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Expanding screening space through the use of alternative reservoirs in vapor-diffusion experiments

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Setting up vapor-diffusion crystallization experiments against four different reservoir solutions showed that the reservoir solution may have a profound effect on the outcome of a crystallization experiment. This suggests that a facile way to increase crystallization space through screening is not to add more crystallization conditions to the process, but to set up the same conditions over different reservoirs.

1. Introduction

Macromolecular crystallization can be considered a two-part process: screening crystallization space for initial hits and refining those hits to produce well diffracting crystals. Crystallization screening is often performed by setting up a subset of the commercially available crystallization screens, looking for promising results and moving into optimization cycles if something noteworthy is found (*e.g.* Page *et al.*, 2003). There is no consensus on the appropriate amount to screen; although a number of about 300 conditions has been suggested (Segelke, 2001), many laboratories simply screen with everything that they happen to have in-house. There are reports in the literature that different experimental setups can give different results in both the number and the type of hits from the same screening conditions (see Hansen *et al.*, 2002). Two of the most common crystallization methods are vapor diffusion and microbatch (Unge, 1999). Although microbatch experiments have a loyal following of adherents (D'Arcy *et al.*, 2003), most of the macromolecular crystallization performed today is some variant of vapor diffusion. A cursory glance through the crystallization papers formerly published in this journal (now published in *Acta Crystallographica Section F*) suggests that the most common screening protocol is to set up Crystal Screen and Crystal Screen II (Hampton Research) using Linbro-style crystallization plates with reservoirs consisting of the screen condition and crystallization drops of 3 µl protein plus 3 µl reservoir dispensed onto siliconized cover slips. Of course, crystallization papers tend to reflect what was the norm a couple of years ago. It is likely that many laboratories have embraced some of the newer protocols: using more modern screens, 96-well sitting-drop crystallization plates and considerably smaller droplets. Either way, the popularity of vapor diffusion is undeniable. One of the strengths of vapor diffusion as a technique is that a path is tracked through crystallization space as the droplet equilibrates with the reservoir, increasing the amount of crystallization space sampled in a single experiment. Depending on the relative ionic strength of the sample and the crystallization reservoir, the droplet can either grow or more often shrink with time until equilibrium is reached between the two liquids. Nucleation and crystal growth can occur anywhere along this time-path and the rate at which drops equilibrate will depend to a large extent on the reservoir: crystallization conditions containing predominantly salts equilibrate faster than those consisting of high percentages of polyethylene glycols (Luft & DeTitta, 1995). Altering the ratio of protein solution to reservoir solution in the droplet will also affect the rate at which the droplet equilibrates with the reservoir, as well as changing the end point of the equilibration.

Crystallization experiments are notoriously tedious to set up, as the experimental plates have to be labeled, crystallization solutions have to be transferred from tubes into individual reservoirs, the drops have

Table 1
Comparison of base costs of 96 vapor-diffusion screening experiments, excluding labor, protein and the cost of any equipment used to set up the experiments.

The conditions are assumed to be Crystal Screens I and II (Hampton Research) and the prices are taken from the Hampton Research 2005 catalogue. It is assumed that the screens are purchased in tubes for the hanging-drop experiments and in 96-well blocks for the sitting-drop experiments. These prices are intended to serve as a comparison only, but it can be seen that the cost of the crystallization chemicals is a substantial part of a screening experiment.

Hanging drops	4 × pregreased VDX plates	$13.92
	96 siliconized cover slips	$29.60
	0.5 ml (5%) of Crystal Screen and CSII	$24.40
	Total fixed cost	$67.92
Sitting drops	1 × 96-well sitting-drop plate	$13.00
	ClearView seal	$1.58
	0.1 ml (10%) of Crystal Screen HT	$13.30
	Total fixed cost	$27.88

to be formed and placed and the experiments sealed. Furthermore, these plates have to be examined many times afterwards to track the course of the experiment. Sophisticated automation has been developed to simplify this process, largely in response to the structural genomics initiatives in the public and private sectors. Some of this technology has trickled down into academic laboratories; however, much of the specialized automation is still too expensive and too large to be widely adopted. While most of the cost associated with any crystallization experiment lies in protein production, the plastic plates, sealing tape (or cover slips) and crystallization chemicals also add significantly to the bottom line (see also B. Rupp, http://www-structure.llnl.gov/Xray/101index.html). This is shown in Table 1. Any change to the crystallization protocol that simplifies the setup and reduces the cost of an experiment can only positively affect the process of crystallization.

