Acta Crystallographica Section D
Biological Crystallography
ISSN 1399-0047

Promoting protein crystallization using a plate with simple geometry

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Received 11 July 2013
Accepted 25 November 2013

Increasing the probability of obtaining protein crystals in crystallization screening is always an important goal for protein crystallography. In this paper, a new method called the cross-diffusion microbatch (CDM) method is presented, which aims to efficiently promote protein crystallization and increase the chance of obtaining protein crystals. In this method, a very simple crystallization plate was designed in which all crystallization droplets are in one sealed space, so that a variety of volatile components from one droplet can diffuse into any other droplet *via* vapour diffusion. Crystallization screening and reproducibility tests indicate that this method could be a potentially powerful technique in practical protein crystallization screening. It can help to obtain crystals with higher probability and at a lower cost, while using a simple and easy procedure.

1. Introduction

Protein crystallization is a challenge for protein structure determination by X-ray diffraction (Chayen & Saridakis, 2002; Moreno *et al.*, 2002; Newman, 2011). To achieve successful crystallization, two steps are typically necessary: screening for initial crystallization conditions (denoted as 'hits') and optimizing these hits in a more defined range (Newman *et al.*, 2005; Newman, 2011; Chayen & Saridakis, 2008; McPherson, 2004). The first step is very important; hence, methods have been proposed to increase the probability of crystallization (D'Arcy *et al.*, 2004; Newman, 2005; Korczyńska *et al.*, 2007; Brzozowski & Walton, 2001). For this step, screening methods such as vapour-diffusion (Nneji & Chayen, 2004; Lu *et al.*, 2010) and microbatch (Brumshtein *et al.*, 2008; D'Arcy *et al.*, 2003) techniques are widely utilized. A number of modifications to the traditional vapour-diffusion method have been developed to increase the chances of crystallization. For example, an evaporation-based crystallization platform has been developed in which the crystallization droplets can be gradually concentrated until the solvent has completely evaporated (Talreja *et al.*, 2005). A crystallization plate (Nextal Crystallization Tool) using screw caps to enable controlled evaporation has been described, and the number of crystallization screening hits was reported to increase (Nneji & Chayen, 2004; Khurshid *et al.*, 2007). The generic reservoir method, in which different reservoirs are substituted by one or several common reservoirs, can be used to alter the outcome of the crystallization experiment quite significantly (Dunlop & Hazes, 2005; Newman, 2005). The so-called crystallization mushroom also enables simultaneous vapour-diffusion-assisted protein crystallization in many drops (Tosi *et al.*, 2011). The desiccation method, in which desiccants are used instead of reservoir solutions, can enlarge the range of concentrations in the

crystallization solution so that the number of hits obtained from the screening process can be significantly increased (Lu *et al.*, 2010, 2012).

In this paper, we propose a simple novel method in which all crystallization droplets are dispensed onto one glass substrate and then sealed in the same space, so that the volatile components in the individual droplets can diffuse throughout the chamber. Because the concentration evolution in the crystallization droplets resembles that in a microbatch method and because of the existence of vapour diffusion among the droplets and the cross-influence (Tomčová & Kutá Smatanová, 2007) among droplets that share the same vapour space, we call this technique the cross-diffusion microbatch (CDM) method. Extensive testing shows that the CDM method dramatically increases the probability of obtaining crystals compared with the conventional vapour-diffusion method. Owing to its simplicity and the good crystallization results obtained using this method, it is also potentially applicable to everyday protein crystallization.

2. Materials and methods

2.1. Materials

In this study, 15 proteins were used. Hen egg-white lysozyme (HEWL; Seikagaku, Japan; catalogue No. 100940, recrystallized six times) was utilized without further purification. Proteinase K (catalogue No. P6556), α-chymotrypsinogen A II (C4879), catalase (C40), concanavalin A VI (L7647), thaumatin (T7638), subtilisin A III (P5380), ribonuclease A XII (R5500), ribonuclease A I (R4875), cellulase (C0615), myoglobin (M1882), α-lactalbumin (L5385), papain (P3125) and haemoglobin (H2625) were obtained from Sigma–Aldrich, USA.

Homoserine *O*-acetyltransferase (HTA; Gene ID 1151404) protein was expressed recombinantly in *Escherichia coli* and purified in our laboratory (Wang *et al.*, 2007). The recombinant bacteria were provided by the Institute of Biophysics, Chinese Academy of Sciences and the structure of this protein has been described previously (Wang *et al.*, 2007).

Sodium chloride (NaCl) was purchased from Tianjin Kermel Chemical Reagents Development Center, People's Republic of China. Acetic acid (HPLC grade) was obtained from TEDIA Co., USA. Sodium acetate and HEPES sodium were purchased from Beijing Chemical Factory, People's Republic of China. The Index crystallization screening kit (catalogue No. HR2-144) was purchased from Hampton Research.

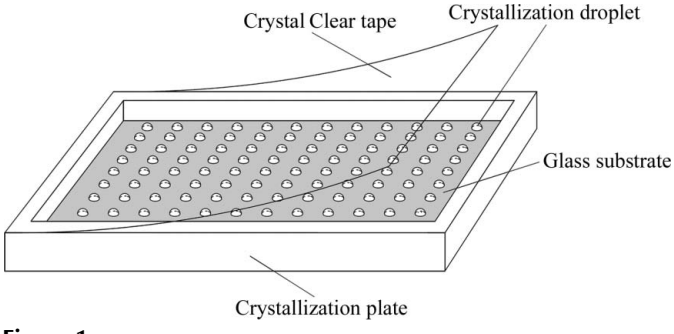

Figure 1
Schematic of the crystallization plate.

A new type of crystallization plate was constructed (Fig. 1). A glass substrate [length 114 mm, width 74 mm, thickness 2 mm, silicified by carbon tetrachloride:dimethyldichlorosilane at 9:1(*v*/*v*); Sinopharm Chemical Reagent Co. Ltd, People's Republic of China] was placed at the bottom of the crystallization plate because glass is easy to clean and reuse. The material of the plate was polymethyl methacrylate (PMMA). The thickness of the PMMA was 6 mm. The size of the plate was made to be compatible with the SBS standard plate (ANSI/SBS 1-2004; American National Standards Institute/Society for Biomolecular Sciences, Danbury, USA; Korczyńska *et al.*, 2007).

2.2. Methods

To test the efficiency of crystallization using the new crystallization plate, two types of crystallization experiments were conducted. The first was a reproducibility study and the second was a screening study. For the reproducibility study, 96 droplets in a 12 × 8 array from the same mother liquor were dispensed onto the glass substrate in the crystallization plate and then sealed and incubated in a temperature controller. After incubation, the crystallization results were examined to verify the reproducibility of crystallization in the plate. For the screening study, the protein solution was mixed with the screening kit on the glass substrate in the crystallization plate to form 96 droplets of different crystallization conditions. The plate was then sealed and incubated in a temperature controller. After incubation, the droplets were examined to obtain the number of crystallization screening hits (crystallization conditions that yielded protein crystals) to check the applicability of the new crystallization plate.

