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A modified vapor-diffusion crystallization protocol that uses a common dehydrating agent

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In the vapor-diffusion protein-crystallization method, a small drop containing protein sample mixed with a crystallization solution is equilibrated against a reservoir solution in a sealed chamber. Whereas the chemical composition of the crystallization solution is critical for success, the primary role of the reservoir solution is to slowly concentrate the crystallization drop in a controlled fashion. Accordingly, it might be possible to use any reservoir solution of appropriate dehydrating strength. The important practical consequence is that many different experiments can share the same reservoir solution. This approach, called the 'shared reservoir solution' method, significantly simplifies manual and robotic experiment setup, reduces cost and allows a completely new design of optically superior and higher density crystallization plates. Although this research was motivated by these practical advantages, recent reports and the authors' results indicate that this method may actually increase crystallization success. The authors suggest that this may indicate that a protein has a preferred water activity for crystallization. Here, present practical and theoretical considerations as well as experimental tests of the shared reservoir solution method are presented.

1. Introduction

The realisation that protein structural knowledge is necessary to understand the molecular mechanisms of protein function has led to rapid expansion of the field of protein crystallography. In addition, there have been large methodological improvements in all aspects of structure determination (Abola *et al.*, 2000), with structural proteomics consortia and the pharmaceutical industry playing a particularly important role in driving the developments. Unfortunately, protein crystallization has largely remained a trial-and-error process and growing well diffracting protein crystals has become a significant bottleneck. The main response has been to increase the number of crystallization trials, exploring more chemical and physical crystallization parameters and targeting multiple related proteins, such as orthologs and mutants (Hui & Edwards, 2003; Mateja *et al.*, 2002). This high-throughput approach has been made possible by the introduction of crystallization robots and automated imaging systems (Hui & Edwards, 2003; Krupka *et al.*, 2002; Spraggon *et al.*, 2002). To maximize the advantages and minimize the limitations of the new technologies, it is important to re-evaluate our crystallization protocols. We recently proposed the dilution method to improve crystallization results, reduce protein consumption and minimize robotic dispensing problems caused by viscosity and evaporation issues (Dunlop & Hazes, 2003). Here, we

revisit the role of the reservoir solution and show that significant practical benefits can be realised when many different crystallization experiments share a common reservoir solution.

The vapor-diffusion crystallization technique is the most widely used protein-crystallization method. Its goal is to bring about supersaturation of a protein solution in a physicochemical environment that allows well ordered crystals to form (McPherson, 1976). Apart from the conditions at the end point of the experiment, the kinetics of change during the vapor-diffusion experiment also affect the outcome, resulting in a complex multi-dimensional parameter space of crystallization (McPherson, 2004). Different chemical environments are explored by mixing the protein solution with different crystallization solutions, typically in a 1:1 ratio. The crystallization solution normally contains a precipitant, buffer and one or more additives. Each resulting crystallization drop is then equilibrated in a sealed chamber against a larger volume of reservoir solution with a higher precipitant concentration than the crystallization drop. (We use the terms crystallization solution and reservoir solution to refer to the solutions added to the protein sample and the reservoir, respectively. We avoid the term 'mother liquor' because it is used indiscriminately for both solutions). As a result, water vapor will diffuse from the drop to the reservoir solution, leading to a slow concentrating effect on the crystallization drop. The vapor-diffusion process continues until the drop and reservoir solution have the same equilibrium vapor pressure, which equates to them having the same water potential. In cases where water is the only volatile component, any solution with the appropriate water potential can be used as the reservoir solution. However, historically the reservoir solution has been the same as the crystallization solution. Before the introduction of pre-made crystallization screens this was convenient because the reservoir well of the crystallization plate could be used to mix precipitant, buffer and additives prior to addition to the crystallization drop. In addition, this procedure ensures that the water activity of the reservoir solution is lower than that of the drop and therefore that the drop concentrates during equilibration. Finally, in this approach non-aqueous volatiles are present in both the drop and the reservoir, and upon equilibrium the composition in the drop will closely match that of the reservoir.

