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Acoustic matrix microseeding: improving protein crystal growth with minimal chemical bias

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A crystal seeding technique is introduced that uses acoustic waves to deliver nanolitre volumes of seed suspension into protein drops. The reduction in delivery volume enables enhanced crystal growth in matrix-seeding experiments without concern for bias from chemical components in the seed-carrying buffer suspension. Using this technique, it was found that while buffer components alone without seed can marginally promote crystal growth in some cases, crystal seeding is far more effective in boosting the number of sparse-matrix conditions that yield protein crystals.

1. Introduction

Multiple seeding methods available for the crystallization of macromolecules have been reviewed (Bergfors, 2003). In one of these methods, 'streak-seeding', a long fiber is used to transfer crushed crystals onto protein drops (Stura & Wilson, 1991). More recently, streak-seeding has been employed in the creation of a new method referred to as 'matrix microseeding', in which the crystal seeds are delivered into similar but non-identical conditions for the improvement of diffraction quality and resolution (Ireton & Stoddard, 2004). Matrix microseeding was later adapted for high-throughput automation with the delivery of microcrystals in liquid suspensions, instead of a long fiber, into broadly varied conditions (D'Arcy *et al.*, 2007). The automated method unintentionally introduces a significant systematic chemical bias originating from the chemical components in the buffer carrying the seed. The bias accounts for 17% of the assembled volume in crystallization drops comprised of 100 nl seed suspension, 300 nl protein and 200 nl sparse-matrix solution, for example.

Subsequent work by another group studied the effect of chemical bias in matrix microseeding using five distinct proteins (St John *et al.*, 2008). This study assembled protein drops using the same liquid volumes as those employed by D'Arcy and coworkers. The authors reported identical results with and without seed, implicating a predominant impact from chemical bias rather than beneficial effects of the seed itself. While this would appear to be promising, the addition of significant volumes of the original crystallization chemical components would limit the opportunity for the discovery of truly improved crystal systems.

Here, we present a technique that enables control of chemical bias by reducing the volume of seed additions to protein drops. The technique employs acoustic liquid dispen-

sing to deliver single-digit nanolitre quantities of seed. In this study, we compare crystallization results from drops supplemented with buffer preparations containing seed (seed suspension), drops supplemented with buffer preparations lacking seed (blank control) and drops without supplementation of any sort, which served as background references (background).

2. Materials and methods

2.1. Growing crystals for subsequent seed preparation

Human brain aquaporin 4 (hAQP4) was expressed, purified and crystallized by Robert Stroud's laboratory at the University of California, San Francisco, California, USA. Crystals were obtained by vapor diffusion at 293 K in sitting drops consisting of 0.5 µl 10 mg ml^{-1} protein in 25 m*M* sodium citrate pH 6.0, 50 m*M* sodium chloride, 5% glycerol, 40 m*M* *n*-octyl-*β*-d-glucopyranoside, 2 m*M* DTT, 0.5 µl Silver Bullet A1 (Hampton Research, Aliso Viejo, California, USA) and 1.25 µl well solution with a composition of 25% PEG monomethyl ether (MME) 2000, 5% glycerol, 40 m*M* *n*-octyl-*β*-D-glucopyranoside, 50 m*M* sodium citrate pH 6.0 (Ho *et al.*, 2009).

The catalytic domain of dengue virus RNA-dependent RNA polymerase type 3 (DEN POL) was expressed, purified and crystallized as previously described (Yap *et al.*, 2007). Purified protein was concentrated to 12 mg ml^{-1} in 25 m*M* Tris–HCl pH 6.8, 250 m*M* sodium chloride, 0.1% CHAPS, 2 m*M* *β*-mercaptoethanol, 1 m*M* EDTA. Apoprotein crystals were obtained at 293 K in sitting drops assembled with 200 nl protein solution and 200 nl well solution consisting of 1.1 *M* potassium/sodium tartrate, 0.1 *M* magnesium sulfate, 0.5% PEG 5K MME, 0.1 *M* imidazole pH 7.0.