With these points in mind, experiments were set up to probe the effect of swapping the crystallization solution reservoir of a typical crystallization experiment with a 'generic reservoir' solution. This is by no means a novel idea: concentrated solutions of sodium chloride and other salts have been used as general desiccants for decades (Luft *et al.*, 1994; McPherson, 1992). However, the development of high-

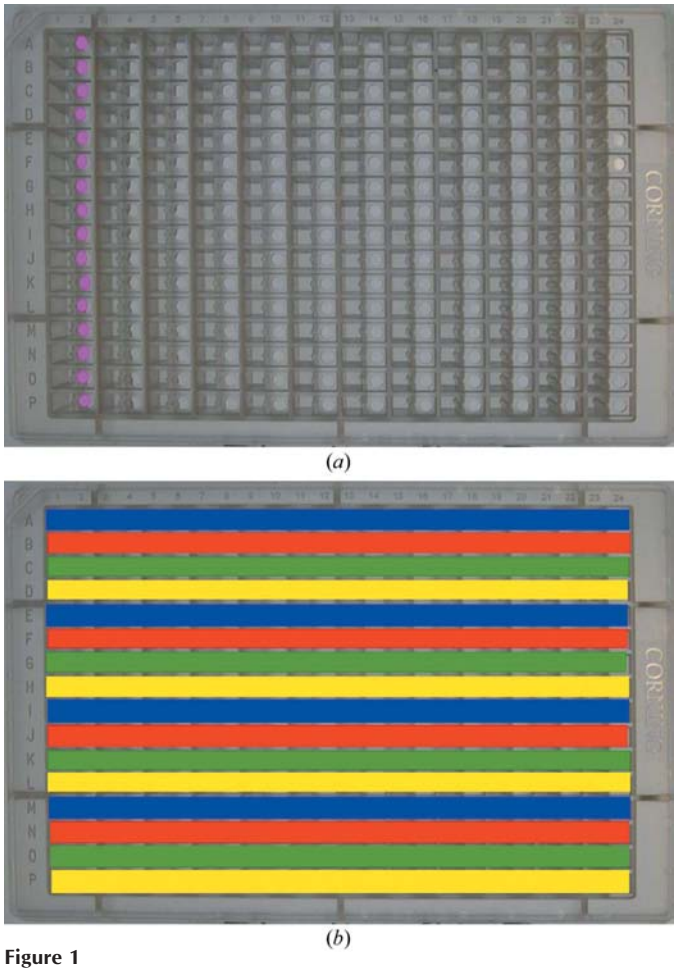

Figure 1
Experimental setup. (*a*) shows how the drops were set up in the crystallization plate; each of the pink spots represents one 200 nl aliquot of the same dirty lysozyme/Hampton condition mixture from the Hamilton syringe. (*b*) shows the organization of reservoir solutions over each of the eight plates in the study. The rows colored blue had reservoir wells filled with 50 µl of the appropriate Hampton condition, the rows colored red had reservoir wells filled with 50 µl 1.5 *M* NaCl, the rows colored green had reservoir wells filled with 50 µl 1 *M* $(NH_4)_2SO_4$ and the rows colored yellow had 50 µl 50% PEG 3350 in the reservoir wells.