The current wide availability of pre-made crystallization screens makes it feasible to add the complex screen solutions just to the crystallization drops and use a more convenient solution of the correct dehydrating strength to fill the reservoir. This is not a completely new idea: published references include McPherson, who used it to pre-screen proteins for favored pH and precipitant type (McPherson, 1992), Berger and coworkers, who developed a nucleic acid crystallization screen that equilibrates 24 conditions against a single reservoir solution (Berger *et al.*, 1996; commercialized as the Nucleic Acid Mini Screen by Hampton Research) and Douglas Instruments, who make the 'Douglas Vapor Batch Plate' that could be used for this method (Mortuza *et al.*, 2004). However, replacing the traditional reservoir solution by an alternate reservoir solution of equivalent dehydrating strength affects crystallization in many ways. Most importantly, the assumption that water is the only volatile component is rarely correct. In addition to obvious volatile components such as 2-propanol, other reagents such as MPD, ethylene glycol and even salts are volatile to various extents. Since the shared reservoir solution does not contain any of these reagents, they will be transferred from the drop to the reservoir. Similarly, a difference in pH between drop and reservoir can drive the transfer of acids or bases, especially when more volatile compounds such as acetate, bicarbonate and ammonia are present. Indeed, transfer of ammonia has been used to induce crystallization (Mikol *et al.*, 1989) and transfer of 2-propanol from the reservoir to the crystallization drop was recently used to crystallize a viral capsid protein (Mortuza *et al.*, 2004). The impact of all these effects on the experiment will depend on the rate of transfer relative to the duration of the crystallization experiment. Changing the reservoir solution can also affect equilibrium kinetics (Forsythe *et al.*, 2002). Although this effect may be dominated by evaporation at the drop–air interface, some effect on water absorption at the reservoir–air interface cannot be excluded.

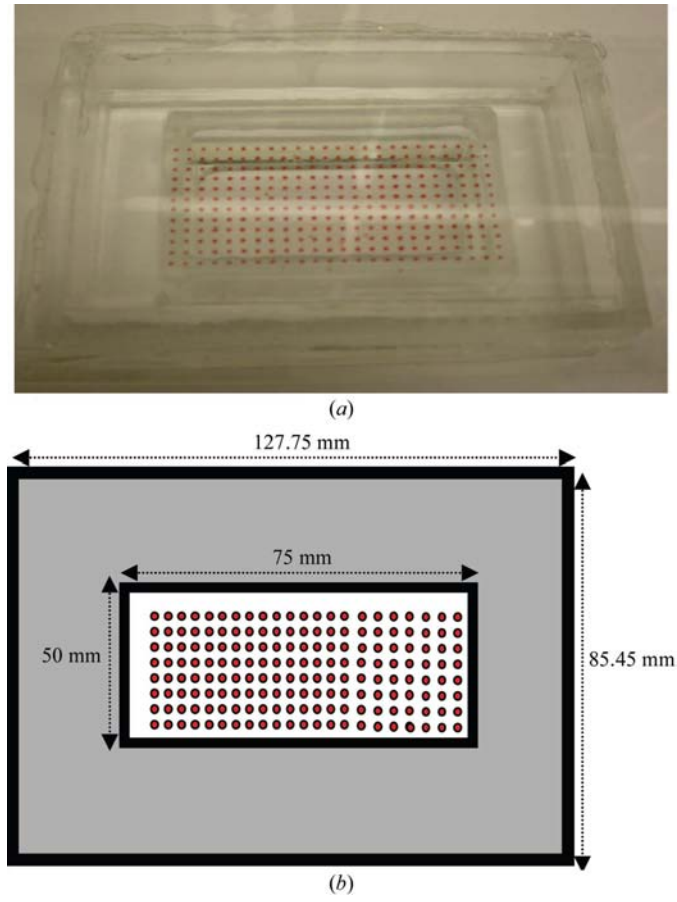

Figure 1
(*a*) Prototype SRS crystallization plate. The 288 200 nl crystallization drops are colored with red food dye for visibility. Drops are spaced on a 3 × 3 mm grid. (*b*) Schematic representation of the top view of the prototype plate. Bold lines represent vertical walls of both the plate and the central pedestal. Solid grey color indicates the plastic bottom and solution in the reservoir. Note that there is no plastic bottom or reservoir solution below the pedestal.