Human IL2-inducible T-cell kinase (ITK) was expressed and purified as reported in the literature (Brown *et al.*, 2004). Crystals were obtained in-house with protein concentrated to 16 mg ml^{-1} in 20 m*M* Na HEPES pH 7.5, 500 m*M* sodium chloride, 0.5 m*M* EDTA, 2 m*M* DTT. Crystals were grown at 293 K in sitting-drop format with 200 nl protein inhibited with small-molecule inhibitor and 200 nl well solution consisting of 2.5 *M* sodium chloride, 100 m*M* Na HEPES pH 7.5.

Human p38 MAP kinase *α* (p38*α*) was expressed, purified and crystallized as reported previously (Trejo *et al.*, 2003). The protein was concentrated to 15 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 0.2 *M* sodium chloride, 10 m*M* magnesium chloride, 10 m*M* DTT. Apoprotein crystals were obtained at 290 K by vapor diffusion in sitting drops of 400 nl final volume formed with equal volumes of protein solution and well solution containing 17% PEG 3350, 20 m*M* calcium chloride, 50 m*M* Na HEPES pH 7.6.

Human immunodeficiency virus reverse transcriptase (HIV RT) was expressed, purified and crystallized as reported previously (Sweeney *et al.*, 2008). The protein was concentrated to 12 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 50 m*M* sodium chloride, 1 m*M* EDTA, 2 m*M* DTT and was inhibited with 0.6 m*M* Nevirapine just prior to setting up drops. Protein drops of 200 nl in volume were mixed with 200 nl well solution consisting of 1.15 *M* sodium malonate, 5% ethylene glycol, 50 m*M* potassium phosphate pH 7.2. The sitting drops were incubated at 293 K.

Human spleen tyrosine kinase (SYK) was prepared and crystallized as described in the literature (Atwell *et al.*, 2004). SYK was concentrated to 10 mg ml^{-1} in 50 m*M* Na HEPES pH 7.6, 10% glycerol, 150 m*M* sodium chloride, 5 m*M* DTT, 10 m*M* L-methionine. Crystals were obtained in drops assembled with 200 nl protein solution and 200 nl well solution at 283 K. The well solution contained 24% PEG 4000, 200 m*M* ammonium sulfate, 100 m*M* sodium acetate pH 5.0.

2.2. Preparation of seed suspensions and blank controls

Crystal seed suspensions for DEN POL, p38*α*, SYK and HIV RT were made in the corresponding well solutions. The crystals from a total of five protein drops were mechanically smashed with a combination of a microspatula and an acupuncture needle (Hampton Research). The smashed crystals

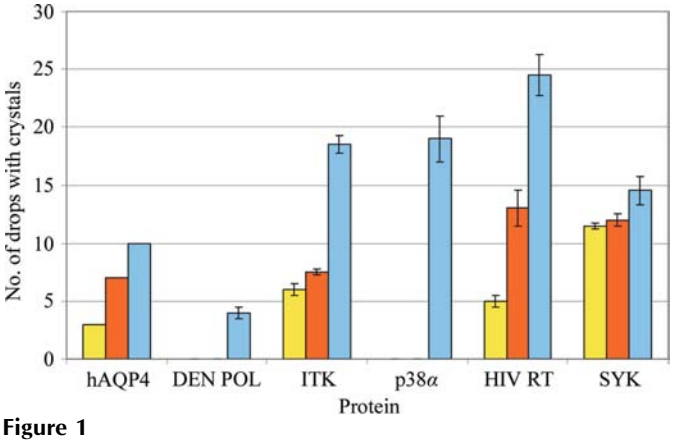

Figure 1
Drops from the Index screen that produced protein crystals 10 ×10 µm in size or larger after 30 days of incubation. With the exception of the protein SYK, drops treated with seed suspension (blue bars) show noticeable enhancement over both background (yellow bars) and blank controls (orange bars). The three treatments were conducted in duplicate, with the exception of those for hAQP4.

