Semi-automatic protein crystallization system that allows *in situ* observation of X-ray diffraction from crystals in the drop

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Semi-automatic protein crystallization device was developed using 96 format Hydra dispenser. Eight extra syringes were added for pipetting protein solution with its own dispensing mechanism. This device allows setting up 96 drops in about three minutes. New crystallization plate has also been developed. The plate allows collecting an oscillation photograph of the crystal directly in the drop. The diffraction quality of the crystals in the drops can be inspected without causing any other physical perturbations to the crystals. With the modified dispenser and this plate, we can perform both hanging and sitting drop vapour diffusion method.

Keywords: protein crystallization; crystallization device; crystallization plate

1. Introduction

The three-dimensional structure analysis of proteins plays an essential role in the biological sciences of the post-genomics era. One superior approach to obtain structure of proteins is X-ray crystallography. Unfortunately, however, the crystallization of proteins is influenced by a number of parameters and it is not so well understood yet about the efficient parameters to get good crystals. Usually, the optimal crystallization conditions are investigated in large screen sets with various parameters of precipitates and buffers by trial and error. Some high-throughput approaches have been proposed (for example Villaseñor, *et al*., 2002), and a robotic crystallization system becomes one of the essential techniques for high-throughput protein crystallography. Even though a nano-droplet crystallization robot has been developed at the Lawrence Berkeley National Laboratory (Stevens, 2000), a micro-liter droplet system might be enough for the majority of laboratories at universities where recombinant protein expression is used for their sample preparation. Several robots for micro-liter droplet are now commercially available, such robots are still expensive, or speed of setting crystallization droplets is not fast enough. Here we have developed a simple and semi-automatic crystallization device using a standard 96-channel liquid dispenser Hydra 96 (Robbins Scientific).

Once the protein forms crystals in some drops, they need to be inspected if they have sufficient diffraction quality. For that purpose, usually one crystal is picked up from the crystallization drop to be exposed to X-ray to check its diffraction power. The X-ray diffraction power of a protein crystal cannot be examined only by optical investigation. Sometimes it is not as good as it appears. However, picking up a crystal from the drop is a delicate process. It requires time, and sometimes a crystal receives considerable physical damage. In order to overcome this difficulty, McPherson (2000) proposed a simple prototype device for *in situ* X-ray data recording. Here we have developed a specialized crystallization microplate that makes it possible to inspect crystals in the drop directly by X-rays. The drop on the plate can be exposed to the X-ray beam with any plate orientation. With this plate, we can examine the intrinsic diffraction quality of crystals without any physical perturbations. In addition, the plate can be used for both hanging and sitting drop vapor diffusion method. In combination with the modified Hydra 96 dispenser, this microplate allows high-throughput screening of protein crystallization conditions.

2. Materials and methods

2.1. Crystallization device

We have used the Hydra dispenser for our crystallization device. Mueller *et al*. (2001) also uses the same microdispenser for protein crystallization. They used HYDRA 96 only for crystallization reagents, and another four channel dispensing system was adopted for deposition of protein solution. On the other hand, we aimed at making deposition of protein droplets and dispensing reservoir solution possible with only one Hydra dispenser. This reduces cost and space for dispensers and operation of the system become simpler. For that purpose, we have modified the Hydra dispenser to have extra 8 syringes on its aspiration/dispense mechanism (Fig. 1). Protein solutions were pipetted by the 8 syringes and the 96 precipitate reagent solutions were pipetted by the original 96 syringes of the dispenser. In order to dispense protein solution without the original 96 syringes touching the plate, the height of the extra syringes was 14.5 mm lower than the original syringes.

