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Protein crystallization for genomics: towards high-throughput optimization techniques

Naomi E. Chayen* and Emmanuel Saridakis

Biological Structure and Function Section, Division of Biomedical Sciences, Faculty of Medicine, Imperial College, London SW7 2AZ, England

Correspondence e-mail: n.chayen@ic.ac.uk

Naomi Chayen is a Principal Research Fellow at Imperial College, London and a visiting Professor at the University of Manchester. She graduated with a BSc in Pharmacy and an MSc in Pharmaceutical Sciences from the Haddassa Medical Centre, Hebrew University of Jerusalem, followed by a PhD in Biochemistry at the Kennedy Institute of Rheumatology, London. In 1984, she joined the Biophysics Section of the Physics Department at Imperial College, working first with Professor John Squire on muscle structure and, from 1986, with Professor David Blow on protein crystals. In 1999, Naomi moved to the Faculty of Medicine at Imperial College, where she heads a crystallization group in the Biological Structure and Function Section. Her scientific interests are focused on the crystallization of proteins and other biological macromolecules; in particular, developing experimental methodology for producing high-quality crystals. The work involves analytical studies into the fundamental principles of the crystallization process and extends to experiments of crystallization in microgravity.

Emmanuel Saridakis was born in Brussels, Belgium of Greek parents and was brought up in Greece. He was awarded his BSc in Physics and PhD in Biophysics at Imperial College, London, where he studied the foundations of macromolecular crystallization under Professor David Blow. He has worked as a Research Associate at the University of Athens, at Imperial College and now at the Institute of Molecular Biology and Biotechnology in Crete, Greece. His principal interests are in the physico-chemical aspects of protein crystallization.

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Protein crystallization has gained a new strategic and commercial relevance in the next phase of the genome projects, in which X-ray crystallography will play a major role. Considerable advances have been made in the automation of protein preparation and also in the X-ray analysis and bioinformatics stages once diffraction-quality crystals are available. These advances have not yet been matched by equally good methods for the crystallization process itself. In the area of crystallization, the main effort and resources are currently being invested into the automation of screening procedures to identify potential crystallization conditions. However, in spite of the ability to generate numerous trials, so far only a small percentage of the proteins produced have led to structure determinations. This is because screening in itself is not usually enough; it has to be complemented by an equally important procedure in crystal production, namely crystal optimization. In the rush towards structural genomics, optimization techniques have been somewhat neglected, mainly because it was hoped that large-scale screening alone would produce the desired results. In addition, optimization has relied on particular individual methods that are often difficult to automate and to adapt to high throughput. This article addresses a major gap in the field of structural genomics by describing practical ways of automating individual optimization methods in order to adapt them to high-throughput techniques.

1. Introduction

Pilot projects in structural genomics show that the success rate of proceeding from cloned protein to structure determination is about 10%. For example, figures taken from the Human Proteome Structural Genomics pilot project (Brookhaven National Laboratory, The Rockefeller University and Albert Einstein College of Medicine; http://proteome.bnl.gov/progress.html, periodically updated on the WWW) show that out of 124 proteins which were cloned, 62 were purified. Of these 62, 33 yielded crystals of some sort, but only 16 of these crystals were of good enough quality to be useful for structure determination. In other words, the largest failure rate is in the step between producing purified protein and obtaining diffraction-quality crystals (Fig. 1). Similar success rates by other pilot projects around the world have been reported at structural genomics conferences and meetings. Clearly, this is highlighting a general problem where even when proteins can be cloned, expressed, solubilized and purified, and even if crystallization trials do yield some crystals, this does not guarantee that the crystals will be good enough for structure determination (Chayen, 2002). For structural genomics to be productive, it is essential that this problem be addressed.

