Bench philosophy (27): Protein crystallization guide

The Art of Protein Crystallization

There is a saying around structural biologists, that protein crystallization is half art and half science. Like science, however, every art is not only based on talent but also on hard work and practice.

In this article we would like to bring the readers' attention to the techniques involved in X-ray crystallography. Before starting the crystallization trials, it is essential to properly prepare the macromolecule – saving time and ultimately, headaches, as proteins can be unfolded, impure, insoluble or unstable. It is also important to ensure that the proteins are active to perform their biological function, instilling the crystallographer with greater confidence that the crystal structure will actually provide relevant biological significance.

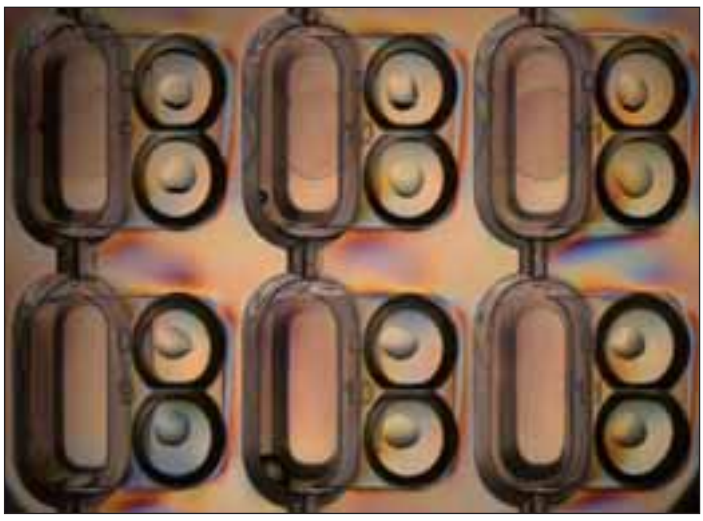
Although the crucial step in protein X-ray crystallography is to obtain well-diffracting crystals, a pre-crystallization characterisation of your macromolecule would dramatically increase the chance of success with the structural biology project. Purity is, of course, the most essential requirement for continuous and homogeneous lattice growth.

Modern recombinant techniques enable the production of massive quantities of protein with useful purification-tags. A simple metal-based elution step can result in protein of more than 95% purity. Cases have been recorded, where using only metal affinity chromatography is sufficient to generate the purity required for growth of well-diffracting protein crystals. However, it is advisable to perform a second purification step, usually by size exclusion chromatography (gel filtration), to obtain a homogeneous pure protein solution.

Protein purity can be simply assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie-blue staining, where the appearance of a single band is indicative of a pure protein. After concentration, normally by dialysis or ultra-filtration, the purity of the concentrated protein solution should be rechecked by SDS-PAGE, as at this stage it is easier to detect minute amounts of impurities. In addition, the sensitivity in detecting minor bands can be improved using silver staining instead of Coomassie. Apart from SDS-PAGE, the homogeneity of the protein solution can also be assessed by using dynamic light scattering (DLS), a method that measures the polydispersity of the protein sample. Usually, a low polydispersity sample has higher conformational purity and, therefore, possesses higher chances of generating crystals.

Check for function

Following the purity and homogeneity experiments, the next step is to check that the protein is properly folded on its active conformation. If a functional assay can be developed (normally for enzymes), it is always good (although not essential) to obtain circular dichroism (CD) spectra for determining the predominant secondary elements. However, when there is no functional assay (structural, membrane proteins or proteins with unknown function) the CD spectra gain extra importance for verifying that the protein is folded, as it can be very difficult to crystallise unfolded proteins.



Crystallization drops, pipetted into a microplate by a robotic system, are checked under the microscope for uniformity.

In general, the more knowledge we gain about our protein, the better the chances to obtain crystals. Important clues become clear if we know whether a protein interacts with any co-factors, substrates or inhibitors, so that they can be included in the crystallization. The use of protein ligands usually increases the probability of getting well-formed crystals, resulting in additional valuable information obtained when the final structure is solved with bound ligands. Methods, such as the thermofluor assay (Ericsson et al., *Analytical Biochemistry*.357, 289-98) can detect, in a high-throughput format, ligands, buffers and additional molecules that increase the stability of the protein under investigation.

Having successfully tested the protein with biophysical methods, we are now keen to go through the crystallization attempt, usually with a concentrated protein solution (10mg/mL or more). Buffers, salts and other solution components are ideal in solubility, stability and activity for each protein studied. Phosphate buffers are generally avoided as they typically form salt crystals (i.e. magnesium or calcium phosphate) in many usual crystallization protocols.