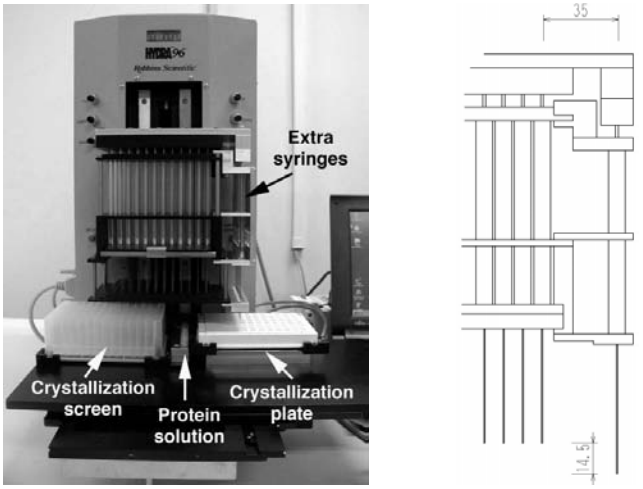

Figure 1

The modified Hydra dispenser with extra 8 syringes. (a) Photograph and (b) drawing of the extra 8 syringes.

The plate positioner of the dispenser was also modified. The protein storage block was placed at the center of the platform. An 8-tube PCR strip can be used for the protein solution. The height of the destination plate position, where the crystallization plate will be placed, was raised by 15 mm. This modification allows the use of any 96 well crystallization microplate of 9 mm standarized format, for example CrystalQuick Plate from Greiner Bio-One. The crystallization screens are stored in 96-well storage blocks at the source plate position, left side, of the platform.

2.2. Crystallization plates

The photograph of the crystallization plate is shown in Fig. 2. The plate consists of 12 strips, each of which has 8 crystallization positions, like CrystalClear Strips from Hampton Research. Each position consists of a protein well to hold a protein drop and a

reservoir to hold mother liquor (Fig. 3). The bottom of the protein well, indicated as D in Fig. 3, is made of thin film, in order to maintain both X-ray and optical transparency. It might be possible to use thin film or plate of glass, plastic or Mylar etc. We use Power Seal (Greiner Bio-One) at present. For sealing the top of the plate after all drops are finished, Crystal Clear Sealing Tape (Hampton Research) is used.

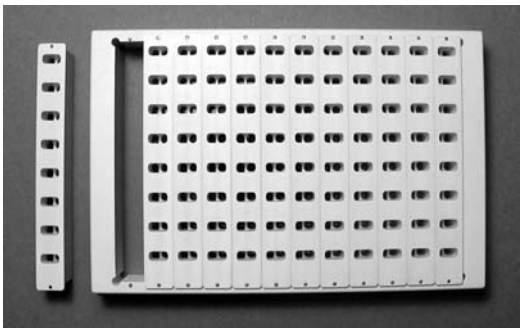

Figure 2

Photograph of the crystallization plate. The plate consists of 12 strips that are contained in a frame to keep 96 standarized format.

(a)

(b)

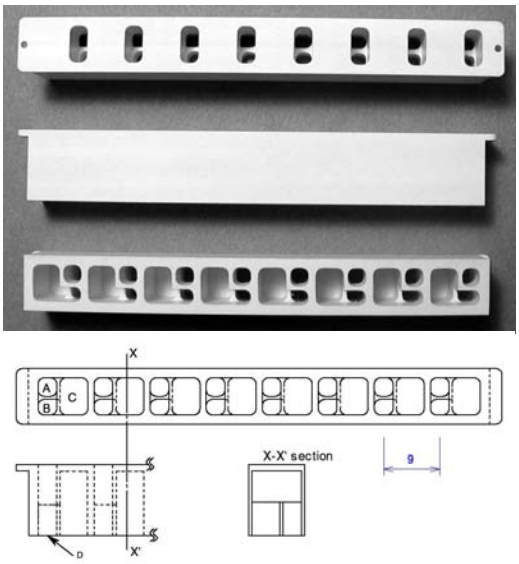

Figure 3

Detail of one strip of the crystallization plate. (a) Top, side and bottom views of the strip and (b) drawing of the strip. A: protein well, B: injection hole for mother liquor, C: reservoir for mother liquid, and D: thin floor for protein drop. All three compartments A, B and C are connected for the vapor exchange.