For practical reasons, we are particularly interested in protocols where all experiments share the same reservoir solution, because this allows the design of novel crystallization plates where many drops are equilibrated against one reservoir solution in large open crystallization chambers (Fig. 1). We have named this modified vapor-diffusion crystallization approach the shared reservoir solution (SRS) method. However, because each crystallization solution has a different water activity, the water activity of a shared reservoir solution is no longer directly linked to that of the drop and drops will therefore concentrate or even dilute to different extents. Adoption of the new plate format will also change some basic parameters of the experiment. Although the plates can be designed so that the volume of reservoir solution, drops and air space are proportionally the same as in traditional plates, the distance of drops to the reservoir will be longer, which can affect equilibration kinetics (Fowlis *et al.*, 1988). There is also potential for chemical cross-talk between drops and the presence of a large open air space may itself have subtle effects.

Ultimately, the usefulness of the SRS method depends on the success rate of finding protein-crystallization conditions, combined with the practical benefits for robotic or manual screening. This can only be determined experimentally. One very recent study, evaluating the method on lysozyme, was accepted for publication while we completed our manuscript (Newman, 2005). That study, which used conventional crystallization plates, indicates that the SRS method actually performs significantly better than the traditional protocol. Here, we present a broader test of the method using our novel plates as well as conventional plates. Our results complement and extend the observations by Newman and further indicate that the success rates with our new plates and the conventional plates is equivalent. Furthermore, we discuss the practical consequences for high-throughput protein crystallization in both experiment setup and visualization.

2. Experimental

2.1. Protein preparation

Lysozyme (hen egg-white, Sigma; MW = 14.3 kDa) was dialyzed against 0.1 *M* sodium acetate pH 4.8, whereas glucose isomerase (Hampton Research; MW = 173 kDa) and thaumatin (from *Thaumatoccus daniellii*, Sigma; MW = 22.2 kDa) were dialyzed against water. UV–Vis spectroscopy gave final concentrations for lysozyme, glucose isomerase and thaumatin of 50, 26 and 50 mg ml^{-1}, respectively (using A_{280} values of 1.04, 2.68 and 1.25, respectively).

2.2. Crystallization

All crystallization experiments were set up at 295 K using sitting-drop vapor diffusion in either CrystalQuick conical bottom 96-well plates or using SRS prototype plates (see Fig. 1). The Greiner plates have three sitting-drop wells per reservoir, which were used to set up the experiment in triplicate. The experimental solutions were dispensed with a Honeybee crystallization robot (Genomic Solutions) using 50 µl reservoir solution and crystallization drops made up of 100 nl protein sample and 100 nl crystallization solution. The results of the crystallization experiments were reported based on experiments at day five.

To demonstrate that the SRS method can reproduce crystallization results obtained with traditional methods, we used both methods to crystallize the three test proteins using known crystallization conditions. For lysozyme, the crystallization solution consisted of 8%(*w*/*v*) sodium chloride and 0.1 *M* sodium acetate pH 4.8 (modified from Drenth, 1994). For thaumatin a crystallization solution consisting of 32% sodium tartrate, 5% ethylene glycol and 0.1 *M* HEPES pH 7.2 was used (modified from Rigaku MSC; http://www.rigakumsc.com/protein/crystallization.html). For glucose isomerase two crystallization conditions were used: (1) 25% MPD, 0.2 *M* magnesium chloride, 0.1 *M* Tris pH 7 and (2) 0.8 *M* sodium citrate pH 6.2.

To evaluate the performance of the SRS method in typical crystal screens, we compared it with the traditional method using the PEG/Ion (Hampton Research) and Cryo I (Emerald BioStructures) screens and a locally developed salt/pH grid screen. The grid screen explores six salts, each at four different concentrations (sodium citrate, 0.3–1.35 *M*; sodium malonate, 1.0–3.1 *M*; sodium bromide, 1.5–3.6 *M*; magnesium chloride, 0.5–1.71 *M*; calcium chloride, 0.8–2.61 *M*; lithium chloride, 1–7 *M*) and four buffers (citric acid pH 4.29, MES pH 5.5, Tris pH 6.8, Tris pH 8.3) to make a total of 96 different conditions.

3. Results

3.1. Choice of precipitant and concentration to use as shared reservoir solution

We hypothesized that any precipitant could be used as SRS, as long as it is of the correct dehydrating strength and water is the only volatile component. To test this hypothesis, we compared traditional experiments using known crystallization conditions with the SRS method. The SRS trials included the known crystallization solution in the crystallization drop and one of three common precipitants as the SRS, *viz*. $(NH_4)_2SO_4$, NaCl and PEG 3350. To determine how sensitive the method is to the precipitant concentration of the reservoir solution, a broad range of concentrations were used. The experiments that used $(NH_4)_2SO_4$ as the SRS did not yield any crystals. The results for the other two SRS choices are given in Table 1.