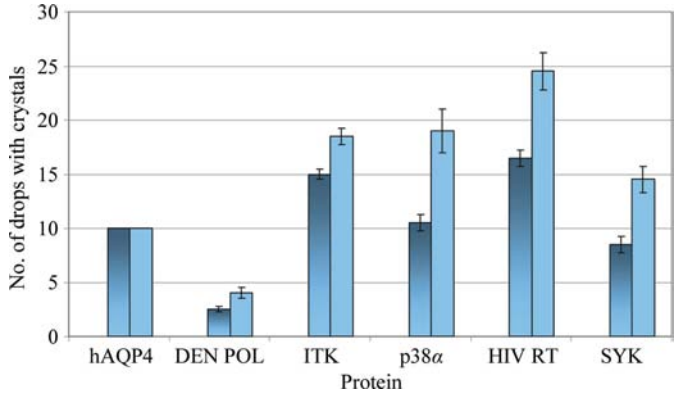

Figure 2
The number of seeded drops (in Index screen) showing protein crystals 10 × 10 µm or larger on days 0, 7 (blue gradient) and 30 (solid blue). The bulk of crystal growth occurred within the first 7 days after seed delivery.

were aspirated and dispensed into a tube containing a final volume of 100 µl well solution and vortexed for 90 s with a Teflon bead available from Hampton Research and previously described in the literature (Luft & DeTitta, 1999). The suspension of hAQP4 crystal seeds was made by vortexing the crystals from one drop in a tube containing a Teflon bead, 50 µl well solution and 20 µl Silver Bullet A1 (Hampton Research). The ITK seed suspension was prepared using a buffer consisting of 1.0 *M* sodium chloride, 1.0 *M* ammonium sulfate, 50 m*M* magnesium sulfate, 50 m*M* sodium acetate pH 5.7. The crystals from five ITK drops were vortexed in 100 µl of this buffer. The seed suspensions for all proteins in this study were partitioned into 10 µl aliquots, flash-frozen in liquid nitrogen and stored at 193 K for subsequent use. Blank controls were made using the same buffers as the seed suspensions but without the addition of crystal seed.

2.3. Automated preparation of crystallization drops

Protein crystallization drops were assembled in sitting-drop vapor-diffusion format in Corning 3785 plates (Corning, New York, USA) with the large reservoirs containing 25 µl of the