To further examine crystallization using the new plate, the effects of the droplet location, the incubation time and the volume of the droplets on the crystallization results were also investigated. Because diffusion among the droplets in the new plate is important, the processes of evaporation and vapour diffusion in the plate were investigated using pure water, NaCl and protein (lysozyme and proteinase K) solutions.

2.2.1. Reproducibility study for lysozyme crystallization. Lysozyme and NaCl were dissolved in 0.1 *M* sodium acetate buffer pH 4.6 at initial concentrations of 40 and 70 mg ml^{-1}, respectively. The two solutions were mixed in a 1:1 volume ratio to prepare the mother liquor, which was then dispensed into an array of 12 × 8 droplets (the volume of each droplet was 2 µl; the distances between the droplets are the same as in standard Intelli-Plate 96-well crystallization plates) on the glass substrate in the crystallization plate by a crystallization robot (Screenmaker; Innovadyne Technologies Inc., USA). The crystallization plate was then sealed using Crystal Clear Tape (Hampton Research, catalogue No. HR4-506) and finally placed into a temperature controller (with 77% relative humidity) for incubation at 293 K for 2 d. The same

Table 1
Different arrays (12 × 8 arrays) of the crystallization reagents from the Index screening kit in the crystallization plate.

(*a*) The first array.

	1	2	3	4	5	6	7	8	9	10	11	12
A	55	56	57	58	59	60	49	50	51	52	53	54
B	67	68	69	70	71	72	61	62	63	64	65	66
C	79	80	81	82	83	84	73	74	75	76	77	78
D	91	92	93	94	95	96	85	86	87	88	89	90
E	7	8	9	10	11	12	1	2	3	4	5	6
F	19	20	21	22	23	24	13	14	15	16	17	18
G	31	32	33	34	35	36	25	26	27	28	29	30
H	43	44	45	46	47	48	37	38	39	40	41	42

(*b*) The second array.

	1	2	3	4	5	6	7	8	9	10	11	12
A	32	40	22	34	92	91	35	6	55	3	96	68
B	16	69	11	54	30	45	77	60	74	78	72	62
C	70	51	33	7	86	38	58	76	81	89	42	28
D	17	41	47	80	14	46	56	63	93	8	67	84
E	90	83	59	79	5	48	53	29	21	25	52	37
F	64	31	49	27	61	88	50	87	26	43	94	19
G	44	15	73	1	36	82	71	23	65	2	4	18
H	85	75	24	95	39	13	9	66	20	57	10	12

(*c*) The third array.

	1	2	3	4	5	6	7	8	9	10	11	12
A	45	6	73	15	55	13	37	16	31	18	48	88
B	70	32	22	81	47	8	72	33	69	3	77	85
C	50	90	4	93	96	57	94	43	63	52	78	49
D	84	91	34	38	40	61	58	86	44	29	28	79
E	12	87	51	7	11	24	17	39	66	59	74	2
F	92	89	95	42	83	26	41	23	65	68	76	14
G	67	64	10	21	56	27	36	1	20	19	30	35
H	82	75	5	54	60	53	25	9	80	62	46	71

(*d*) The fourth array.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

reproducibility crystallization experiment was repeated 50 times (*i.e.* 50 plates were utilized) and images of the droplets were captured by an automated crystal image reader (Xtal-Quest Inc., People's Republic of China).

In the reproducibility study, the experimental results were very simple and only two types of crystallization results were observed. Either well defined faceted lysozyme crystals grew in the droplets, or the droplets remained clear and no crystals were observed. Therefore, it was easy to obtain the crystallization success rate by summing the number of times crystals were produced at each droplet location and dividing it by the total number of experiments.

2.2.2. Crystallization screening study. To test practical crystallization screening using the CDM method, we carried out crystallization screening experiments using the new crystallization plate and compared the crystallization results with a control in which standard sitting-drop vapour-diffusion (SDVD) crystallization plates were used.

In the CMD, 14 model proteins and recombinant HTA protein were used. The model proteins were dissolved in 25 m*M* HEPES sodium buffer pH 7.0 at initial concentrations of 5, 10, 15 and 20 mg ml^{-1}. The concentration of the HTA protein obtained from the purification was 2 mg ml^{-1}. The protein solutions were mixed with the screening kit (Index, which was arbitrarily chosen from the commercially available crystallization screening kits) at a ratio of 1 µl:1 µl to prepare the crystallization trials using the crystallization robot. After setting up the plates, the crystallization trials were placed into a temperature controller for incubation at 293 K for 3, 6 and 10 d.

For classical SDVD, Intelli-Plates (Hampton Research, catalogue No. HR3-143) were used and the preparation of the crystallization droplets and incubation followed the same procedures as in the CDM. The volume of the reservoir was 80 µl.

2.2.3. Investigation of the effect of droplet location on crystallization screening results. To observe the effect of the location of the droplets on the screening results from the CDM method, we rearranged the locations of the droplets and repeated the screening study. Four different droplet arrays were used (Table 1). The first array was obtained by exchanging the positions of the inner droplets with those of the outer droplets. The second and third arrays were obtained by randomly allocating the positions of the droplets using the *MATLAB* software (The MathWorks, USA). The fourth array was obtained using the standard sequence (*i.e.* with no rearrangement of the standard array). The detailed arrangements of the positions in the different arrays are shown in Table 1. After the arrays had been determined (as in Table 1), the corresponding array of the screening reagents was manually set up in a deep-well plate. Finally, the crystallization trials were set up using the crystallization robot by following the procedures described above in §2.2.2.

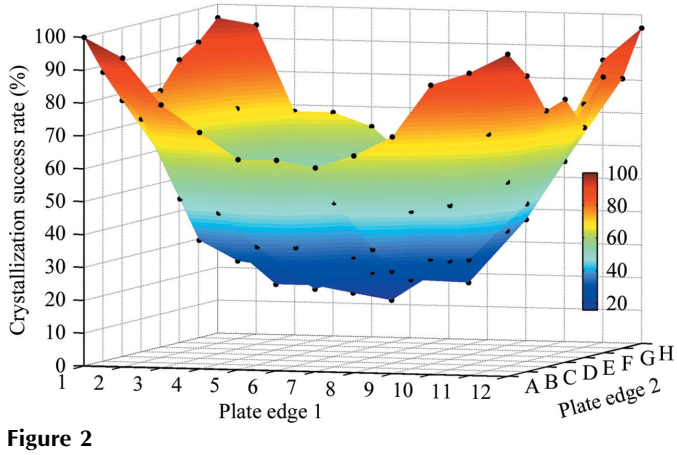

Figure 2
A three-dimensional surface plot of the crystallization success rate for each droplet location on the CDM crystallization plate. The experiment was repeated 50 times. The incubation time was 2 d.