2. Automation of screening procedures

In the last decade, major advances have been made both in the automation of protein expression and purification methods (Stevens, 2000*a*) and in X-ray analysis (Abola *et al.*, 2000) and modelling (Sánchez *et al.*, 2000). In the case of crystallization, now that commercial screening kits and computer algorithms for designing arrays of potential conditions are readily accessible, it is no longer a problem to conduct trials automatically (*e.g.* Chayen *et al.*, 1994; Stevens, 2000*b*). Automatic generation of high-throughput screening crystallization trials is under way (Abola *et al.*, 2000; Stevens, 2000*b*; Segelke & Rupp, 2000; Luft *et al.*, 2001; Mueller *et al.*, 2001; Segelke, 2001) as well as automated follow-up and analysis of the results (Luft *et al.*, 2001; Elkin & Hogle, 2001; Kim & Stewart, 2000; Asanov *et al.*, 2001; Jurisica *et al.*, 2001). Some proteins will surely crystallize during this initial screening, but most trials are likely to yield microcrystals or low-ordered crystals. The conversion of such crystals into useful crystals requires intellectual input and individualized optimization techniques. Such techniques do not lend themselves readily to automation and they have yet to be adapted to cope with the huge volume of experiments required by genome projects. Consequently, apart from the obvious steps of merely changing the concentrations, pH, additives *etc.* around the initial conditions found by screening, the subject of optimization has been neglected (however, see Bray *et al.*, 1998). The obvious need for high throughput at every stage in structural genomics has prompted us to design automated optimization methods which go beyond the usual fine-tuning of conditions.

This article highlights several simple optimization methods, some of which have resulted in significant improvement of crystal quality (*e.g.* Mayans *et al.*, 1998; Saridakis & Chayen, 2000; Nield *et al.*, 2002). These have not yet been adapted as high-throughput techniques, but they have now been automated, thus making them easily adaptable to high-throughput trials.

2.1. Application of the automated microbatch technique for high-throughput screening and optimization

The first semi-high-throughput experiments for both screening and optimization were designed in 1990 as microbatch trials under oil (Chayen *et al.*, 1990). Microbatch trials consisting of 0.5–2 µl drops of a mixture of protein and crystallizing agents are generated by an automated system called IMPAX and are dispensed and incubated under oil in order to prevent evaporation. The IMPAX system has two modes of action: one is used to automatically screen numerous potential crystallization conditions and the other is used for optimization of the most promising screening conditions by changing concentrations and pH in small steps (Chayen *et al.*, 1992, 1994). Many target proteins have been successfully crystallized using the microbatch method (*e.g.* Barrett *et al.*, 1998; Chayen, 1998; Stock *et al.*, 1999; Zhang *et al.*, 2001). The microbatch method has recently been adapted for high-throughput screening experiments, using a large bank of syringes dispensing 0.4 µl volumes into 1536-well microassay plates (Luft *et al.*, 2001), and for dispensing crystallization trials on a nanolitre scale (Abrahams *et al.*, 2002; DeLucas *et al.*, 2002; Juárez-Martínez *et al.*, 2002).

3. Development of optimization procedures

As well as screening, in our laboratory we have now automated a variety of optimization methods, which up until now have only been performed manually. These methods are reported below.

3.1. Crystallization in gels

Growth of crystals in a gel medium can improve the quality of crystals in comparison with solution media because convection and sedimentation are reduced (Robert *et al.*, 1999). The application of gels in the crystallization of macromolecules, particularly proteins, has been pursued for more than ten years, yet the method remains underused. This may be because of the relatively complicated procedures required when applying gels to crystallization trials and also to the large quantities (mostly $\geq$10 µl) of sample needed. The Granada Crystallization Box (Hampton Research) overcomes some of these problems; however, to date, crystallization in gels has not been automated.

Two advances are described here: (i) automation of crystallization in gels and (ii) generation of gelled drops using very small volumes ($\geq$0.3 µl). These have been achieved using the IMPAX robot.

IMPAX works as a five-channel system where precipitant, buffer, protein *etc.* are put into different channels and dispensed through a fine multi-bore tip by the action of motorized syringes. By placing a gel solution in one of the channels, it is possible to automatically dispense microbatch trials which form the gel/crystallization mixtures in final volumes of 0.3–2 µl. This can be achieved with the same ease as conventional automated microbatch trials. The gel solution is loaded into the liquid-handling apparatus in the same way as the other ingredients of the crystallization trial. Then, while still a low-viscosity liquid, it is dispensed under oil simulta-

