A first commercial device using this technology, called SONICC, is available since 2011. When combined with a LIMS and a visible light imaging station, SHG can be used to automatically score and pre-sort the results for the experimenter.

7.4 Automated diffraction analysis
In parallel with automation taking hold of many crystallisation labs, the last part of the protein structure analysis pipeline, diffraction analysis in the X-ray beam, is increasingly automated as well. Not so long ago, a crystallographer would either measure his/her crystals at a home X-ray source or would travel to a synchrotron facility to do so. The process involved manually harvesting of the crystals, preparing them for the X-ray beam (mounting in a capillary or in a cryoloop, freezing), mounting them manually in the X-ray beam, gathering a few trial images to determine optimal settings for exposure, distance etc. and finally recording a set of diffraction images to solve the structure. The most time consuming steps have now been automated (Cork et al., 2006, Song et al. 2007). Most notably, crystal mounting robots will now automatically take samples out of a liquid nitrogen dewar and place them in the X-ray beam, eliminating the need for the user to enter the X-ray hub of the synchrotron after every crystal. At the home lab, the mounted crystals are packed in specific dewar compatible with the robot arms at the beamline, and they are mailed to the synchrotron. In many synchrotrons, the user now has a choice of having a local operator collecting the data, or to drive the computers at the facility remotely from their own lab, there is no need to travel to a remote synchrotron anymore. In the near future, the automation can be improved by automatic crystal centering routines (Vernede et al., 2006). With e.g. the use of SHG (see 7.3) crystals can automatically be located inside the cryo loops, and this information can be used to automatically center the crystal in the beam.

8. Conclusions
The chapter covers some of the main aspects of protein nucleation and crystallization. Different diagnostic tools, crystallization techniques and strategies are explained. New tendencies in the field such as combining heterogeneous nucleants and high throughput methods are also presented.

9. References
Matthews, B.W. (1968), Journal of Molecular Biology, Volume 33, Issue 2, pp. 491-497
In modern research and development, materials manufacturing crystal growth is known as a way to solve a wide range of technological tasks in the fabrication of materials with preset properties. This book allows a reader to gain insight into selected aspects of the field, including growth of bulk inorganic crystals, preparation of thin films, low-dimensional structures, crystallization of proteins, and other organic compounds.

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