The four proteins used in this part of the study were HEWL, α-chymotrypsinogen A II, catalase and concanavalin A VI. These proteins were dissolved in 25 m*M* HEPES sodium buffer pH 7.0 to an initial concentration of 10 mg ml^{-1}. The protein solutions were then mixed with the crystallization reagents from the Index screening kit in a volume ratio of 1 µl:1 µl.

2.2.4. Protein crystallization screening *versus* incubation time. Owing to the permeable nature of the plate, the droplets in the plate will dry out completely given a sufficiently long incubation time. Therefore, it is necessary to examine the screening results by incubation time. Four proteins (HEWL, α-chymotrypsinogen A II, concanavalin A VI and catalase) were used in this part of the study. The proteins were dissolved in 25 m*M* HEPES sodium buffer pH 7.0 to an initial concentration of 10 mg ml^{-1}. The protein solutions were mixed with the crystallization reagents from the Index screening kit in a 1:1 volume ratio (the volume of each droplet was 2 µl). The screening experiments then followed the procedures described above for the screening study. Both the CDM and the conventional methods were studied. The droplet images were captured every day for 15 d.

2.2.5. Protein crystallization screening with different droplet volumes. To determine a suitable droplet volume for the CDM method, we carried out screening experiments using different initial droplet volumes. All of the procedures followed the screening experiment protocols described above, except that the initial volume of the droplets was varied (0.4, 0.8, 1.2, 1.6 and 2 µl) for this part of the study.

2.2.6. Evaporation and vapour diffusion in the CDM method. We also investigated the evaporation of water using the CDM method. To test this, we prepared an NaCl solution

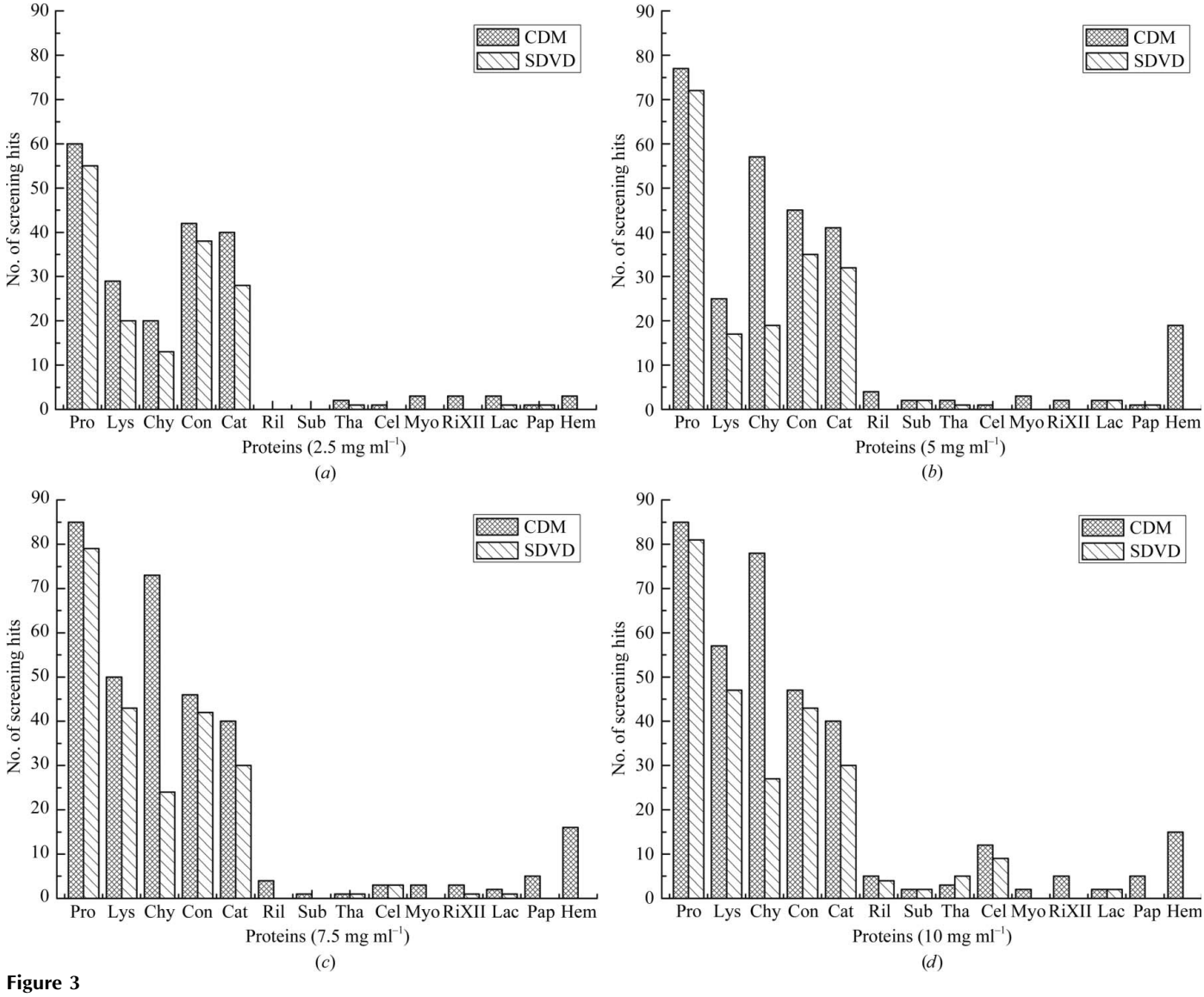

Figure 3
Comparisons of screening hits using the CDM and conventional methods with different initial protein concentrations. Pro, proteinase K; Lys, lysozyme; Chy, α-chymotrypsinogen A II; Con, concanavalin A VI; Cat, catalase; RiI, ribonuclease A I; Sub, subtilisin A III; Tha, thaumatin; Cel, cellulase; Myo, myoglobin; RiXII, ribonuclease A XII; Lac, α-lactalbumin; Pap, papain; Hem, haemoglobin. (*a*) 2.5 mg ml^{-1}. (*b*) 5 mg ml^{-1}. (*c*) 7.5 mg ml^{-1}. (*d*) 10 mg ml^{-1}.

at a concentration of 35 mg ml^{-1} in 0.1 *M* sodium acetate buffer pH 4.6. We then dispensed the NaCl solution as a 12 × 8 array of 2 µl droplets onto the homemade crystallization plate and incubated it at 293 K for 2 d. Pure water (triple distilled) was also dispensed as a 12 × 8 array of 2 µl droplets onto the homemade crystallization plate and sealed for incubation for 2 d using the same conditions as used for the NaCl droplets.