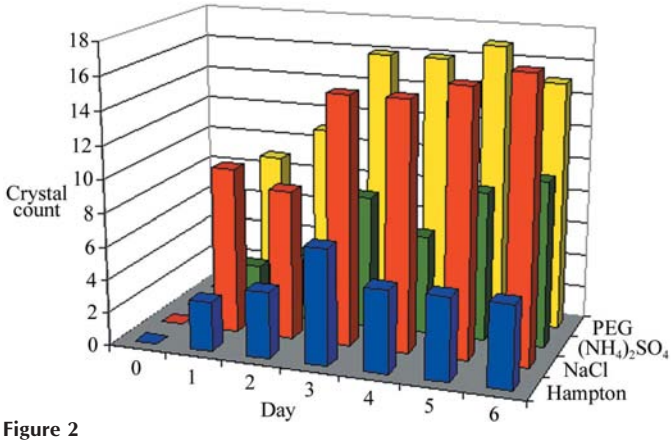

Figure 2
A graphical representation of the time course of the crystallization experiment. The blue bars show the progression of crystal growth for the Hampton reservoirs, the red bars show the crystal count against the 1.5 *M* NaCl reservoir, the green bars show the results of the 1 *M* $(NH_4)_2SO_4$ reservoirs and the yellow bars show the results for the 50% PEG system. Crystal count here means the count of droplets that show crystal growth, rather than the total number of crystals found. It can be seen that the crystal counts do not increase monotonically; this is likely to be a function of the manual scoring of the experiment, rather than the disappearance of crystals during the course of the experiment.

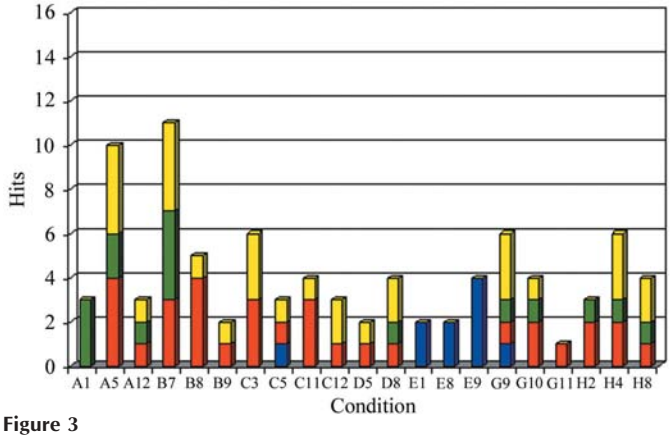

Figure 3
Summary of the experiment on day 10. The color composition of each bar of the histogram shows which reservoirs supported crystal growth for that condition: red is NaCl, blue is the Hampton condition, green is $(NH_4)_2SO_4$ and yellow is PEG 3350. The length of the colored bar shows how many of each reservoir trials showed crystal growth. Only one condition (G9) showed crystal growth when equilibrated against all four reservoirs and no condition yielded crystals in all four trials where crystals were found in one, although conditions A5 and B7 were the most reproducible of the conditions that supported growth.

Table 2
Summary of the reproducibility of crystallization using the results from the 1536 images (96 conditions × four reservoirs × fourfold redundancy) collected on day 10.

The count is the average number of drops containing crystals in the four identical trials if at least one crystal was seen in one of the trials.

Reservoir	Count of redundant crystals	Standard deviation of redundancy
Crystal Screen	2.0	1.2
1.5 *M* NaCl	1.9	1.1
1.0 *M* $(NH_4)_2SO_4$	1.6	1.1
50% PEG 3350	2.0	1.1

density crystallization plates and hardware to image these experimental plates have allowed more detailed probing of this idea. The effect of different reservoirs was tested against a standard protein by setting up the same sample, consisting of a protein solution mixed with a screening solution, against four different reservoirs: three 'generic reservoirs', sodium chloride (NaCl), ammonium sulfate [$(NH_4)_2SO_4$] and polyethylene glycol (PEG 3350), as well as the crystallization condition itself. Each reservoir was tested against the same droplet four times, so as to gauge the reliability of the crystallization process with the different reservoirs.

