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Microbatch crystallization under oil – a new technique allowing many small-volume crystallization trials

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An approach to rapid protein crystallization using very small samples is described. A computer controlled microdispenser is used to make crystallization samples as microbatch droplets under oil. Samples of 1–2 μl are dispensed ready-mixed and with good precision. The samples are protected from evaporation, contamination and physical shock by the oil. When favourable conditions for crystallization have been found using one mode of the system, the size and quantity of crystals are optimized by a second program which generates a set of conditions throughout the area of interest. Crystals of diffraction size and quality have been grown in 1 μl drops.

1. Introduction

When trying to crystallize a protein, the initial aim of the crystallizer is to screen rapidly many conditions with a minimum of labour, and using as little protein as possible.

To ease the laborious and time consuming task of crystallization, several automatic systems have been introduced [1–5] – but all previous automation procedures are directed toward vapour diffusion methods. We are presenting here an automated technique for batch crystallization which fulfils the following requirements:

(a) Dispensing of very small samples;
(b) high level of accuracy;
(c) rapid screening to determine solubility properties;
(d) homing in on conditions that produce crystallization;
(e) automatic execution in a quick and simple way;
(f) flexible changes of operation mode for different types of survey.

2. Crystallization under oil

To reduce the consumption of material, very small samples must be dispensed. This leads to two major problems: (a) evaporation; (b) inaccuracy of dispensing.

The problem of evaporation is solved by dispensing and incubating the crystallization samples under oil. Samples are dispensed as droplets into the wells of tissue culture plates (of the kind known as Terazaki plates, Sterilin, UK) where they are isolated by a layer of oil (fig. 1). The

droplets can be dispensed under oil manually or automatically.

High accuracy is achieved by using a microdispenser comprising a bank of Hamilton syringes driven by stepper motors under computer control, to dispense liquid under oil through a fine multi-bore tip [6]. The high accuracy of the motorized syringes delivers accurate volumes at the tip. Delivery from a fine tip under oil cleanly removes the delivered volume as the tip is drawn out from the oil, and eliminates "carry-over". Tests show that 1 μl droplets can be dispensed with a standard deviation of less than 0.02 μl.