The narrow strip structure of the plate allows it to be mounted onto the goniometer of laboratory X-ray diffractometers (Fig. 5). The diffraction quality of the crystals in the drops can be inspected by direct X-ray exposure without any other physical perturbations to the crystals. In addition, using this plate, both hanging and sitting drop vapor diffusion methods are possible with the same dispenser. If one needs to perform hanging drop method, one can put the plate upside down just after dispensing all drops and solutions.

2.3. Performance test of the system

Unknown function protein PH1136 from *Pyrococcus horikoshii* (131 amino acid, 14,728 Da and 20 mg/ml in 10 mM Tris-HCl, pH 8.5) and 96 crystallization screens (No. 1 - 48 of Crystal screen I and all of Crystal screen II, Hampton Research) were used in the crystallization trials with this system. The semi-automated crystal screen preparation with this system was accomplished as follows. (*i*) Place the protein tubes at the center of the platform and 13 µl protein solutions were aspirated by the extra 8 syringes. (*ii*) One microliter of protein solution is arrayed in all 96 wells of the crystallization plate by combination of dispensing and horizontal movement of the platform. (*iii*) After putting the 1 µl remaining in the syringes back into the protein stock tube, 101 µl of all 96 screens were aspirated by the original 96 syringes from the storage block. (*iv*) One microliter of solution was delivered to each protein drop simultaneously, and then 100 µl residuals were dispensed to each reservoir. (*v*) The plate was sealed manually with Crystal Clear Sealing Tape. (*vi*) Finally, the plate was placed upside down and stored in an incubator to perform hanging drop vapor diffusion (Fig. 4). The 96 wells could be set up within about 3 min.

The quality of the growing crystals was inspected directly by exposing crystallization drops to X-rays and taking oscillation photographs. As described above, it is possible to mount it onto the goniometer. In the present experiment, a DIP-R300 image-plate system was used with a rotating anode X-ray generator of Cu Kα radiation, operated at 50 kV, 90 mA (MAC Science). The diameter of the collimator used was 0.5 mm. Photographs were taken at two degrees oscillation with the crystal-to-detector distance set to 150 mm. An exposure time was 60 min. The photographs were processed using *DENZO* (Otwinowski & Minor, 1997).

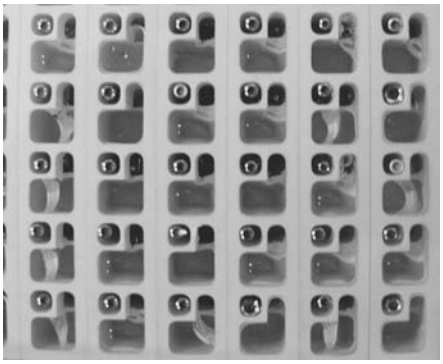

Figure 4

Magnified view of the part of the plate. In this case, the plate was put upside down to perform hanging drop vapor diffusion. The drops are hanging under the Power Seal.

3. Results and discussion

Table 1 summarizes the crystallization results for PH1136 protein after two days. Crystals were obtained under 12 different conditions. Large crystals grew at three conditions, D1 (0.1 M Tris hydrochroride pH 8.5, 0.4 M ammonium sulfate), A5 (4.0 M sodium formate) and F5 (0.1 M HEPES-Na pH 7.5, 1.4 M tri-sodium citrate dihydrate). Photographs of these drops are shown in Fig. 6. Small crystals were observed in the drops at F1, A3, F3, C6, D7, D8, C11 and E12. Interestingly, the protein formed a phase separation at wells F3 and C11 when the plate was prepared manually.

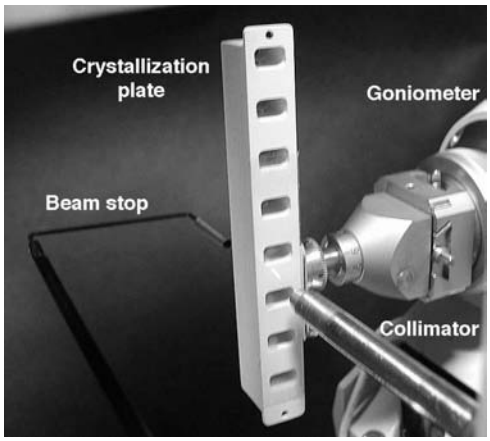

Figure 5

Photograph of the crystallization strip mounted on the goniometer.