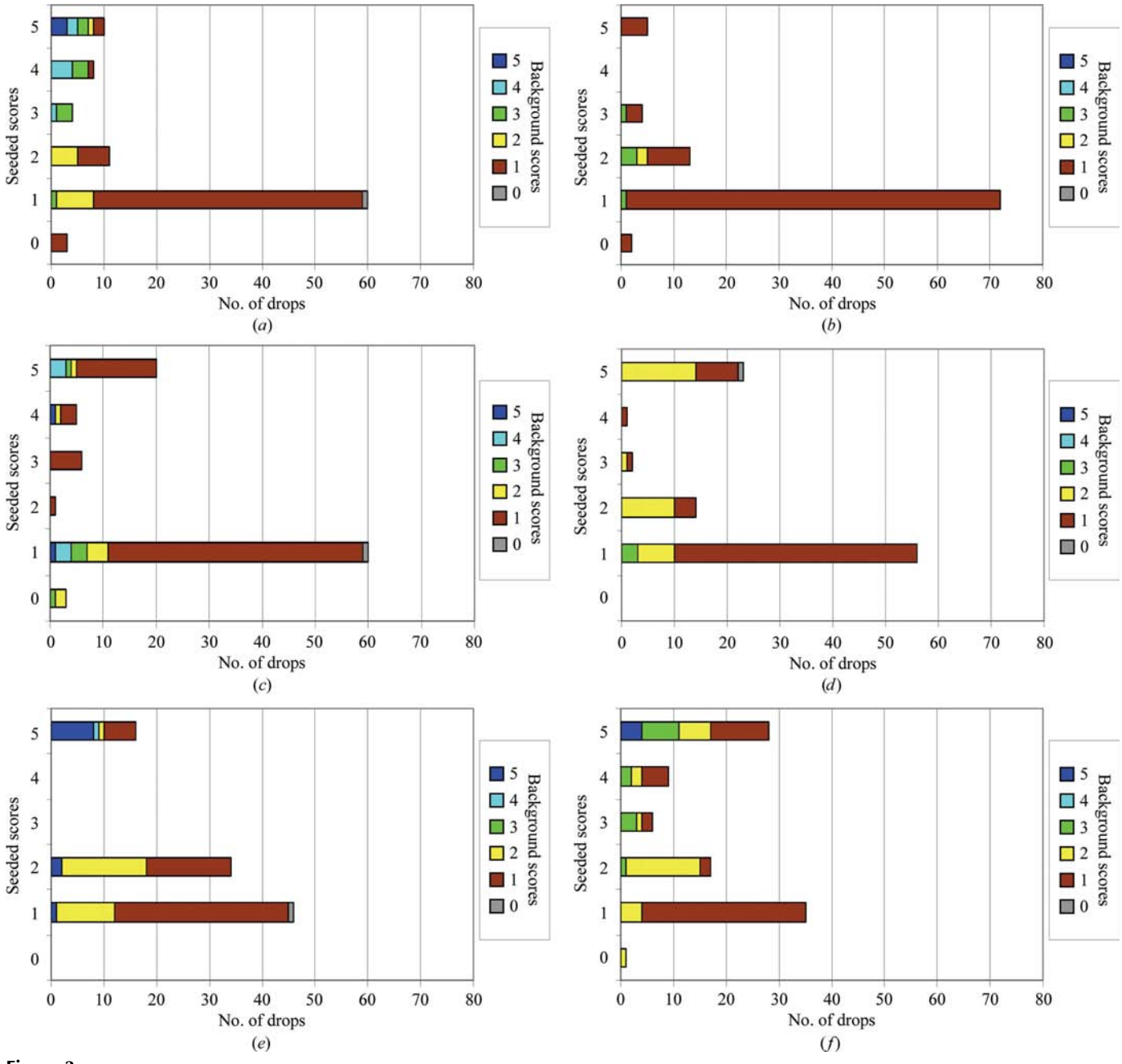

Figure 3
A direct comparison of seeded *versus* background trials for (*a*) hAQP4, (*b*) DEN POL, (*c*) ITK, (*d*) p38α, (*e*) SYK and (*f*) HIV RT. The color-coded scores pertain to empty well (0), non-translucent precipitate (1), clear drop (2), translucent precipitate (3), microcrystals (4) and crystals 10 × 10 µm or larger (5). The scores shown on this figure were collected from one of two plate duplicates of each protein.

Index sparse-matrix screen (Hampton Research). Protein drops consisting of 200 nl protein solution and 200 nl well solution were dispensed by a Crystal Creator liquid dispenser (Cybio-AG, Jena, Germany) integrated with a Seal-IT 100 plate sealer (Abgene, Epsom, England), plate stackers (Cybio-AG) and a double-jointed Kinedx robot (Peak Robotics, Colorado Springs, Colorado, USA). A total of six plates were set up for each protein to accommodate two duplicate plates for addition of seed suspension, two plates for addition of blank control and two plates as background references. For hAPQ4, three plates were prepared without duplicates. All plates were sealed by the SealIT 100 with adhesive crystallography tape (Thermo Scientific, Waltham, Massachusetts, USA) and kept sealed at room temperature only momentarily (30–60 min) while the seed stocks were thawed in preparation for seed delivery onto protein drops.