When the protein has been properly prepared, we are finally in a position to start. These days, it is a relatively simple procedure to screen many conditions with the availability of high-throughput robotic systems. You are first expected to identify any initial condition where the protein crystallizes. If you are lucky, the crystals will grow and diffraction patterns will be collected. A more common scenario is for the first crystals not to produce a high resolution diffraction pattern (for X-ray crystallography we are aiming for a resolution beyond 3Å), they are not three dimensional, anisotropic or simply do not diffract!

In addition, there are difficult proteins that may resist our attempts to crystallise them; however, what appear to be nice looking crystals can turn out to be salt crystals. That is why some people say "Crystallization is half science and half art"; perhaps

implying that crystallization has something of a magical and unexpectedly bizarre air about it, due to difficult and sometimes non-reproducible experiments.

Crystallization screens

A good way to start is to use sparse matrix screens, which are in fact a collection of diverse conditions of buffers, precipitants and other additives. The Joint Consortium of Structural Biology has prepared a popular screen named JCSG (Newman et al, 2005, *Acta Crystallogr*. D61: 1426-31), designed to cover the most common conditions where most proteins crystallise. Several companies produce crystallization set-ups for initial screening, such as those from Molecular Dimension (structure screen I and II), Emerald Biosciences (wizard screen I and II) and Hampton Research (index, crystal screen and crystal screen 2).

Most of these screens are available in a 96-well format to facilitate the use of a robotic system. The JCSG screen is often combined with the systematic screen PACT where conditions, such as pH, anions and cations are tested in an organised manner. The combination of a sparse matrix (JSCG) and systematic (PACT) screens on the initial experiments provides significant information regarding the behaviour of the protein to be crystallized, even if crystals are not formed.

There are several approaches for protein crystallization. The most popular are the vapour diffusion methods, either on a sitting or hanging drop, depending on the way the drop is placed (whether it 'sits' or 'hangs') over the well containing the crystallization solution (known as mother liquor). The most critical parameters that may be varied are the protein and the precipitant concentrations. In most crystallography books you can find a diagram with a plot of the protein concentration versus the precipitant concentration showing zones where nucleation and crystal growth occur (Rupp, *Biomolecular crystallography*, 2010, Garland Science, p. 103). Normally, in the vapour diffusion method, we aim to get the soluble protein solution to a state, where nucleation begins by diffusion of vapour out of the protein drop, raising the protein and precipitant concentrations to the critical value.

Crystals growing in a drop.

The initial crystal trials are performed by mixing the pure protein (at least 10 mg/mL) with the PACT/JCSG+ screens conditions in a 96-plate format using a robotic system. The use of additional screens at this point is optional and depends on their availability, budget and amount of protein necessary. We suggest that a pH systematic screen and a precipitant screen could be used for identifying the best pH and precipitant combination. As we are not sure that the starting concentration of the protein is appropriate, it is useful to apply two different ratios of protein: crystallization solution (1:1 and 1:2). The volumes are in nanolitres (~100 nL of protein per well) giving us the opportunity to perform several experiments with a small amount of protein.

Following the crystal set-up, we seal the wells and observe the drops under a stereo microscope to check whether they are uniform (indication that the mosquito has done the pipetting properly) and for any effect of rapid precipitation. We then incubate the plates at a specific temperature (16 °C).

No general rules

As with several aspects in the crystallization procedure, there is no general rule about the optimal temperature. A popular temperature is 16 °C; however, people also choose 19 °C or even room temperature. Lower temperatures such as 10 °C and 4 °C have also been successfully applied. Sometimes the choice of temperature depends on the equipment in specific labs (i.e. cold room, dedicated incubators for crystallization plates). If you observe an array of microcrystals or what is known as a "crystal shower", then it is advisable to lower the nucleation point by reducing either the protein concentration or the precipitant concentration. After modifying a single parameter, it is essential to carefully observe the plates to compare the outcomes and record any interesting phenomena happening on the drops. This process can be time-consuming but it is essential for further optimisation. Good laboratory notes need to be kept at this point!

Different results

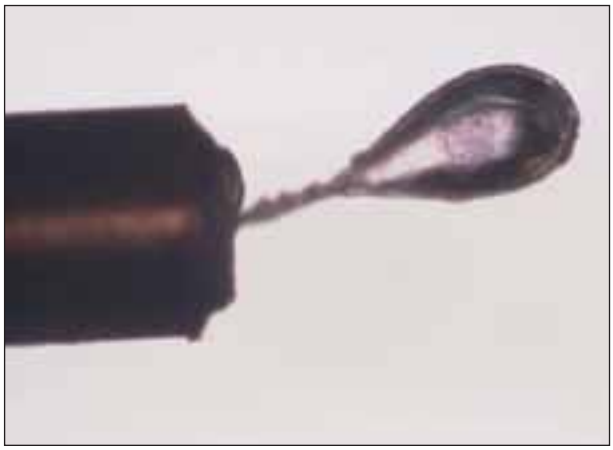
Possible results might be: 1) the drop is clear, 2) the drop has amorphous/gelatinous precipitate, 3) the drop has heavy precipitate, 4) the drop has oily structures and – the preferred result – 5) the drop contains crystal-like material or well-formed crystals! If the drop is clear, it means that the protein is readily soluble under this condition. This is an important observation as it can tell, which condition enhances protein solubility. Another interpretation could be that there is less protein concentration than required (especially if the drop with the concentrated protein: crystallization solution (1:1) remains clear) so the next trial should start with a more concentrated stock of the protein.