The microtip is automatically moved using a

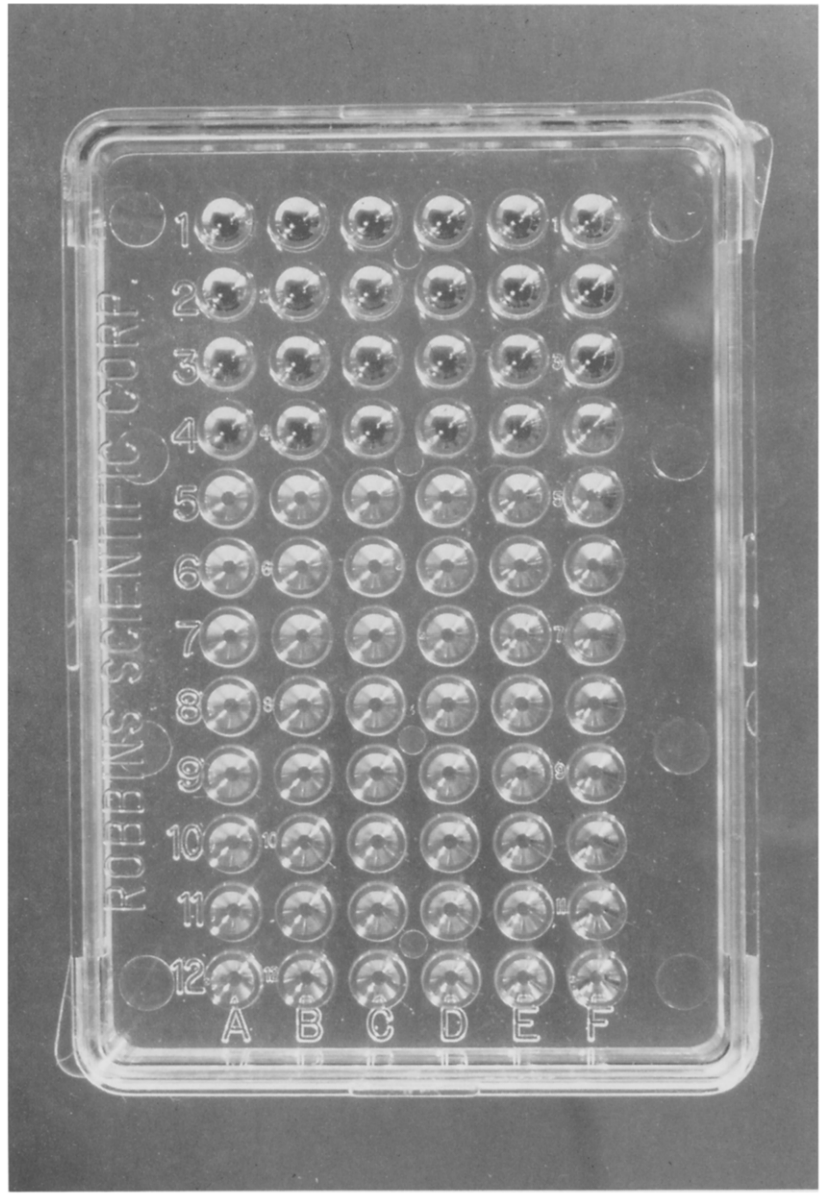

Fig. 1. 24 droplets of a coloured solution dispensed under oil in a Terazaki plate. The actual size of the plate is 55 × 80 mm.

robot arm (which moves vertically) with a moving table (which moves horizontally in two directions). Because the robot arm does not move horizontally, flexing of the tubing is kept to a minimum, which improves the accuracy of dispensing.

We have found that this method of crystallization has the following advantages:

(1) Low protein consumption. Because evaporation is reduced, dispensing under oil enables the use of 0.5–0.7 μl of protein, with a final droplet volume of 1–2 μl. Crystals produced in such small droplets are of the same or better quality for diffraction, and of equal or greater size than those grown in much larger drops (10 μl) by means of other methods.

(2) Very stable crystals. In several cases (e.g. reverse transcriptase [7]), crystals grew in hanging drops but dissolved several days later (probably due to small temperature changes which cause condensation). This does not happen in the microbatch under oil.

(3) Batch method. The sample is mixed fully when it is dispensed. In the microbatch, concentration gradients and convection flows are kept to a minimum, and there is less tendency to form skins. The volume and composition of a droplet is known exactly, which is useful for theoretical studies of the crystallization process. This contrasts with the vapour diffusion methods where the physical complication of the methods means that the precise conditions of crystallization are not known.

(4) Time saving method. Methods for crystallizing small samples usually involve careful manipulation. This technique is mechanically simpler than both vapour diffusion and dialysis.

Fig. 2. Crystals of carboxypeptidase G2 in a 2 μl droplet under oil. Crystals were grown from 1.6M ammonium sulphate and 8mM zinc sulphate. The actual size of the largest crystal is $700 \times 100 \times 90$ μm.

(5) Protection of sample. Drops are buoyed up by the viscous oil, they are therefore easily transportable. The layer of oil also protects the droplets from dust and contamination.
(6) Reduced consumption of disposables. Linbro trays and coverslips are not required; pipettes and chemicals are used in small quantities.
(7) Compact. The use of the small Terazaki plates saves on storage space.

So far, in all cases tried, a crystal form obtained by means of vapour diffusion has been grown by microbatch with only slight modification of the vapour diffusion conditions. However, on more than one occasion this method has given crystal forms which could not be obtained by means of vapour diffusion, e.g. reverse transcriptase [8], carboxypeptidase G2 (fig. 2) and others.

Harvesting the crystals does not cause problems: The droplets are first enlarged by adding about 30 μl of a harvesting solution (which contains precipitant at a slightly higher concentration than that in the droplet), crystals are detached, if necessary, by stirring with a glass fibre, drawn up and transferred to a depression glass, containing more harvesting solution, using a micropipette.

3. Rapid screening for crystallization conditions

In an alternative configuration, the microdispenser can be used to carry out large numbers of crystallization trials using pre-mixed standard stock solutions of precipitating agents and buffers.

In this case, a 2-bore microtip is used and the experiment is carried out automatically using the robot arm with two motorized syringes (fig. 3). One motorized syringe is used to aspirate typically 1 μl of stock solution from the wells of a microtitre plate and transfer it into wells of a Terazaki plate in sequence. After each transfer of solution, the tip is rinsed by aspirating and dispensing a few microlitres while remaining in a well containing distilled water. A second motorized syringe is then used to dispense typically 1 μl of protein solution into each of the crystallization wells of the Terazaki plate.