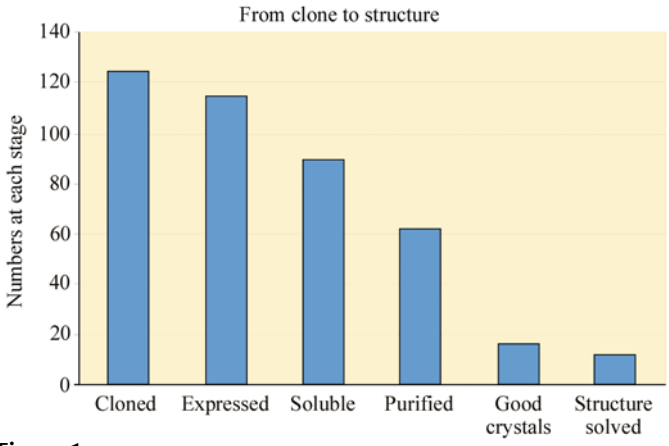

Figure 1
The steps leading to X-ray structure determination and their success rates. Histogram showing the different stages from clone to structure.

neously with all the other ingredients. After a known time, polymerization occurs and the drop gels (Moreno *et al.*, 2002). Agarose gels and two silica gels, one made by neutralization of sodium metasilicate and the other by hydrolysis of tetramethyl orthosilane (TMOS), have been tested with trypsin, thaumatin, concanavalin A, lysozyme and C-phycocyanin (Table 1).

The results show that a higher proportion of the total crystal yield consisted of large usable crystals in the gelled drops with at least one of the gels (Table 1). Trials were dispensed in volumes of 0.3–3 µl. The best results were observed between 0.7 and 3 µl (Fig. 2). In order to test whether the gels would make a difference even under suboptimal (*e.g.* initial screening) conditions, concanavalin A was crystallized in the presence of either agarose or TMOS gels under conditions which normally gave many small crystals. The presence of TMOS gel made a striking difference: fewer, larger crystals were obtained (Table 1; Figs. 2*e* and 2*f*). TMOS at a concentration of 0.2% has consistently given the best results with all five proteins tested.

3.2. 'Containerless' crystallization

Heterogeneous nucleation, which is often detrimental to the production of diffraction-quality crystals, can be induced by the contact of a crystallization trial with the walls of its supporting vessel (Yonath *et al.*, 1982). Crystallization in a 'containerless' set up, in which a crystallization drop is suspended between two oils of different densities, results in reduction of heterogeneous nucleation (Chayen, 1996; Lorber & Giegé, 1996; Chayen, 1997*a*), thus leading to the production of a smaller number of high-quality crystals. This two-oil method was useful for manually setting up a small number of experiments, but it took time to layer the oils, it was difficult to harvest the drops and the method was not amenable to automation.

High throughput can now be achieved by a modification of the original method whereby the lower layer, previously consisting of a heavy fluorosilicone oil, is replaced with a much cheaper hydrophobic surface of high-vacuum silicone grease. The grease covers the bottom of the crystallization plate onto which the trial drops (0.3–2 µl) are automatically dispensed using a robot. MicroWell Modules (Nunc Intermed, Denmark) and Linbro plates have been used, but most other crystallization plates are suitable (Fig. 3). A low-density oil, usually paraffin (0.84 g cm^{-3}) is applied as the top layer. The greased surface has an added advantage over the original two-oil set up, in that the grease provides a stable interface to the upper layer. This prevents crystals from migrating to the walls, making them much easier to harvest. The crystals could be lifted directly out of the drop with a loop or even a spatula. This method was tested for several proteins including a newly crystallized C-phycocyanin. In the 'containerless' setup, prisms of C-phycocyanin measuring 0.1 × 0.1 × 0.1 mm were obtained from crystallization conditions which yielded mostly thin plates in standard microbatch drops. In some cases, the drops in contact with the vessels yielded crystals measuring only 0.06 mm in the largest dimension.