It is usually good when the drop contains an amorphous or gelatinous precipitate as those conditions can lead to crystal formation by slightly altering the parameters. On the other hand, if precipitation is excessive, the protein rapidly leaves the soluble phase and, therefore, those conditions do not favour crystallization. If many drops have heavy precipitate, the protein concentration is probably higher than required. Oily structures in the drop can indicate a phase separation (the protein molecules tend to dissociate from the mother solution but the interaction energy is not sufficient for nucleation). There are several factors that affect nucleation, such as concentration of protein/precipitant, temperature, thermodynamic parameters and the number of nucleation points.

Two-dimensional crystals are not suitable for diffractions.

Ideal crystals should be three dimensional with sharp edges. The first crystals don't often look like emeralds and are rath-

er two or even one-dimensional, like plates or needles. Optimising such crystals can be challenging, requiring a refinement of the existing conditions or the addition of extra components. The seeding method may also be used. A crystal is collected using a loop and then being smashed (seeding solution). We then use an aliquot of this solution in the next crystallization set-up (i.e. 10nL per drop). In addition, if we have clear drops, we can use a loop or even a hair, dipped into the seeding solution and then pass it into the clear drops. The theory behind seeding is to add nucleation points to the wells, so the equilibrium is driven into crystal formation and growth.



Crystal mounted on a cryogenic loop.

You can also optimise the crystals by generating grid screens. These are systematic screens where all parameters are varied (pH, salt, concentration of precipitant) around the initial hit. Emerald Biosystems maintain a useful blog (*http://web.emeraldbiosystems.com*) of crystallographic news, methods, tips and techniques, where a scheme for grid screen optimisation design by Paul Reichert can be found (Rational Protein Crystallization Optimization Schema). Using that method in a 96-plate enables all three conditions (pH, precipitant and salt concentration) to be tested. From our experience with a mycobacterial protein, a 0.25 increase of the pH was sufficient to produce high diffraction crystals, diffracting at 2Å resolution.

One important factor of the optimisation experiments is to use the same batch of purified protein, as it will establish whether the results observed are because of the optimisation and not the protein. A useful tip is to dispense small aliquots (20μL) of the concentrated purified protein into thin-wall PCR tubes and flash freeze them. When ready for a crystal set-up, just remove one or more aliquots and rapidly thaw by touching it between your fingers for 10 to 20 secs and use that sample for the optimisation trials. Once the desirable conditions have been determined, we can scale up the crystallization process (in cases where we have enough protein) by setting it up in a 24-well plate, where a drop of 1μL protein and 1μL mother liquor is used. With this method larger crystals are usually obtained, which are easier to scoop into the mounting loop.

Bring in the harvest

Once the crystal has grown to a considerable size, it is necessary to harvest the crystal from the growth solution, transfer it to a loop or tip and freeze it immediately (flash freezing) in liquid nitrogen (-196 °C) to preserve the crystal before the X-ray diffraction experiment. Scooping the crystal is a crucial step (especially difficult for beginners!) as it can be easily damaged or smashed (against the walls of the wells). The procedure involves carefully picking one of the crystals from the growth solution with a nylon loop by looking through a stereo microscope. It requires patience and refined motor skills, and it has to be done quickly as the crystals are fragile and sensitive to changes in their surroundings.

Once the crystal is in the cryogenic loop, it is transferred to a drop of crystallization solution containing cryoprotectant (glycerol, ethylene glycol, high molecular weight polyethylene glycol) before being scooped up into the loop again and rapidly frozen by plunging the cryogenic pin (loop+microtube+magnetic base, see figure) into liquid nitrogen. The pin is appropriately stored in specific dewars and maintained at cryogenic temperatures until data collection.

There is a catch to all these processes; cryocooling must be done extremely fast in order to prevent the formation of ice crystals. The crystals are embedded in their mother solution (otherwise they will dry out, melt or stop diffracting) and the cryoprotectants help to discourage the formation of water ice in the process of cryocooling. Some specialised techniques have been used in order to crystallize directly in the loop and then dip into liquid nitrogen directly. Others are now developing the technology to harvest crystals using robots in a high-throughput format (Viola et al., *J Struct Funct Genomics*, 8, 145-152).

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