2.4. Delivery of seeds and blank controls by acoustic liquid transfer

An ATS-100 acoustic liquid dispenser (EDC Biosystems, Milpitas, California, USA) was employed to dispense 15 nl seed suspension or blank control onto pre-dispensed 400 nl protein drops. The acoustic instrument is a true non-contact dispenser that uses a focused burst of ultrasound emitted from a transducer located beneath the liquid sample to eject single-digit nanolitre droplets from the top surface of the sample onto a destination target well plate (Wong & Diamond, 2009). In this study, we placed 10 µl sample (seed suspension or blank control) in a single source well in an Aurora 384 IQ-LV plate (Aurora Biotechnologies, Carlsbad, California, USA) seconds before the start of acoustic liquid handling to keep seed sedimentation to a minimum. Three 5 nl droplets were then ejected upward from the source well through a 20 mm vertical trajectory to make contact with the target, a 400 nl protein drop hanging from a crystallization plate turned upside down. The process of delivering 15 nl onto each of 96 protein drops on a plate took 90 s, after which the plate was inverted back to upright orientation and immediately sealed with ClearSeal Film (Hampton Research). All completed plates were incubated at 293 K, except for the ITK plates which were incubated at 278 K.

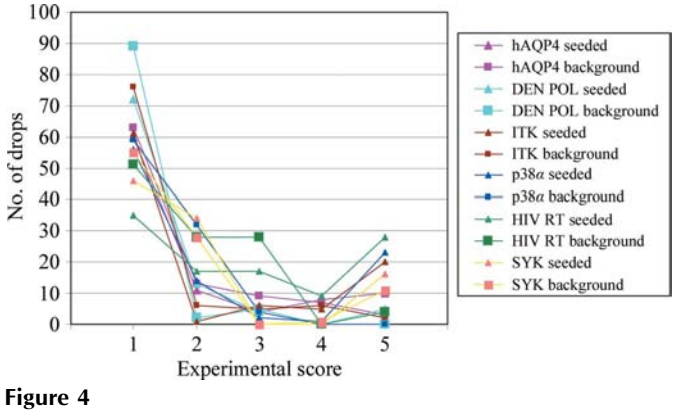

Figure 4
Proteins showing fewer drops with non-translucent precipitate (score 1) in the background experiments delivered a greater number of drops with crystals (score 5) in the seeded trials. The numeric scores correspond to non-translucent precipitate such as heavy, moderate and light precipitate (1), clear drop (2), translucent precipitate (3), microcrystals (4) and crystals of 10 × 10 µm or larger (5). The scores shown here were collected from one of two plate duplicates of each protein.

2.5. Experimental observation

All protein drops were digitally imaged in the Crystal Farm (Nexus Biosystems, Poway, California, USA) on days 0, 1, 2, 7, 15, 21 and 30 after setup. In order to capture the end result of crystal seeding, the final images from day 30 were scored numerically on observation of the following outcomes: empty protein well (0), non-translucent precipitate ranging in intensity from light to heavy (1), clear drop (2), translucent/shiny precipitate (3), microcrystals (4) and crystals of mountable size: 10 × 10 µm or larger (5). A numeric score was not assigned for phase separation because this event can occur in outcomes 1–5.

3. Results

Fig. 1 shows the average number of drops containing crystals of 10 × 10 µm or larger after 30 days of incubation. As shown in the figure, DEN POL background and blank-control trials did not exhibit crystal formation in any of the 96 Index screen conditions. However, trials supplemented with seed suspension resulted in an average of four drops containing crystals of mountable size (*i.e.* 10 × 10 µm or larger, corresponding to a score of 5). Similarly, the p38α background and blank-control trials resulted in zero drops containing crystals. The seeded trials, on the other hand, delivered an average of 19 out of 96 drops containing crystals of mountable size. The hAQP4 screens delivered three, seven and ten drops containing mountable crystals from the background, blank-control and seed-suspension trials, respectively. ITK experiments resulted in six, seven and 18 drops containing crystals from the background, blank-control and seed-suspension trials, respectively. The HIV RT trials were the only case in this study in which the blank control delivered a noticeable boost over the background; it resulted in 13 drops containing crystals, while the background delivered only five. Nevertheless, the seeded HIV RT drops provided the greatest enhancement, with an average of 24 drops containing crystals of mountable size. The SYK trials presented a unique case in which all three treatments delivered essentially equivalent results, with 11, 12 and 14 drops containing crystals from the background, blank-control and seeded trials, respectively.