It was also noticed that many drops could be dispensed within a small flat area coated with the grease, doing away

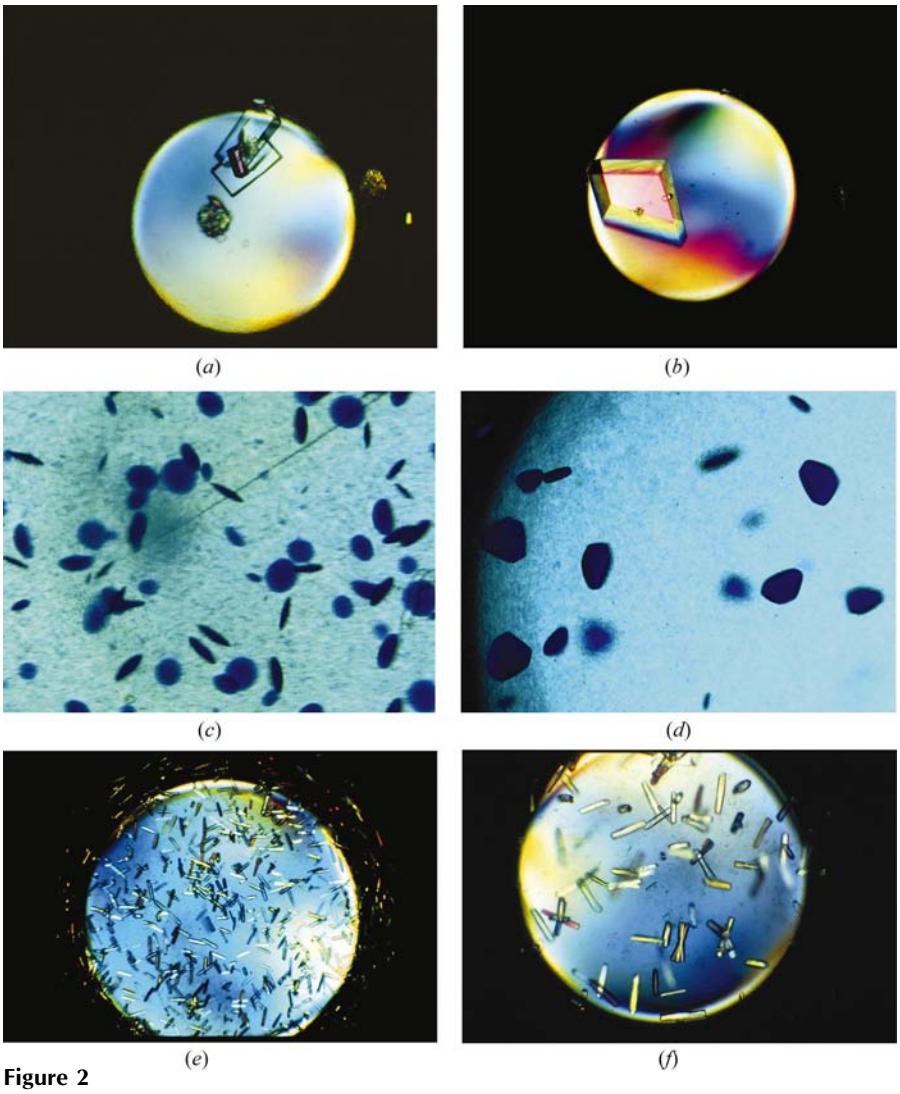

Figure 2
Standard and gelled 2 µl microbatch drops. (*a*) Trypsin crystals in a standard drop. Scale: 1 cm = 0.4 mm. (*b*) Trypsin crystal in a gelled (TMOS) drop under otherwise similar conditions as in (*a*). 1 cm = 0.36 mm. (*c*) C-phycocyanin crystals in a standard drop. 1 cm = 0.32 mm. (*d*) C-phycocyanin crystals in a gelled (TMOS) drop under otherwise similar conditions as (*c*). 1 cm = 0.32 mm. (*e*) Concanavalin A crystals grown in suboptimal conditions in a standard drop. 1 cm = 0.44 mm. (*f*) Concanavalin A crystals grown in TMOS gel under the same conditions as (*e*). 1 cm = 0.44 mm.

Table 1
Crystals in standard and gelled microbatch drops.