2. Materials and methods

The protein sample used was 'dirty lysozyme', a mixture of 25 mg ml^{-1} hen egg-white lysozyme (Sigma, L4631) and 5 mg ml^{-1} bovine serum albumin (Sigma, A1470). This mixture has been shown experimentally to behave more like a 'normal' protein in standard screens than pure lysozyme, which does tend to either crystallize or stay in solution (data not shown). Crystallization samples were created by mixing 10 µl dirty lysozyme with 10 µl of each of the 96 crystallization conditions from Crystal Screen HT (CS HT; Hampton Research). A 10 µl positive-displacement syringe with a ratchet device (Hamilton PB600) was used to aspirate approximately 5 µl of a dirty lysozyme/CS HT condition sample and place 200 nl drops of each of the crystallization samples in the sample wells of one column of a 192-well sitting-drop crystallization plate (Corning CrystalEx 384), after which the syringe was rinsed five times with water. As each column was completed, it was covered temporarily with Scotch adhesive tape. After all the drops on a plate had been set up, the temporary tape was removed and the plate sealed with a ClearView Seal (Hampton Research). As each plate consists of 16 rows by 12 columns, eight plates were required to set up all 96 crystallization samples. Furthermore, each column of any plate contained 16 droplets of identical composition. The reservoirs of the experimental plates had previously been filled with 50 µl of the different reservoirs. Rows A, E, I and M contained the appropriate Crystal Screen HT condition, rows B, F, J and N contained 1.5 *M* NaCl, rows C, G, K and O contained 1.0 *M* $(NH_4)_2SO_4$ and the remaining four rows (D, H, L and P) contained 50%(*w*/*v*) polyethylene glycol (PEG) 3350. See Fig. 1.

The three generic reservoir solutions were chosen as they are common, cheap and might be expected to produce different results: the NaCl should act as a simple desiccant, $(NH_4)_2SO_4$ should both desiccate and affect (most often raise) the pH of the droplet through the gaseous NH_3 which is in equilibrium with the NH_4^+ ions in solution and PEG 3350 should desiccate to some extent, although the kinetics should be quite different from the NaCl solution, as PEG solutions alter the water activity within the solution (and thus the water vapor pressure above the solution) to a relatively small degree; Luft & DeTitta, 1995).

Table 3
Summary of the manually assigned scores for the reservoir experiment.

'Clear' describes drops which have nothing in them. 'Precipitate' covers all types of precipitate, including grainy, denatured *etc*. 'Crystalline' applies to drops that contain microcrystals or crystals. 'Other' applies to drops where it is unclear exactly what is in them: fibers, dust *etc*. 'Null' applies to a drop which was either missed or wicked to the side of the sample well and could not be imaged or that was fogged with condensation and the image could not be interpreted. No time course was used as reference during the manual scoring.

Classification of drops	Percentage
Clear	48
Precipitate	37
Crystalline	4
Other	3
Null	7

Not just the composition but also the concentration of the reservoir can influence the result of the crystallization experiment. The concentration of NaCl was chosen by systematically comparing different concentrations of NaCl in the reservoirs (from 0.5 to 3 *M*) against reservoirs consisting of Crystal Screen HT for plates made up of dirty lysozyme/Crystal Screen HT droplets. Visual inspection showed that drops equilibrating against 0.5 *M* salt tended to get larger over time and drops equilibrating against 3 *M* NaCl rapidly dried out almost to completion, but that drops equilibrated against 1.5–2 *M* NaCl looked about the same as those equilibrated against the Crystal Screen reservoirs. The concentrations of the $(NH_4)_2SO_4$ solution and the PEG solution were chosen based on experience and comparison with the NaCl reservoir concentration tests (data not shown). A comparison of equilibration rates of PEG 8000 with sodium chloride and ammonium sulfate (Arakali *et al.*, 1995) suggests that a higher concentration of ammonium sulfate might also be appropriate in the reservoir.

The completed crystallization plates were stored at room temperature and were imaged using an automatic imaging system (RoboMicroScope II, RoboDesign International Inc.) after setup and on days 1, 2, 3, 4, 5 and 10 after setup. Each image was examined manually and assigned one of five scores: clear, precipitate, crystal, other or null.