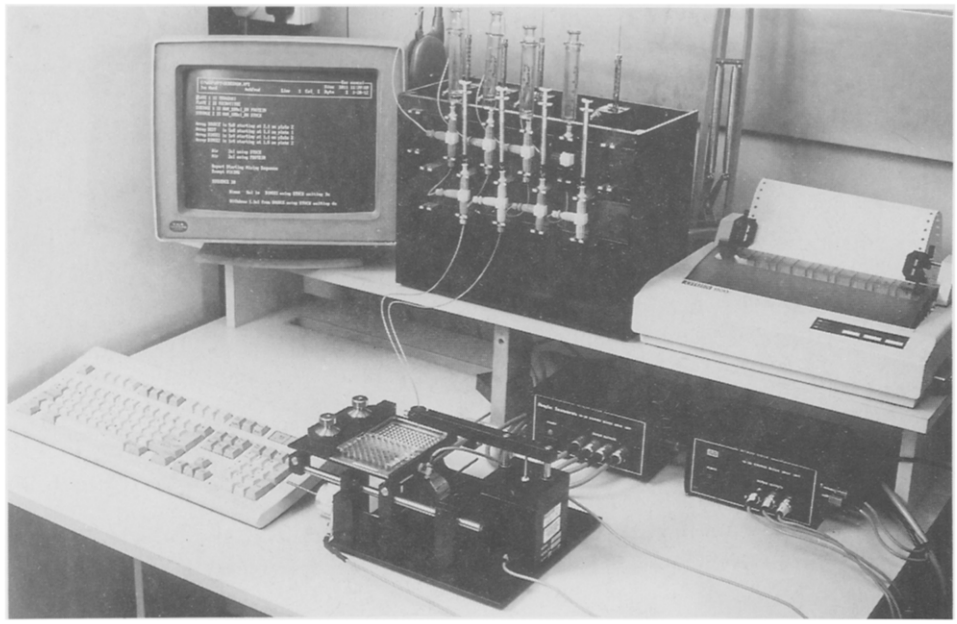

Fig. 3. A view of the apparatus comprising the liquid handling system, stepper motor drivers, PC, printer, microtip, robotic arm and the moving table. Two sets of wells are placed on the moving table: a plate with microtitre strips containing stock solutions for crystallization and a Terazaki plate. The microtip is in the act of dispensing a solution into the Terazaki plate, using the ASP program. A similar configuration is used with X-Step, but with only one set of wells, and a four-bore microtip.

Both stock solutions and crystallization drops are situated under oil. These stock solutions are prepared in approximately 20 ml volumes, and stored in a freezer. They may be chosen to scan a range of conditions in a systematic way, or they may contain precipitants, additives and buffers chosen at random using a random number generator, in the style of Carter and Carter [9]. Forty solutions can be screened in one run using approximately 400 μg of protein. This run takes approximately 1 h but need not be supervised. The program is named ASP (Automated Sample Preparation), and is written in ordinary sentences, which makes it easy for anyone to use. In the same manner, up to forty additives can be automatically transferred to crystallization trials which are otherwise identical.

4. Optimization of conditions

To generate a set of crystallization conditions evenly spaced throughout the area of interest, the program X-Step previously described by Chayen et al. [6] is used. Two parameters are varied in a single run, and a four-bore microtip is used. In successive runs conditions are fine-tuned, until the best crystals are produced. This program displays a four by six array of cells. The conditions in the top left and bottom right cells can be edited, then all other conditions are automatically determined by the program by linear interpolation of concentrations or pH values. Each cell corresponds to a single microbatch droplet. During execution the droplets from the entire array are automatically dispensed in sequence.

5. Summary

Our approach to the crystallization of proteins starts with the rapid determination of the ranges of conditions where precipitation can be achieved, using automatic dispensing and a minimum amount of protein. The system described above is routinely used in the following order: When a new protein is to be crystallized, a standardized initial screen is performed first with the use of the ASP program. In this way, conditions that look favourable for crystallization are found. In the next step, the X-Step program [6] is used to fine-tune the conditions and produce large crystals for X-ray diffraction.

Several new forms of crystals were obtained by using the system in this way. Among them are carboxypeptidase G2 (fig. 2), reverse transcriptase [8] and a protein which regulates the amidase operon of *Pseudomonas æruginosa* [10].

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