Crystals were obtained for each protein with both NaCl and PEG 3350 as SRS precipitant. In some cases, but not all, crystallization success appears to be relatively independent of precipitant concentration in the reservoir solution. There is no clear trend differentiating the traditional and SRS methods in crystal size or number.

3.2. Effects of non-aqueous volatile components in the crystallization drop

The failure to obtain crystals when using $(NH_4)_2SO_4$ as SRS precipitant most likely results from the fact that NH_4^+ is in

Table 1
Evaluation of NaCl and PEG 3350 as shared reservoir solutions.

Results are listed as the number of crystals per drop/the longest crystal dimension (μm)/the number of drops out of the triplicate that contained crystals.

Precipitant	Lysozyme	Glucose isomerase 1	Glucose isomerase 2	Thaumatin
Control†	20/132/3	1/170/1	3/50/3	3/60/2
0.5 *M* NaCl	0	2/99/2	2/90/2	3/40/2
1 *M* NaCl	30/120/2	2/125/1	1/80/3	12/180/3
1.5 *M* NaCl	30/120/3	0	2/70/3	10/350/3
2 *M* NaCl	1/519 cluster/2	0	2/50/2	200/120/3
3 *M* NaCl	0	0	3/50/2	0
4 *M* NaCl	0	0	4/40/2	10/20/1
5.00%(*w*/*v*) PEG 3350	0	2/60/2	1/100/3	20/5/1
10.00%(*w*/*v*) PEG 3350	0	0	1/100/2	0
15.00%(*w*/*v*) PEG 3350	0	2/85/2	2/75/3	4/10/3
20.00%(*w*/*v*) PEG 3350	0	2/100/3	5/60/3	10/20/3
30.00%(*w*/*v*) PEG 3350	20/40/2	2/100/3	6/110/3	12/80/3

† Control experiments use the same solution for drop and reservoir.

equilibrium with NH_3 and since NH_3 is volatile it can be transferred to the crystallization drop (Mikol *et al.*, 1989). To test if the same is true when $(NH_4)_2SO_4$ is present in the crystallization solution and not in the reservoir solution, we attempted to crystallize glucose isomerase using a third known crystallization condition [19% $(NH_4)_2SO_4$ with 0.1 *M* HEPES pH 7.2; Rigaku MSC; http://www.rigakumsc.com/protein/crystallization.html]. Crystals were obtained using the traditional vapor-diffusion method, but not when NaCl or PEG 3350 were used as SRS (results not shown). We also tried using $(NH_4)_2SO_4$ as SRS for this condition and even though $(NH_4)_2SO_4$ was now present in both the drop and reservoir, no crystals were obtained. This may be a consequence of pH differences, because NH_3 would diffuse towards the solution with the lowest pH, where it is captured by protonation to the non-volatile NH_4^+ state. Our experiments suggest that $(NH_4)_2SO_4$ is not suitable for the SRS method. Further experiments will be needed to test whether this effect is seen for all volatile reagents or only when the reagent also acts as a base or acid.

3.3. Crystal screening

The most interesting application of the SRS method is to use it for crystallization screening, where it has the potential to increase the set-up efficiency of large numbers of experiments. To compare the success rate of the SRS method with the traditional vapor-diffusion method, we screened glucose isomerase, lysozyme and thaumatin with the PEG/Ion (Hampton) and Cryo I (Emerald BioSystems) screens and a home-made salt/pH grid screen. Because all PEG/Ion screen conditions contain 20% PEG 3350 plus 200 m*M* of various salts as additive, we used 20% PEG 3350 with 200 m*M* NaCl as the SRS. This ensures that the dehydrating strength of the SRS is very similar to the individual crystallization solutions of the screen. The Cryo I screen uses a great variety of precipitant types and concentrations and therefore no single reservoir solution can match their dehydrating strength. In addition, 66% of conditions contain a potentially volatile component that would be absent in the SRS. Nevertheless, we again used 20% PEG 3350 plus 200 m*M* NaCl (SRS A in Table 2), as well as a simpler SRS containing just 20% PEG 3350 (SRS B in Table 2). As a final test, we screened with a home-made salt grid screen using 1.25 *M* NaCl as the SRS. 1.25 *M* NaCl was chosen because results from Table 1 and data not shown suggested that it had wide applicability. The results of these experiments are presented in Table 2.