The crystallization results mentioned above (Fig. 1) essentially show that chemical bias was not a key driver of crystallization in this study. Namely, the blank control did not outperform both the background and seeded trials in terms of crystal production. Furthermore, the seeded trials delivered more drops containing crystals than the background and blank controls for five of the six proteins, thereby validating the seed preparations employed. Therefore, the results from blank-control trials are no longer of value from this point forward in this communication. Instead, we have chosen to compare

and contrast results from seeded drops with those from the background-control trials.

A time-course illustration of the seeded drops (Fig. 2) shows that the bulk of crystal growth occurred during the first week after setup. Images collected on day 0 after seed delivery show an absence of crystals in all trials. Subsequent images show the greatest number of drops containing crystals was observed on day 30, while more than half of the final total was achieved by day 7.

In order to follow crystallization trends, a direct drop-to-drop comparison was conducted between seeded drops and their background counterparts. The comparison, summarized in Fig. 3, shows that crystal growth in seeded trials occurred across the spectrum of possible results represented by scores 1 to 5. Fig. 3(*a*) shows a total of ten seeded hAPQ4 drops yielded crystals of mountable size (score of 5), while the same drops in the background trials produced results that included non-translucent precipitate (score 1), clear drops (score 2), translucent precipitate (score 3) and microcrystals (score 4). Fig. 3(*b*) shows DEN POL seeded trials produced five drops with mountable crystals, while the same drops in the background set yielded a score of 1. The ITK results (Fig. 3*c*) show a total of 20 drops with mountable crystals. The majority of the 20 background counterparts produced non-translucent precipitate, with a small set of drops showing soluble protein, translucent precipitate and microcrystals.

23 seeded drops of p38α produced mountable-sized crystals (score 5) as shown in Fig. 3(*d*). Over half of the corresponding background trials exhibited clear drops and less than half exhibited non-translucent precipitate. Fig. 3(*e*) shows results from the SYK experiments, in which 17 seeded drops produced crystals of mountable size. The matching background drops, on the other hand, delivered two main results, namely non-translucent precipitate (score 1) and mountable protein crystals (5). Fig. 3(*f*) shows that HIV RT seeded trials delivered 28 drops with a score of 5, while the corresponding background drops returned four results; namely, non-translucent precipitates, clear drops and translucent precipitate, with only a small number of drops showing mountable crystals.

The compiled results from all proteins (Fig. 4) illustrate a trend in which proteins with a smaller number of drops showing non-translucent precipitate in the background experiments yield a greater number of drops containing protein crystals in the seeding experiments. DEN POL had the greatest number of drops (90) in background trials containing non-translucent precipitate (top left of the figure), while the seeded trials yielded only five drops with a score of 5 (bottom right of the figure). At the other extreme, HIV RT exhibited the least number of drops in background trials with non-translucent precipitate (51), while the seeded HIV RT experiments produced the greatest number of drops (28) with mountable crystals. The other proteins followed the same trend, with drop numbers between the two mentioned extremes.

A set of examples in which seeding delivered improved results are shown in Figs. 5 and 6. The seeded hAQP4 drop (Fig. 5*c*) shows the growth of single individual crystals, while the drop treated with blank suspension (Fig. 5*b*) delivered a cluster of needle crystals and the background drop (Fig. 5*a*) did not produce crystals. DEN POL, ITK, p38α and HIV RT experienced crystal growth in seeded drops (Figs. 5*f*, 5*i*, 6*c* and 6*f*, respectively) but not in the corresponding background and blank-control treatments (see the treatment-specific images in Figs. 5 and 6).