To further examine the evaporation and vapour diffusion among the droplets in the homemade crystallization plates, we carried out a screening experiment using proteinase K as a model protein. Proteinase K was dissolved in 25 m*M* HEPES sodium buffer pH 7.0 to an initial concentration of 20 mg ml^{-1}. The protein solution was mixed with the crystallization reagents from the Index screening kit in a 1:1 volume ratio (the volume of each droplet was 2 µl).

The rest of the procedures followed the methods described for the screening study. The crystallization plate was incubated at 293 K for 6 d.

3. Results

3.1. Reproducibility study results for lysozyme crystallization

The crystallization reproducibility of lysozyme was used as an initial test of the crystallization plate. 96 identical crystallization droplets were dispensed onto the plate from the same mother liquor and then incubated. Fig. 2 shows the crystallization success rate for all 96 of the droplet locations. It is clearly observed that the crystallization success rate at locations nearer to the edges was higher than that at locations close to the centre. This phenomenon (the 'edge effect') could be found down and across all droplets, *e.g.* A1–A12, H1–H12, A1–H1, A12–H12, A1–H12, H1–A12 *etc*. Thus, the highest success rate could be found at the corners and the lowest success rate was at the centre of the plate.

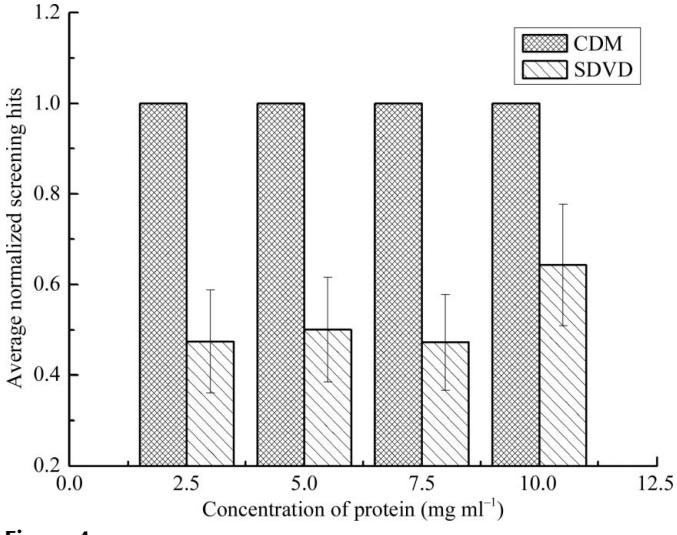

Figure 4
Statistical comparison of the average normalized screening hits between the CDM method and the conventional method (n = 14). The number of hits was normalized on the basis of the data for the CDM method.

3.2. Crystallization screening results for the CDM and the conventional methods

The crystallization reproducibility study showed that the new method could affect the crystallization results; therefore, we tested this novel method in crystallization screening and compared it with the conventional vapour-diffusion method using 15 proteins. Fig. 3 shows the crystallization screening hits for the 14 commercial proteins using both methods at four different initial protein concentrations (2.5, 5, 7.5 and 10 mg ml^{-1} after mixing). The results showed that in almost all cases the CDM method produced more crystallization hits than the conventional sitting-drop method. To evaluate the significance of the improvement using the new method, we performed a paired-samples t-test, and the results showed that the difference between the two methods was statistically significant (n = 14, P = 0.003, 0.001, 0 and 0.02 < 0.05 for initial protein concentrations of 2.5, 5, 7.5 and 10 mg ml^{-1}, respectively).

In the screening test of recombinant HTA protein, 26 and six screening hits were obtained in the CDM and conventional methods, respectively. If these two methods were combined, 27 crystallization conditions could be obtained. This result shows that the two methods were complementary to each other in terms of screening hits. To check the combined effect of using both methods for the other proteins, we list the crystallization conditions found only by the CDM or only by the conventional method in Table S1 (Supporting Information[1]). It can be observed from Table S1 that there are only a few examples where the conventional method yielded more hits than the new method. The CDM method generally showed a higher likelihood of finding hits than the conventional method. The crystallization conditions found by the two methods are complementary to each other; therefore, a combination of the two methods should identify more crystallization conditions than either method alone.

To verify the significance of the improvement, we normalized and averaged the data of the hits from the experiments and the controls. The results are presented in Fig. 4. This figure shows that in all of the tested cases the average normalized screening hits were greater in the CDM method than in the conventional method. The average normalized screening hits were 110.73, 99.81, 111.65 and 55.47% higher in the CDM method than in the conventional method at initial protein concentrations of 2.5, 5, 7.5 and 10 mg ml^{-1}, respectively.

3.3. Taking advantage of the edge effect to maximize the crystallization hits by combining multiple arrays of droplet locations

From Fig. 2, we see that the location of the droplet affects the crystallization success rate in reproducibility experiments for the CDM method. Thus, it was logical to also check the effect of droplet location on the hits determined from the screening experiment. We rearranged the locations of the droplets in three different arrays and carried out the crystal-

[1] Supporting information has been deposited in the IUCr electronic archive (Reference: BW5419).

lization experiments, checking the number of screening hits every 3 d for 18 d. Fig. 5 shows the results for four proteins. From the figure, we observe that although the number of screening hits was different for the different droplet arrays, the trend of the hits over time was the same when different arrays of the same protein were compared. The different number of hits indicated that the different droplet arrays were complementary to each other and that a combination of droplet arrays could yield a larger number of screening hits than a single array alone. Table 2 shows the number of nonredundant hits under all possible array combinations using the four arrays of location arrangements. It can be observed that the use of more arrays provides a higher probability of obtaining more hits. However, when three arrays were used, the total number of hits was nearly the same as that for four arrays. This result indicates that a combination of three different arrays will produce satisfactory screening results. In other words, three different arrays were sufficient to achieve the best result with the CDM method. We also found that different proteins may have different sensitivities to changes in droplet location. For example, in the cases of lysozyme and α-chymotrypsinogen A II we found many additional crystallization conditions by changing the locations of the droplets. However, in the cases of the other two proteins (catalase and concanavalin A VI) the number of additional crystallization conditions that were identified was small.