The traditional and SRS methods have very similar success rates when using the PEG/Ion screen. The small differences do not appear to be significant given the observed variation amongst the triplicate experiments. However, despite the similarity in crystallization success, the set of crystallization-drop conditions that gave hits in the traditional and SRS methods are not entirely identical. For lysozyme and glucose isomerase, only 80% of the successful conditions gave crystals with both methods. For thaumatin, this number was just 60%. Thaumatin also shows the largest variation amongst the triplicate experiments, indicating that the lack of correlation between the traditional and SRS methods may be a consequence of the random variations inherent in crystallization experiments. However, even robust conditions that gave crystals in each of the three triplicates using one method did not always give crystals with the other method. For the Cryo I screen, the success rates of the traditional and SRS methods are more variable, as might be expected given the broad range of precipitant strengths and volatile components. For lysozyme and thaumatin, there is a considerable difference when using 20% PEG 3350 plus 200 m*M* NaCl (SRS A) or just 20% PEG 3350 (SRS B) as reservoir solution. This result is not surprising given that a 130 m*M* concentration of NaCl approximates to a 20%(*w*/*v*) concentration of PEG (Arakali *et al.*, 1995) indicating that the 200 m*M* NaCl has a significant effect on the dehydrating strength. Interestingly, for both lysozyme and thaumatin one SRS choice outperforms the traditional method. The success rates for glucose isomerase do not differ significantly for the two SRS choices and both are somewhat below the rate for the traditional method. We were interested to determine whether conditions with volatile components in the Cryo I screen have a reduced relative success rate in the SRS mode. We found that for lysozyme, glucose isomerase and thaumatin the percentage of successful crystallization conditions that contained a volatile component was 66, 100 and 63%, respectively. Since 66% of conditions in the Cryo I screen contain a volatile component, there does not appear to be a bias against these conditions when using the SRS method. Finally, for the salt/pH grid screen our choice of 1.25 *M* NaCl as SRS outperformed the traditional method for all three proteins.

Table 2
Comparison of the SRS and traditional methods in crystal screens.

The first three numbers indicate the number of conditions that gave crystals in 1, 2 or 3 drops of the triplicate experiments, respectively. The last number (in bold) is the sum total.

	Lysozyme	Glucose isomerase	Thaumatin
PEG/Ion SRS	3/2/20/**25**	4/0/10/**14**	7/6/12/**25**
PEG/Ion traditional	5/2/19/**26**	2/4/12/**18**	4/8/12/**24**
PEG/Ion overlap†	20	11	15
Cryo I SRS A	0/0/9/**9**	0/1/3/**4**	0/0/8/**8**
Cryo I SRS B	0/1/2/**3**	0/0/5/**5**	1/1/22/**24**
Cryo I traditional	0/0/6/**6**	1/0/7/**8**	3/2/7/**12**
Cryo I overlap†‡	4	3	6
Salt grid SRS	1/0/79/**80**	4/0/6/**10**	7/0/20/**27**
Salt grid traditional	1/4/52/**57**	0/1/6/**7**	1/0/19/**20**
Salt grid overlap†	56	1	16

† Number of conditions where both methods gave crystals. ‡ The SRS used in this calculation was SRS A.

Table 3
Comparison of the SRS and traditional plates using crystal screens.

The first two numbers indicate the number of crystal hits in the PEG/Ion screen and the Cryo I screen, respectively, and the last number (in bold) is the sum total.

	Lysozyme	Glucose isomerase	Thaumatin
Traditional plate	29/8/**37**	19/4/**23**	44/10/**54**
SRS plate	32/7/**39**	23/5/**28**	40/15/**55**

3.4. SRS crystallization plate

The traditional vapor-diffusion method uses identical crystallization and reservoir solutions. This requires that each experiment be set up in individual sealed chambers. The SRS method makes it possible to use a single reservoir solution for many different crystallization experiments. We have exploited this feature in a novel plate design that makes it possible to incubate many crystallization solutions in a single open chamber. We have named this open chamber plate type the SRS plate. To test the practicality and crystallization behavior in the SRS plate, we have constructed a prototype (Fig. 1).