3. Results

As the dirty lysozyme solution was pre-mixed with each of the 96 conditions from Crystal Screen HT, this enabled the 16 drops of any given protein/condition to be set up from a single aspiration into the Hamilton syringe. This was to ensure that the variation seen between the 16 equivalent protein/condition drops should mostly be the effect of the different reservoirs. It could be that differences between the drops were the result of pipetting variations, either in drop volume or profile; however, given that each drop was produced using a ratchet device attached to a microlitre syringe, volume variations should have been minimal.

Each reservoir was tested in fourfold duplicate, spaced out in the experimental plates to try to avoid edge effects. Ideally, if a crystal grew under one condition, that same crystal form should be observed four times. Only about half the number of expected crystals based on this redundancy in setup were observed and this seemed to be independent of the reservoir (see Table 2).

Overall, the plates were imaged seven times each, so that close to 11 000 images were collected and examined. The overall results for the eight plates are shown in Table 3.

None of the crystallization drops showed any signs of crystals in the images taken directly after setup. There was a rapid increase in the

number of conditions that showed signs of crystal growth until day 5; between days 5 and 10 there was no great increase in the number of crystals seen in the images (Fig. 2). Fig. 2 also shows that the different reservoir solutions produced quite surprising differences in the number of crystals produced. Most surprising was that the drops equilibrated against the crystallization cocktail solution were the least productive in producing crystals. My original expectation was that a crystallant would produce crystals somewhat independent of the reservoir used to dehydrate the droplet and that the reservoir was mostly just a 'water sink' that would allow the drop to shrink with the concomitant concentration of protein and precipitant. Thus, the expected result for the summary graph would have been a number of conditions, each of which showed crystal growth against all four reservoirs. Furthermore, in a system which is not nucleation-limited there should be crystals seen in each of the 16 identical droplets (*i.e.* crystals in each drop of a column of drops, if one drop in the column showed crystal growth). Fig. 3 shows a summary of the conditions and reservoirs under which crystal growth was observed. Only one condition of the 21 that supported crystal growth showed crystal growth against all four reservoirs and there was no condition under which all 16 identical droplets showed crystal growth. The results of the PEG and the NaCl reservoirs were very similar, whereas the $(NH_4)_2SO_4$ reservoir and the Hampton reservoir showed quite different distributions of successful conditions. Although there were only five conditions that supported crystal growth when equilibrated against the Hampton condition, three of the five were unique to the Hampton reservoir, whereas only one of the 17 conditions under which crystal growth was observed when equilibrated against 1.5 *M* NaCl was unique to that reservoir. However, if the overlaps between the NaCl and the PEG reservoirs are excluded, then seven of the 17 NaCl conditions were unique. Only one condition of the ten which were found to support crystal growth when equilibrated against the 1 *M* $(NH_4)_2SO_4$ was unique and all of the conditions that supported crystal growth against the PEG reservoir also gave crystals against the NaCl reservoir.

4. Conclusions

The experiment described above tested the effect of equilibrating crystallization droplets against different generic reservoirs, comparing the results against a standard crystallization experiment where the crystallant portion of the crystallization droplet was the same as the reservoir against which the droplet was equilibrated. From Table 1 it can be seen that the reservoir contributes at least 35% to the fixed cost of a crystallization experiment and that the substitution of a simple salt would shave 30% or more off the fixed cost of each crystallization setup.

The experiment shows that for a test protein the reservoir has an effect on the number of screening conditions that produce results noteworthy enough to push into the optimization stage of crystallization. The 1.5 *M* NaCl reservoir or the 50% PEG reservoir yielded similar results, whereas the 1 *M* $(NH_4)_2SO_4$ reservoir or a reservoir of the crystallant pulled different positive conditions out of the same set of screening conditions. This limited experiment suggests that substituting different reservoirs in the same vapor-diffusion experiment can alter the outcome of the crystallization experiment quite significantly. Furthermore, it was seen with this model protein system that simply duplicating the same experiment a number of times increases the chances of finding a crystal hit: this result is likely to hold true for any system that is reluctant to nucleate. In order to obtain the most hits out of any screening set it would seem that simply setting up duplicate droplets over different reservoirs can profoundly increase the success rate of the screening process.

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