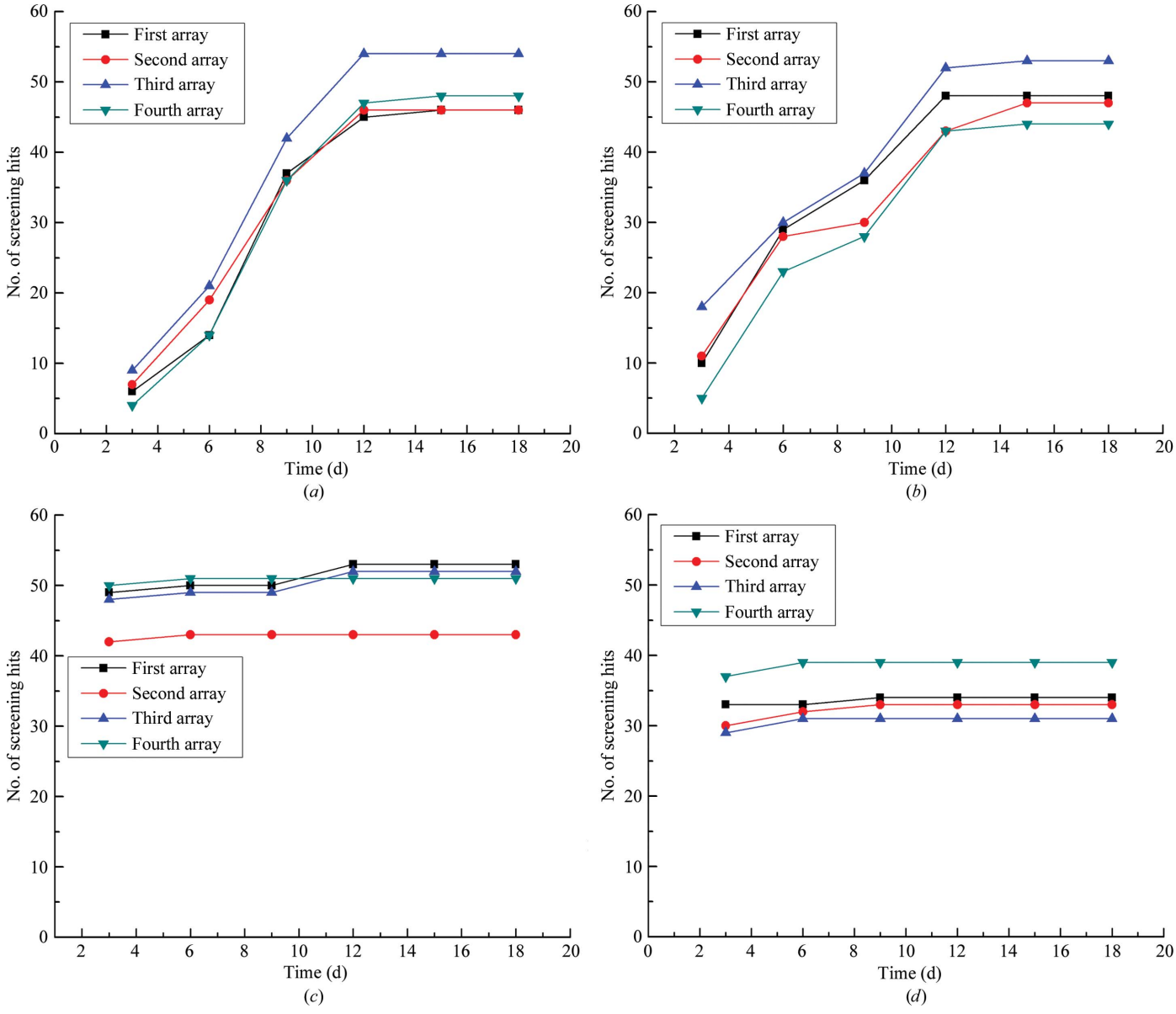

Figure 5
The number of screening hits for different proteins in different droplet arrays of the 96 screening hits. In the first array, the position of droplets in the inner parts was exchanged with that in the outer parts. In the second and third arrays, random arrangements were used. In the fourth array, the standard arrangement of the crystallization reagents, *i.e.* the same sequence as in a routine protein crystallization screening experiment, was used. (*a*) Lysozyme. (*b*) α-Chymotrypsinogen A II. (*c*) Concanavalin A VI. (*d*) Catalase.

3.4. Effect of incubation time on protein crystallization screening

We compared the number of crystallization screening hits over time for the CDM and the conventional methods. Fig. 6 shows that although towards the beginning of the crystallization time course the number of hits was higher with the conventional method (Figs. 6*a* and 6*b*), the final number of hits from the CDM method was eventually higher than that of the

Table 2
Number of nonredundant hits under combinations of droplet-location arrays.

Note: the first row ('Combinations') represents the combinations of different arrays. For example, '1' represents array 1, '12' represents a combination of arrays 1 and 2. The other numbers in the table represent the number of nonredundant crystallization hits, *i.e.* the overall number of crystallization conditions obtained under different combinations. This table shows that the combination of the three arrays can provide satisfactory crystallization results.

Combinations	1	2	3	4	12	13	14	23	24	34	123	234	124	134	1234
Protein															
HEWL	48	47	53	43	52	56	52	58	54	58	59	62	56	60	63
α-Chymotrypsinogen A II	46	46	54	48	58	62	54	60	53	56	67	61	60	64	68
Catalase	34	33	31	39	40	39	41	40	40	41	41	42	42	41	42
Concanavalin A VI	53	43	52	51	54	56	54	55	51	55	56	55	54	56	56