Our prototype open plate has a single chamber in a standard SBS format. The reservoir is filled with a volumetric pipette and the sitting-drop platform is made from a single piece of glass with dimensions of 75 × 50 mm. These dimensions are slightly larger than the 36 × 72 mm taken up by eight rows and four columns of a standard 96-well plate. To close the plate, we use a lid that is also made of glass. The seal between both glass plates and the plastic plate is made with vacuum grease. In our experiments, we have dispensed 288 drops of 200 nl each using 3 mm spacing between drops (see Fig. 1). To compare the success rate of SRS experiments in this plate against a traditional plate, we screened glucose isomerase, lysozyme and thaumatin with the PEG/Ion (Hampton Research) and the Cryo I (Emerald Biostructures) screens. The plates compared were the Greiner CrystalQuick conical bottom 96-well plate and prototype SRS plates. We used 20% PEG 3350 with 200 m*M* NaCl as the SRS in all experiments. Triplicate experiments were carried out in each well of the CrystalQuick plate. 96 triplicate experiments were set up in each SRS plate. The results of these experiments are presented in Table 3.

The screening success rates for the SRS and traditional plates are very similar and the differences do not appear to be significant given the observed variation amongst the triplicate experiments.

3.5. Optical quality of SRS plates

One of our motivations to make the SRS plates was to obtain the best possible imaging of crystallization drops. Design goals were a completely homogeneous background, no crystallization drop well that can distort drop shape and position and no birefringent material in the optical path. We have accomplished this by using glass for both the sitting-drop surface and the lid. Fig. 2 shows images of drops using the CrystalQuick and SRS plates with and without polarization.

The images in Fig. 2 show several common crystallization-drop and crystal-visualization problems caused by current crystallization plates of various brands. Concave wells have a lens effect and drops often cling to the sides (Fig. 2*d*), obscuring potential crystals from being imaged. Flat-bottom wells give a better drop shape (Fig. 2*a*), but drops with larger volume or lower surface tension often touch and draw up the well edge (not shown). Most common plates use plastics that are birefringent and give strong colored background in polarized light (Fig. 2*a*). In addition, production methods frequently create visible lines in the plastic of the plate (Fig. 2*a*) that would give a false positive during automatic classification using edge detection (Spraggon *et al.*, 2002). On SRS plates, drops cannot creep up the crystallization drop well edges because the crystallization drops are dispensed onto a large flat surface. In addition, both large and small drops can be accommodated by simply selecting appropriate drop spacing, something that is not possible in conventional plates. Drop shape can be further adjusted by using either plain glass or siliconized glass for the drop platform. The use of glass gives optical qualities that are highly favorable. There are no lines or other visual artefacts arising from the moulding process of plastics and unlike most plastics the use of glass allows us to use cross-polarizers (Figs. 2*b* and 2*c*). Finally, this type of plate can be recycled by cleaning or replacing the glass slide that forms the sitting-drop platform.

4. Discussion

4.1. Choice of SRS precipitant

The main requirements of the SRS method reservoir solution are that it has appropriate dehydrating strength and does not contain volatile components. In our experiments, an