Protein	Most effective types of gel	Conditions used for crystallization	Protein concentration (mg ml^{-1})	Typical crystal numbers and sizes (µm) in microbatch	
				Gelled	Standard
C-phycocyanin (126 kDa)	TMOS 0.2%(*v*/*v*)	0.7–1.0 *M* ammonium sulfate, 40 m*M* MES pH 6.1, 1.5 m*M* dodecyl maltoside	10–20	40, 140 × 140 × 110	150, 140 × 100 × 80 (often smudged)
Thaumatin (22 kDa)	TMOS 0.2%(*v*/*v*), metasilicate 0.2%(*v*/*v*)	5–13%(*w*/*v*) Na/K tartrate, 50 m*M* PIPES pH 6.8	20–25	10, 500 × 300 × 250	25, 400 × 200 × 150
Trypsin (24 kDa)	TMOS 0.2%(*v*/*v*), agarose 0.1%(*w*/*v*)	33–36% saturated ammonium sulfate, 100 m*M* Tris pH 8.5	20–35	1, 700 × 200 × 150	5 (frequent clusters), 700 × 200 × 100 (generally flat)
Lysozyme (14.5 kDa)	TMOS 0.2%(*v*/*v*)	4.6–7% saturated sodium chloride, 40 m*M* sodium citrate pH 4.6	20	1, 400 × 300 × 300	5, 400 × 300 × 300
Concanavalin A (102.5 kDa)	TMOS 0.2%(*v*/*v*)	(*a*) 3–10%(*w*/*v*) PEG 6K, 100 m*M* Na cacodylate pH 5.6, (*b*) 52–60% saturated ammonium sulfate, 100 m*M* phosphate buffer pH 7	(*a*) 5–10, (*b*) 10	50, 160 × 40 × 40	150, 80 × 20 × 20

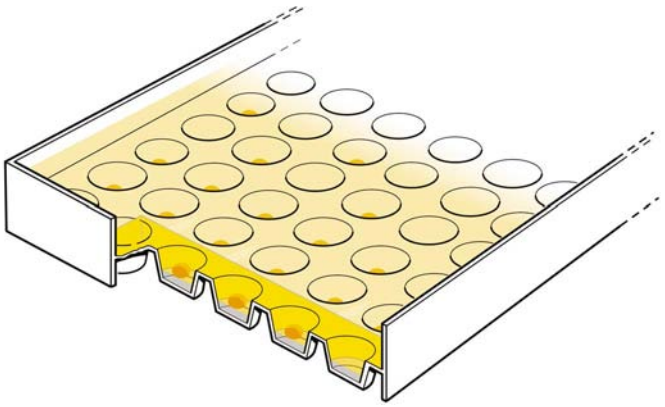

Figure 3
'Containerless' crystallization setup. Schematic diagram of crystallization drops positioned between silicone grease (in grey) and low-density oil (yellow) inside a microbatch plate.

with the need for wells (*e.g.* ten 2 µl drops were dispensed on a 0.95 cm^2 flat area).

3.3. Control of evaporation kinetics

It is well known that nucleation is a prerequisite for and is the first step in crystal growth, yet excess nucleation yields a large number of small crystals instead of a small number of useful ones. A means of controlling nucleation by reaching nucleation slowly and then stopping it before it becomes excessive can now be carried out in the microbatch method. This is achieved by controlled evaporation, and therefore concentration, of the drops through a thin oil layer. Evaporation is later arrested by increasing the thickness of the oil layer.

The paraffin oil generally used in standard microbatch trials is not completely impermeable to the aqueous solution which constitutes the crystallization drops. The conventional microbatch method involves using a layer of oil thick enough (4 mm, corresponding to 8 ml covering all 72 wells of a microbatch plate measuring 8 × 5.5 × 0.9 cm) to render evaporation through it negligible within the timescale of a crystallization experiment (typically one week to one month). However, if controlled evaporation is required, the thickness of the layer can become an active parameter of the process. Instead of setting the microbatch conditions well inside the nucleation zone of the phase diagram, conditions are set to be undersaturated or metastable and water is allowed to evaporate slowly through a thin oil layer (1.2–2 mm for 2 µl drops, Fig. 4*a*). This optimum range for the oil-layer thickness has been determined by extensive trials and seems to be independent of the protein used. It is, of course, a function of the volume of the original drop. The solution therefore arrives at the nucleation zone in a controlled way. The thickness of the oil layer is then increased, rendering evaporation negligible, and the experiment progresses along the conventional batch route (Fig. 4*b*). Assuming that the evaporation has been arrested at the early stages of nucleation, this means that the trial spends most of its lifetime in the metastable zone of conditions, as the protein becomes absorbed into the forming crystals. This procedure can be conducted automatically with an extra arm added to the crystallization robot for dispensing oil after set time intervals.