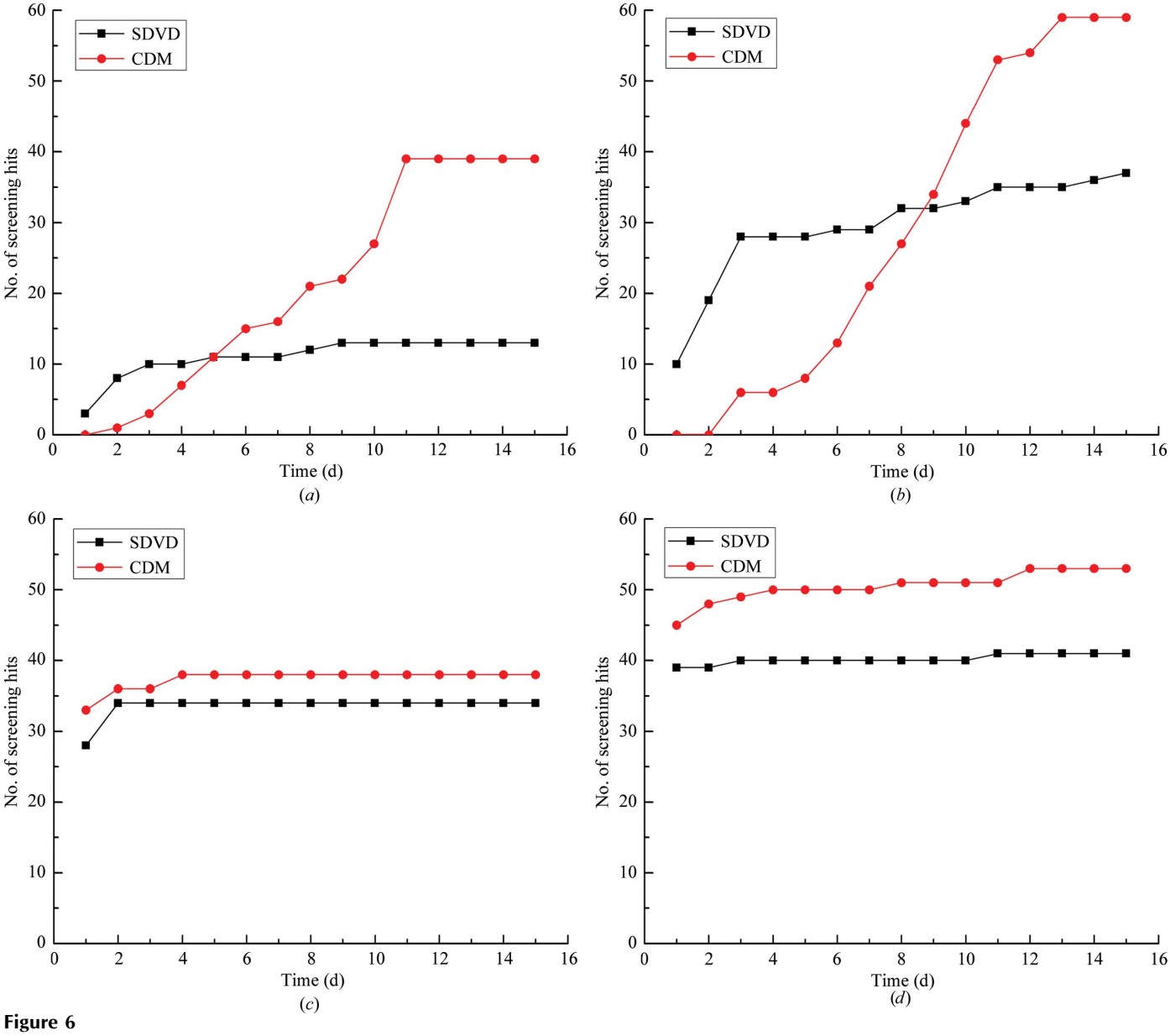

Figure 6
Comparison of the number of crystallization screening hits over time between the CDM and the conventional methods. (*a*) α-Chymotrypsinogen A II. (*b*) Lysozyme. (*c*) Catalase. (*d*) Concanavalin A VI.

conventional method. Note that in the case of catalase and concanavalin A VI, the number of hits was initially higher in the CDM method than in the conventional method (Figs. 6*c* and 6*d*).

3.5. Effect of different droplet volumes on protein crystallization screening

We noticed in our experiments that the drop volume could affect the crystallization results. Therefore, we conducted a crystallization screening of lysozyme using different initial drop volumes ranging from 0.4 to 2 µl. Fig. 7 illustrates that a larger drop volume was preferable for obtaining more crystallization conditions. A smaller drop volume means that the concentration changes more rapidly for a given evaporation rate. Therefore, when the droplet volume was 0.4 or 0.8 µl, the concentration process was fast and crystallization initiated immediately. Because the concentration process was so fast, some droplets yielded amorphous precipitates and ultimately reduced the final number of screening hits (*e.g.* volumes of 0.4 or 0.8 µl). For higher drop volumes, the number of hits was small initially but reached higher final values at later times than the smaller droplets (*e.g.* a volume of 2 µl). In addition to the aforementioned explanation, another important mechanism is that larger droplets have a greater chance of nucleation because they contain more molecules.

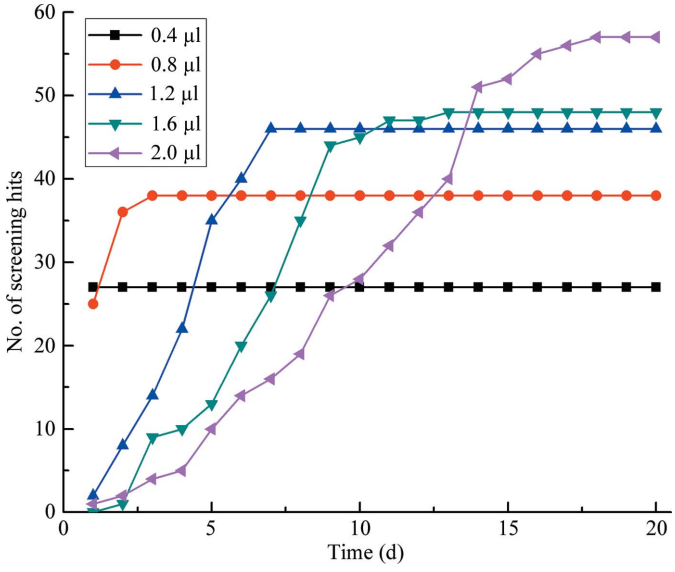

Figure 7
Comparison of the number of crystallization screening hits of lysozyme at different initial drop volumes.

4. Discussion

4.1. Edge effect and the effect of variation of supersaturation and concentration on the reproducibility of the study

Using the CDM method, proteins crystallized more easily near the edges of the plate compared with in the centre parts of the plate. The difference in the crystallization success rates at different locations indicated that the supersaturation in the droplets varies with the location of the droplet on the plate. Fig. 8 shows three crystallization droplets located at different positions. Droplet A1 is at the corner, droplet A6 is at the middle part of one edge and droplet D5 is in the centre part of the plate. It could be observed that the sizes of the droplets differed from each other after 2 d of incubation. After 2 d, the corner droplet was the smallest and the droplet in the centre part of the plate was the largest. This phenomenon indicated that the droplet at the corner reached a higher supersaturation level than those in the centre; therefore, a higher crystallization success rate could be expected near the edges.

The crystallization of lysozyme is very sensitive to variations of concentration or supersaturation. In fact, a small difference in supersaturation (as small as 0.018) can result in an observable difference in the crystallization success rate (Chen *et al.*, 2012). According to the empirical equation (1) for the crystallization success rate R against the supersaturation σ (Chen *et al.*, 2012),

$$R = 333.62\sigma^3 - 5665.4\sigma^2 + 32085\sigma - 60541, \qquad (1)$$

we can obtain the supersaturation of each crystallization droplet (Fig. 9). The difference in the supersaturation of neighbouring droplets from the edge to the centre part of the plate was mostly greater than 0.018, which means that a detectable difference in crystallization success rate can be observed. We performed a one-sample t-test to evaluate the significance of this difference. From the edge to the centre part of the plate, the difference in the supersaturation of every row and every column of neighbouring droplets was statistically significant (n = 80, P = 0 < 0.05; n = 72, P = 0 < 0.05).