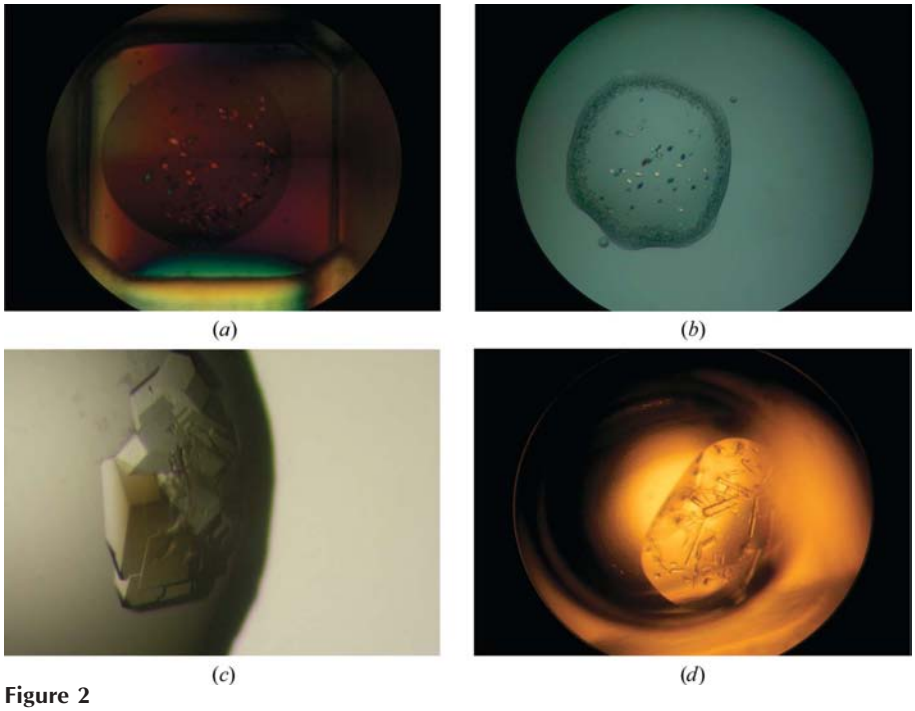

Figure 2
(*a*) Polarized light image of thaumatin crystals in a flat bottom CrystalQuick plate. (*b*) Polarized light image of thaumatin crystals in a SRS plate at low magnification. (*c*) Polarized light image of glucose isomerase crystals in a SRS plate at high magnification. (*d*) Unpolarized image of a crystallization drop in a conical bottom CrystalQuick plate.

ammonium sulfate reservoir solution failed to give crystals for all three test proteins. This is most likely to be because of the ammonium cation, which becomes volatile when it is deprotonated to ammonia. Ammonium sulfate has been used successfully as an SRS (Newman, 2005), but the success rate was lower than with other SRS precipitants. In general, we believe that ammonium sulfate is not a good SRS, although it may actually be beneficial in isolated cases where, for instance, pH change facilitates crystallization (Mikol *et al.*, 1989; McPherson, 1992). PEG 3350 and NaCl were both successful SRS precipitants in our experiments and other non-volatile precipitants would most likely also be viable choices. We prefer the use of NaCl since it is a common, safe and inexpensive reagent that gives non-viscous solutions.

4.2. Effect of volatile reagents in the crystallization drop

Many crystallization experiments use reagents that are highly or moderately volatile. For instance, 66% of the conditions in the Cryo I screen contain components such as 2-methyl-2,4-pentanediol (MPD), ethyleneglycol, ethanol or 2-propanol. Because the SRS lacks the volatile reagent, transfer from the drop to the reservoir should occur. Nevertheless, the traditional and SRS methods gave equivalent success rates for crystallization with the Cryo I screen (Table 2). In a similar study using lysozyme and Crystal Screen HT (Hampton Research), which also contains many conditions with volatile components, the SRS method actually outperformed the traditional method (Newman, 2005). Apparently, the presence of volatile components in crystallization screens is not a problem for the SRS method. Several explanations, or most likely a combination thereof, probably play a role in the outcome: (i) the amount of the volatile component that is transferred to the reservoir within the duration of the experiment may be too small to affect the results, (ii) the concentration of the volatile component is not always an important parameter or (iii) the transfer of the volatile reagent may have affected some experiments in a positive and others in a negative fashion, without affecting the overall success rate. As discussed above, conditions containing ammonium salts may affect crystallization success, but it remains to be determined how general this effect is and whether it occurs mostly for reagents that can act as an acid or base. Importantly, our results and those reported by Newman (2005) suggest that the presence of volatile components does not appear to reduce the success rate in commonly used crystallization screens, which is the most important criterion for crystallization screening. As we gain more experience with the SRS method, we may need to replace some of the volatile reagents in our screens or separate out those conditions into a screen to be set up by the traditional method.