So far, this procedure has been tried with trypsin, lysozyme and C-phycocyanin. The metastable starting conditions are listed in §5. The critical times after setup at which to arrest evaporation by adding more oil were found to be 18–24 h for trypsin, 2–4 h for lysozyme and 4–6 h for C-phycocyanin (for 2 µl drops). Trials set at the same conditions without allowing any evaporation resulted in clear drops. If trials were allowed to evaporate without arresting, showers and eventual drying-out of the drops occurred. Arresting the evaporation at the above times enhanced the size and yield of useful crystals compared with crystals grown by the standard microbatch method.

3.4. Optimization by decoupling nucleation and growth

Nucleation requires different conditions to those of growth. The most common way of decoupling nucleation and growth is by seeding (Stura, 1999). However, quenching of nucleation using dilution is more amenable to high-throughput processing. Dilution can be achieved in both microbatch (Saridakis *et al.*, 1994) and hanging-drop methods (Saridakis & Chayen, 2000). In the case of hanging drops, the cover slips holding the drops are incubated for some time over reservoir solutions that normally give many small crystals. After a given time, the cover slips are transferred over reservoirs with lower precipitant concentrations that would normally yield clear drops. Various studies have shown that there is a time lag between the solution reaching the appropriate supersaturation and the formation of the first post-critical nuclei (*e.g.* Rosenberger *et al.*, 1993). This induction time for nucleation is, however, fairly short compared with the time at which the crystals start becoming visible in a microscope (Saridakis *et al.*, 1994). In practice, for vapour-diffusion experiments one must allow sufficient evaporation against the high-concentration reservoir to take place so that the drop reaches spontaneous nucleation levels and add to that a little more time to allow for the nucleation time lag. To avoid shock to the drop arising from the transfer, the low-concentration reservoir solutions are dispensed at the beginning of the experiment and sealed with self-adhesive tape. This allows a humid atmosphere to develop above the well to which the cover slip will be transferred. The transfer itself lasts 1–2 s. In the case of the microbatch method the drops are diluted by automated means after incubation. We have so far reported successful use of these techniques only in highly individualized experiments where nucleation and metastable conditions, as well as optimal timing, had been fine-tuned to the system in hand (Saridakis *et al.*, 1994; Saridakis & Chayen, 2000; M. Kokkinidis, personal communication). We have now tested the suitability of this approach for high-throughput trials. Hanging drops were transferred at various times (selected by reference to the time which it took to see the first crystals in the preliminary screens) from a standardized set of screening solutions at high concentrations to screens at lower concentrations (see §5). Trials using model proteins indicate that the average number of trials leading to crystals is higher when using this method compared with using a screen consisting of only either the high- or the low-

Table 2
Number of 'hits' using the 3-D Screen in vapour diffusion.

No. of 'hits' are out of 24 protein-containing drops. A 'hit' signifies crystalline material in the drop.

Protein	Protein-stock concentration (mg ml^{-1})	No. of 'hits': standard screens		No. of 'hits': 3-D screen
		Low conc.	High conc.	
Lysozyme (14.5 kDa)	25	1	1	5
C-phycocyanin (126 kDa)	70	6	5	6
Trypsin (24 kDa)	40	2	1	4