By curve-fitting the published data (Forsythe *et al.*, 1999; Chen *et al.*, 2012), we could obtain the relationship between the lysozyme solubility C_s and the NaCl concentration C_{NaCl} with lysozyme and NaCl dissolved in sodium

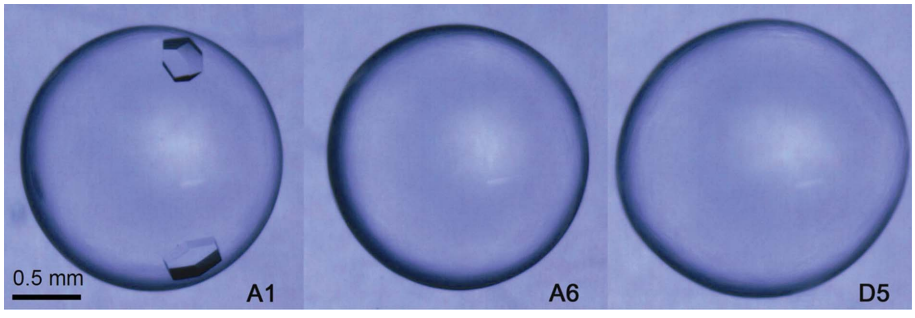

Figure 8
Size difference of crystallization droplets located at different positions. Droplets A1 and A6 are at the edge and D5 is in the centre part of the plate. The size of the droplets in the centre part became larger than those at the edge, indicating that the droplets at the edge can reach higher supersaturation than those in the centre part of the plate.

acetate buffer pH 4.6 at 293 K (NaCl concentration of between 20 and 50 mg ml^{-1}). The relationship can be expressed as

$$C_s = -0.0016C_{NaCl}^3 + 0.2146C_{NaCl}^2 - 9.6437C_{NaCl} + 148.06. \quad (2)$$

When the initial concentrations of lysozyme and NaCl were 40 and 70 mg ml^{-1}, respectively, according to (3) and (4),

$$C = \frac{4}{7}C_{NaCl}, \quad (3)$$

$$\sigma = \frac{C}{C_S}, \quad (4)$$

where C represents the concentration of lysozyme, we can obtain the relationship of the supersaturation σ and C_{NaCl}. Finally, the concentrations of NaCl and lysozyme can be calculated (Fig. 10). From the results, it can be observed that the concentrations of lysozyme and NaCl at the edge were higher than those in the centre part of the plate, indicating that crystallization is indeed easier at the edge.

4.2. Verification of evaporation from droplets using the CDM method

Fig. 11 shows images of water droplets at three points on the plate (A1, A6 and D5) after incubation. The size of the droplets at the edge (droplets A1 and A6) was evidently reduced, while the droplet at the centre (droplets D5) showed little change and the droplet at the corner had almost disappeared. The numerous small droplets near droplet D5 showed that the evaporated water had condensed in the centre part of the plate, indicating that evaporation at the edges was faster than in the centre part. However, when we prepared droplets using NaCl solution and incubated them, the volume difference was not as evident as with pure water.

4.3. Verification of vapour diffusion among the droplets during crystallization screening

During crystallization screening, vapour diffusion from the edge to the centre must have occurred because we observed a higher probability of obtaining crystals near the edges. Apart from this vapour diffusion (from the edges to the centre), another type of vapour diffusion was also noteworthy. Fig. 12 shows several images of droplets found in the screening of proteinase K. It can be observed that some droplets shrank to a smaller size, indicating that the amount of vaporizing solvent was greater than the amount of incoming solvent. On the other hand, some other droplets became larger, showing that more solvent was deposited by vapour diffusion than was escaping. The images provided evidence that vapour diffusion occurred among the droplets depending on their actual vapour pressure, which is mainly determined by the drop composition.

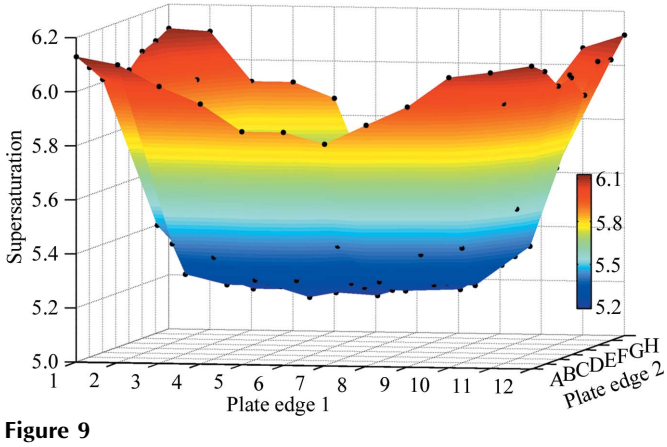

Figure 9
Supersaturation of each crystallization droplet in the crystallization plate.

4.4. Why does the CDM method help to promote protein crystallization?

The experimental results presented above show that the CDM method can help to increase the chance of obtaining protein crystals. To determine why the CDM method helps to promote crystallization, it is necessary to examine the differences between the CDM and conventional vapour-diffusion methods. The following mechanism may occur in the CDM method.

(i) The edge effect. In the reproducibility study, it was clearly observed that crystallization using the CDM method

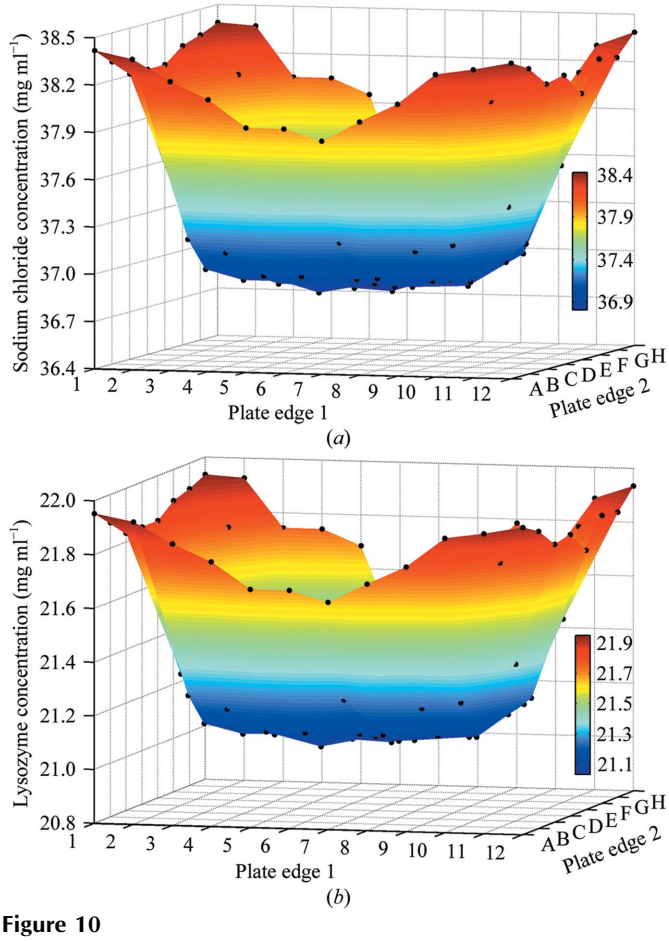

Figure 10
Concentration of (*a*) sodium chloride and (*b*) lysozyme in each crystallization droplet in the crystallization plate.