4.3. Choice of reservoir solution dehydrating strength

In the traditional method, the mixing of protein sample with reservoir solution assures that the crystallization drop always has a lower dehydrating strength than the reservoir. In the SRS method, this relationship between the dehydrating strength of the drop and reservoir solution has been uncoupled. This raises the issue of how to select an SRS with optimal dehydrating strength. For the PEG/Ion screen, the dehydrating strength of all conditions is rather similar since they all contain 20% PEG 3350 plus 200 m*M* of a salt. Our SRS choice of 20% PEG 3350 plus 200 m*M* NaCl should therefore closely match the dehydrating strength of each condition. This is reflected in the similar success rates for the SRS and traditional methods. In most other screens the dehydrating strength varies greatly between the different conditions. We have quantified this by measuring the water activity for many conditions of the Cryo I screen (results not shown). This information could be used to group conditions into clusters of comparable water activity that can then be equilibrated together against an SRS with comparable dehydrating strength. However, our results with the three test proteins indicate that it may not be desirable to match the water activity of the SRS to that of the crystallization solution. Indeed, for both lysozyme and thaumatin we found an SRS

choice that performed better than the traditional method for the PEG/Ion and Cryo I screens. The same was true for our salt/pH grid screen. In a recent study with lysozyme, the benefit of the SRS method over the traditional method was even more pronounced, perhaps because a range of reservoir-solution concentrations were tested for optimal crystallization success with a specific screen (Newman, 2005).

Our results and the work by Newman suggest that the water activity of the reservoir solution is an important parameter for crystallization success. In other words, there may be an optimal water activity for protein crystallization and the optimal value is likely to depend on the target protein and its concentration. This provides a rationale for the observation that the SRS method can outperform the traditional screen, because in the latter many conditions will have a water activity far from the optimal value, whereas in the SRS method all conditions will reach the same equilibrium water activity that is dictated by the reservoir solution. It has been previously suggested that there is a window of protein–protein interaction energies that is suitable for protein crystallization (McPherson, 1999). Since water from the protein hydration layer is released upon formation of protein contacts, the interaction energy should be a function of water activity. Although the net interaction energy is the sum total of many different contributions, there may be a window of water activity that is most likely to yield a net interaction energy that is suitable for crystallization. In this case, water activity would be an important predictor of crystallization probability. Unfortunately, the water activity of crystallization solutions has not been considered to be a relevant parameter and is therefore not normally reported. We have started to measure the water activity for commercial crystallization screen solutions as well as commonly used crystallization precipitants. If we can use this information to calculate an approximate water activity for solutions of given composition, then we can use the existing protein-crystallization databases to search for correlations between water activity and crystallization success.

4.4. Practical advantages of SRS plates

The SRS plate described here is a prototype to show that the crystallization success rates of such plates are equivalent to those of our current multi-chambered plates when using the SRS method. The key advantage is that large flat sitting-drop platforms can be created on which crystallization drops can be placed at high density and without the visualization artefacts often encountered on traditional plates. The flat surface also allows the user to accommodate drops of various sizes by selecting the appropriate drop spacing. Although we believe that a glass sitting-drop substrate has superior optical and drop-shape properties, plates made entirely from plastic would still share many of the benefits and may be more practical in some situations. The design of reservoir wells and crystallization platforms is obviously open to many variations. For our Honeybee crystallization robot we are considering a plate with three chambers. In this design, standard microscope object slides can be used as the sitting-drop platform. Depending on the drop radius, we expect to be able to place between 96 and 216 drops per chamber (using 4.5 and 3 mm spacing, respectively). However, this design is not compatible with crystallization robots that require a symmetric 96-well plate layout, for example the Hydra96+1 (Robins Scientific) and Hummingbird (Genomic Solutions) robots. For these robots, plates with eight or 12 chambers can be made by fusing all wells along each row or column, respectively.

Like other plates that are optimized for high-density crystallization screening, our plates are not ideal for harvesting crystals, because during harvesting all drops in the chamber are disturbed. We do not consider this a serious disadvantage, as finding promising conditions is the principle goal of crystal screening. These lead conditions will be optimized using more traditional techniques that allow convenient harvesting.

5. Conclusions

A shared reservoir solution has been used to a limited extent in the past, but without serious evaluation of its potential. In particular, the practical consequences of using a shared reservoir solution in our current high-throughput environment had not been explored. Our comparisons of the traditional and shared reservoir solution methods indicate that the latter performs well and is, if the appropriate precipitant strength is used, perhaps even superior to the traditional method. This finding agrees with even more striking results for lysozyme using Hampton Research Crystal Screen HT (Newman, 2005). An important and unanticipated outcome is thus that the water activity of the reservoir solution may be an important global crystallization parameter that deserves greater attention in future studies.

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