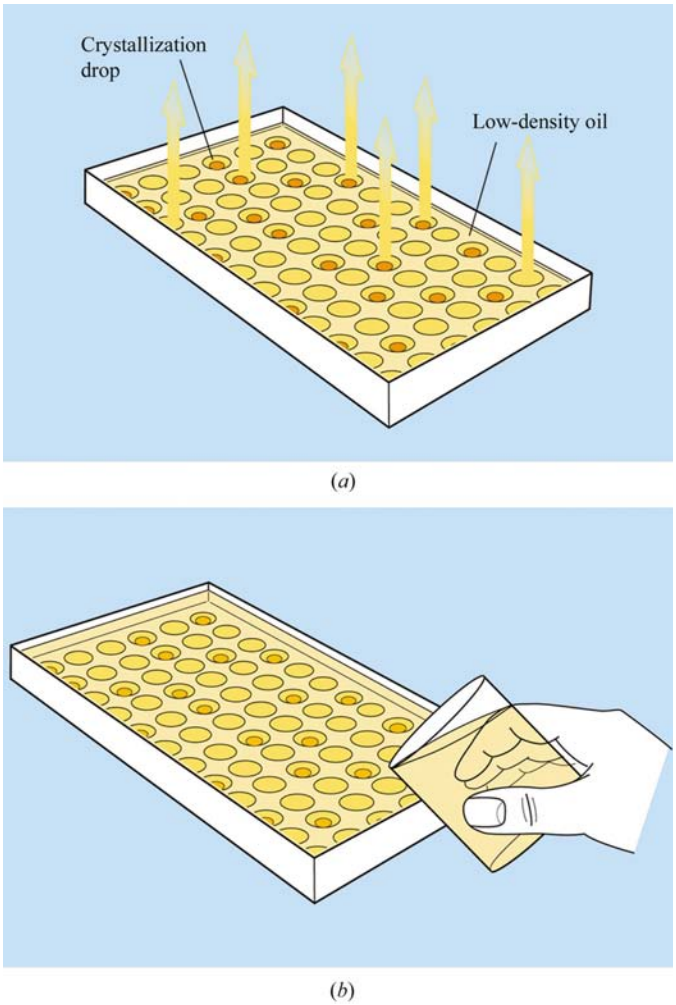

Figure 4
Schematic display of controlled evaporation. (*a*) Microbatch drops under a thin layer of oil which allows their concentration (symbolized by the arrows). (*b*) Arrest of evaporation/concentration by topping up the oil to produce a thicker layer above the drops. From then onwards, the experiment follows its 'conventional' route.

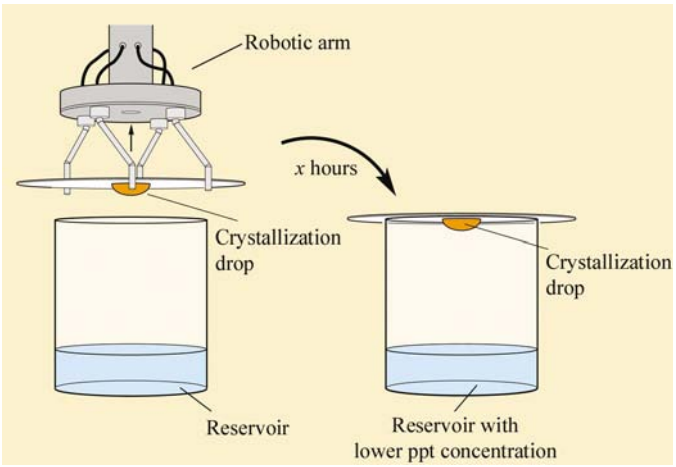

Figure 5
Automatic transfer of hanging drops from nucleation to growth conditions.

concentration sets of conditions (Table 2). For a decoupling experiment to be successful, it must be ensured that the solution is diluted to the metastable zone of conditions after incubation under spontaneous nucleation conditions. It was found, however, that when these conditions are not known beforehand, as is the case in a high-throughput environment, the best results were obtained when standard screening kits solutions were diluted to between 60 and 80%. These observations have led to the development and use of a '3-D screen' which has an in-built first optimization step (see §5). Protocols for conducting these experiments automatically (Fig. 5) are currently being investigated by manufacturers of crystallization robots.

3.5. Slowing down vapour diffusion with an oil barrier

A means of slowing down the equilibration rate and thereby approaching supersaturation more slowly in order to avoid crystal 'showers' is by placing a paraffin/silicone oil mixture as a barrier over the reservoir of a hanging or sitting-drop trial (Chayen, 1997*b*). The mix of paraffin and silicone oil can be varied as needed. It was found that volumes of 250–500 µl placed over 1 ml reservoirs in standard Linbro plates (corresponding to layer thickness of 1.25–2.5 mm) were most efficient. This method has been shown to work well for several proteins (*e.g.* Chayen, 1997*a*; Mayans *et al.*, 1998; Mandelman *et al.*, 2002). The advantage of this technique is that no change is required to the crystallization conditions nor to the method used. It can be applied in Linbro, VDX, Cryschem or any other vessel. The insertion of an oil barrier has recently been automated by adding one extra step to the procedure used by the Cyberlab robot.