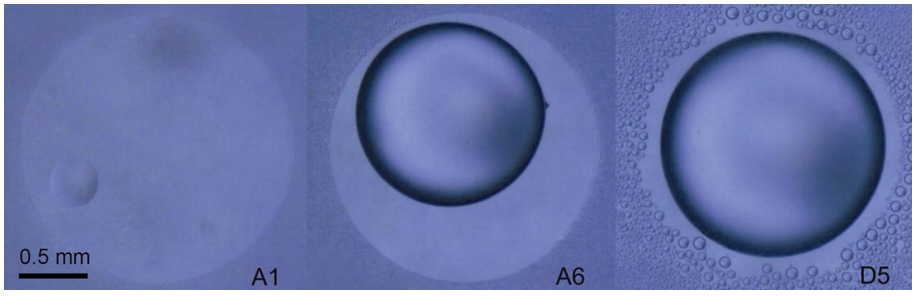

Figure 11
Volume change of the water droplets caused by evaporation upon incubation at 293 K for 2 d. Droplet A1 is at a corner of the plate, droplet A6 is at the centre of the edge and droplet D5 is approximately at the centre of the plate. The size evolution of the droplets from an identical volume to different volumes during the incubation period indicates that the evaporation rate at the edges is faster than in the centre part of the plate.

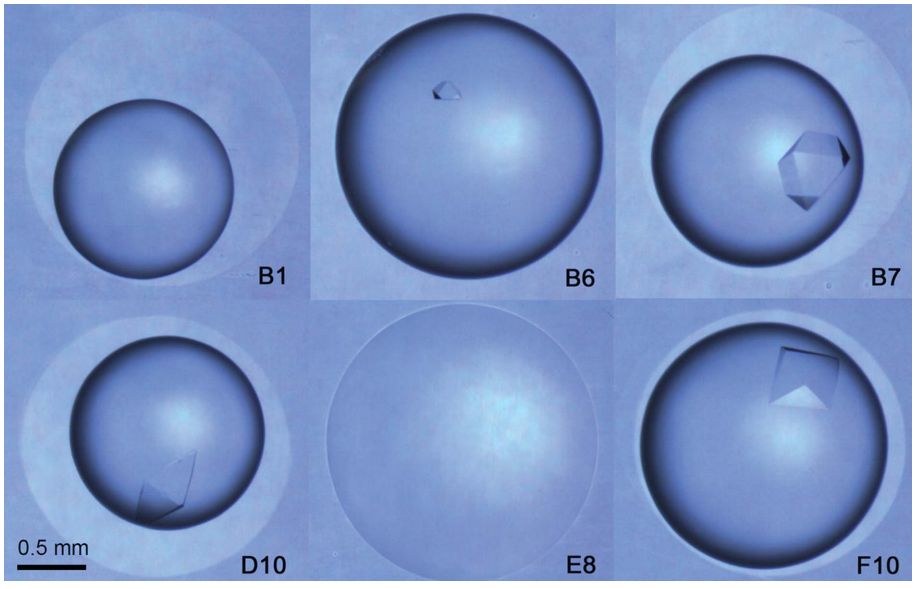

Figure 12
Volume change during crystallization screening of proteinase K. The images were taken 6 d after starting the experiment. The screening kit was Index.

was easier near the plate edge than in the centre part of the plate. The sizes of the droplets near the edges differed from the sizes of the droplets in the centre part of the plate. Lysozyme crystallization was also found to be easier at the edge of the plate. In the screening experiment a similar edge effect was also observed, which caused a higher likelihood of crystallization at the edges, but reduced the chance of obtaining crystals in the centre part of the plate. By rearranging the positions of the droplets and combining the final hits, more crystallization hits were obtained using the CDM method than the conventional method.

(ii) A new vapour-diffusion environment. In the CDM method, all sorts of volatile components (including solvents) from one droplet can diffuse to any other droplets because all of the droplets are within the same sealed space. The number of incoming water molecules will be larger for droplets with a lower vapour pressure than for those with a higher vapour pressure. This determines whether the drops become larger or shrink. Therefore, the concentration of the droplets will increase or decrease during the CDM method depending on the actual vapour pressure, while in the conventional method vapour diffusion only occurs between the droplet and its corresponding reservoir, and the concentration in the droplet only increases before reaching a limited value. This new vapour-diffusion environment provides more variable crystallization conditions with larger ranges of protein and precipitant concentrations, and thus enables more possible crystallization hits.

(iii) Permeable plate. The material of the crystallization plate is PMMA, which is water-permeable and gas-permeable (Yeh *et al.*, 2004). This property enables the droplets in the CDM method to gradually reach a higher supersaturation level than in the conventional method, so that clear drops in the conventional method may yield crystals in the new method.

4.5. Advantages of the CDM method

There are several advantageous features of the CDM method. Firstly, the method is very simple and easy to execute, and the plate geometry is compatible with the prevailing crystallization robots; therefore, there are no obstacles to its application. Secondly, the new method can enable wider ranges of protein and precipitant concentrations and more recipe combination possibilities. Crystallization is therefore easier to achieve with this method and more crystallization conditions can be obtained. Thirdly, there is no need to use a reservoir; only droplets of a mixture of protein solution and precipitants are needed. Fourthly, a very low protein concentration is possible with this method. Fifthly, the crystallization plate is reusable; therefore, the cost of crystallization plates can be greatly reduced. Finally, both sitting-drop and hanging-drop setups can be applied to the CDM method by simply inverting the plate (Luft & DeTitta, 1992) and no modifications to the plate are required.

There is one inconvenience to using the CMD method that needs to be addressed, which is that it may be difficult to reproduce crystallization during the optimization stage. However, a very simple solution to this inconvenience is to use the initial crystals as seeding crystals for subsequent optimizations. Another concern is how to harvest the crystals. In our experience, the solution to this problem is simple. Firstly, we used a syringe needle to slice the tape and open a small

window above the drop and we then harvested the crystal as in any other method. After this, the window can be resealed with a small piece of Crystal Clear tape. The disturbance owing to evaporation through the opened window was greatly reduced compared with opening up the entire plate and exposing all of the droplets to the open air.

5. Conclusions

In conclusion, the present study suggests that the CDM method can increase the number of crystallization screening hits compared with the conventional method. These advantageous features indicate that the CDM method could be suitable for routine protein crystallization.

This work was supported by the National Natural Science Foundation of China (grant Nos. 31170816 and 51201137), the National Basic Research Program of China (973 Program; grant No. 2011CB710905), the Doctorate Foundation of Northwestern Polytechnical University (grant No. CX201327) and the Ministry of Education Fund for Doctoral Students Newcomer Awards, China.

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