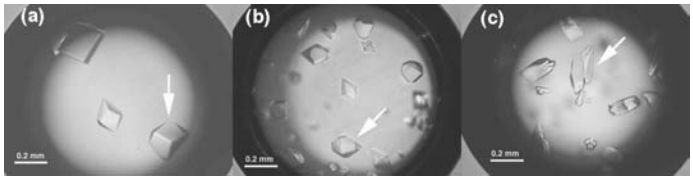

Figure 6

PH1136 crystals grown in (a) well D1, (b) A5 and (c) F5 of the plate. Crystals aligned at the centre of the X-ray beam are indicated by white arrows.

Oscillation photographs of these crystals taken by directly exposing drops to X-rays are shown in Fig. 7. Diffraction spots to 2.9 Å and 3.2 Å were observed for crystals grown in well D1 and F5, respectively. However, the crystal in A5 diffracted only to 8.5 Å. The crystals in A5 look better than those in F5, but the crystal in F5 gave better diffraction spots than that in A5. These results indicate that the X-ray *in situ* observation of the diffraction quality of the crystals is superior to optical observations by microscopes. With this system, one can decide which crystallization condition is better for an X-ray study rapidly and efficiently without picking crystals out from the crystallization drops. It took more than half an hour for exposure in this experiment. It will be much faster, if one can use synchrotron radiation for the X-ray source. Once the crystal has enough quality for data collection, one can pick up other crystals from the same drop and use it for the next experiment such as screening of the cryo-cooling conditions.

As shown in Table 1, there was no hanging drops formed at some wells successfully, especially if the precipitant contained high percentage of organic solvent, such as 2-methyl-2,4-pentanediol and iso-propanol. We are still continuing to improve the design of the crystallization plate.

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Table 1

Summary of the crystallization results for PH1136 protein in 96 well plate. Legends are: C, large crystals; c, crystals; p, phase separation; N, no crystallization drop left after two days. There was no drop found at X from the beginnings. Order of screen solutions starts from A1 to H1 then A2 to H2..., and A12 to H12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	p	c		C	p			X			
B		p	p	N		p				p		
C			X	N		c				p	c	X
D	C	N	p	p			c	c		p		
E						p		p			p	c
F	c		c	p	C	p			p		p	
G		p		p		c	p					
H											N	

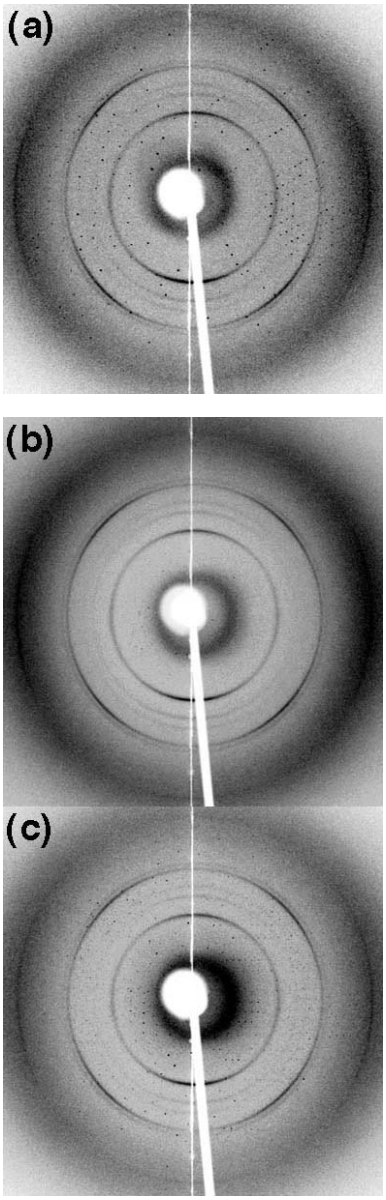

Figure 7

Oscillation photographs of PH1136 crystals. (a) Crystal grown in well D1, (b) A5 and (c) F5 of the plate.

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