4. Conclusions

Numerous recent articles and special issues of scientific journals have highlighted the importance of structural genomics in the post-genome era (*e.g.* Abbott, 2000; *Nature Structural Biology*, 2000; Stevens *et al.*, 2001; Vitcup *et al.*, 2001). However, surprisingly little attention has been given to improving methods of protein crystallization and optimization. The statistics shown in Fig. 1 and the enormous number of proteins which need to be dealt with indicate that, like screening, optimization must be adapted to high throughput. Without this we will soon run out of resources and be left with a backlog of useless microcrystals. The combination of automated screening with further development of automated crystal optimization methods will remove the main bottleneck in structural genomics and equip the genome projects to move forward into the post-genomic era.

5. Experimental procedures

Porcine pancreatic trypsin (T-0134), thaumatin from *Thaumatococcus danielii* (T-7638), jack bean type IV concanavalin A (C-2010), hen egg-white lysozyme (L-6876), buffers and salts were purchased from Sigma. C-phycocyanin from *Synechococcus elongatus* was purified in-house (J. Nield, personal communication). All experiments were performed at 291 K.

5.1. Preparation of gels

2 ml stock solutions of gels were prepared to load the robot for each series of experiments.

(i) Agarose 1%(*w*/*v*) stock. 20 mg 'Wide Range' agarose powder (Sigma cat. No. A2790) was progressively dissolved into 2 ml deionized water previously heated to approximately 353 K and continuously stirred. The optimal concentration of agarose in the crystallization drop was 0.1–0.15%(*w*/*v*) (Moreno *et al.*, 2002). Higher concentrations often caused precipitation.

(ii) TMOS 5%(*v*/*v*) stock. 0.1 ml TMOS solution (Fluka cat. No. 87682) was added to 1 ml distilled water in a glass tube and the solution vigorously stirred to disperse it. It was then topped up to 2 ml and vigorously stirred for an additional 10–15 min, keeping the vessel covered. The optimum final concentration in the crystallization drop was found to be 0.2%(*v*/*v*) (Moreno *et al.*, 2002).

(iii) Sodium metasilicate 5%(*v*/*v*) stock. Silicate solution (Sigma cat. No. 33844–3), which has an initial pH of 11.6, was diluted with distilled water at almost the desired concentration and acidified to pH 6.5 by addition of 1 *M* acetic acid solution while stirring. The optimum final concentration in the drop was 0.2% as for TMOS (A. Moreno, personal communication).

5.2. Control of evaporation

0.3–2 µl microbatch drops were set up at the following conditions (where the pH indicates pH of the buffer and not of the final mixture and the saturation temperature of the ammonium sulfate solution was 293 K).

(i) Lysozyme: 20 mg ml^{-1} protein, 50 m*M* sodium acetate–HCl pH 4.6, 0.7 *M* sodium chloride.

(ii) C-phycocyanin: 15 mg ml^{-1} protein, 40 m*M* MES–NaOH buffer pH 6.1, 1.5 m*M* dodecyl maltoside, 0.6–0.72 *M* ammonium sulfate.

(iii) Trypsin: 20 mg ml^{-1} protein, 50 m*M* Tris–HCl buffer pH 8.4, 39% saturated ammonium sulfate.

5.3. Decoupling of nucleation and growth

The 3-D Screen (MD1-13; Molecular Dimensions, UK) is made up of 48 solutions. 24 contain sparse-matrix screening conditions; the other 24 are a 70% dilution of the precipitants in these (buffer and additive concentrations are kept constant). Using this screen, all hanging drops were first incubated for 3–6 h over the solutions at high concentrations. The cover slips holding the drops were then transferred over the reservoirs at 70% dilution. The cover slips were sealed with Apiezon C oil (D. Bewhay Ltd, Borehamwood, Hertfordshire, UK), not with grease. This makes it easy to transfer cover slips from one reservoir to another.

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