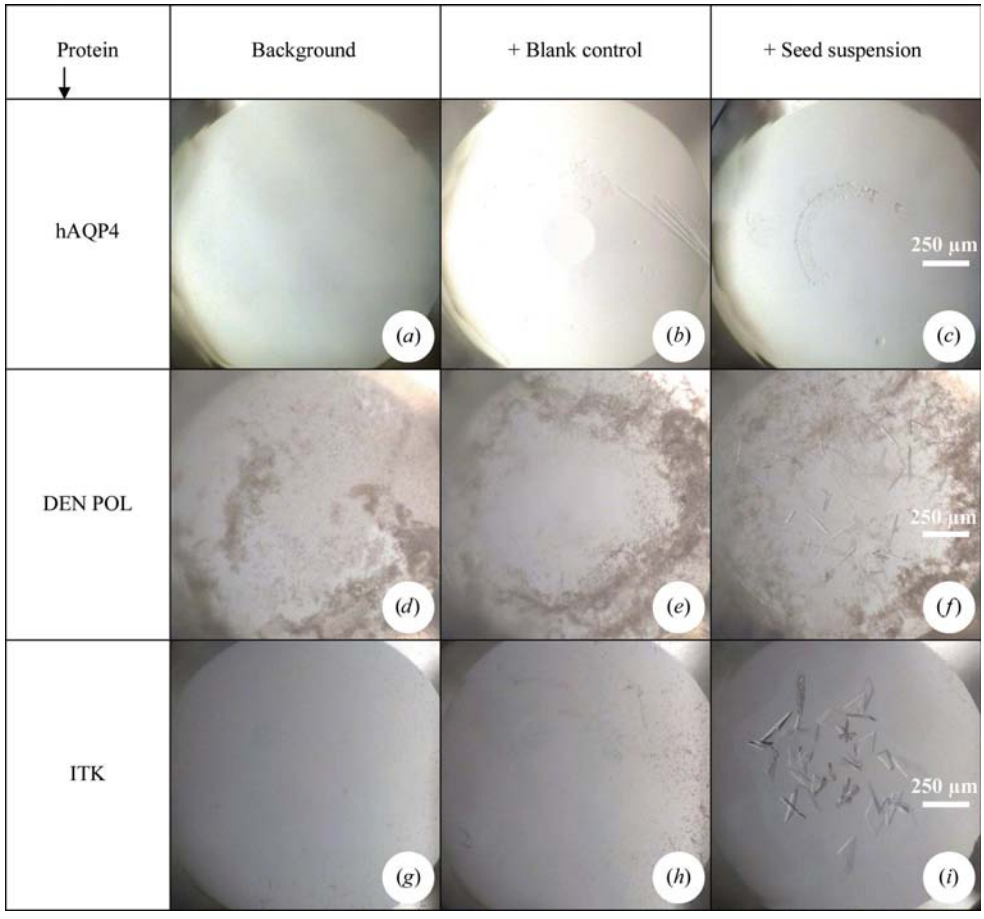

Figure 5
Direct comparison of background, blank-control and seeded drops. Seeded drops of (*c*) hAPQ4, (*f*) DEN POL and (*i*) ITK delivered enhanced results in relation to corresponding background and blank controls. All images in this figure were collected from 30-day-old drops.

A detailed description of the buffer conditions supporting crystal growth in seeded trials is given in Table 1 and is compared with the original starting condition in which the seeds were grown. Seeding delivered growth of hAQP4 crystals in the pH range 5.5–8.5. Five new conditions were identified for DEN POL crystallization, which involved three new precipitants and three new salts. The ITK experiments identified eight new precipitants for crystal growth. The p38α trials identified six new precipitants and the HIV RT trials identified five.

4. Discussion

Conventional crystallization screening campaigns rely on spontaneous nucleation and frequently suffer from low rates of successful crystal growth. Increased rates of crystallization upon seed addition have identified the crystal nucleation event as the most environmentally sensitive phase of the crystallization process, being confined to a relatively narrow band of solubility space. Providing exogenous crystal seeds as nucleants grants access to a broader range of buffer conditions that do support crystal growth, even if not in a regime capable of germinating spontaneous nucleation events. This promise led to the implementation and extension of crystal seeding in a broad manner (Ireton & Stoddard, 2004), with considerable enrichment of crystal growth in automated matrix-seeding (D'Arcy *et al.*, 2007). However, it was subsequently suggested that the higher rates of crystallization obtained using the automated method may have been a consequence of the significant volume of the original crystallization buffer inadvertently biasing the new well condition back towards the starting known chemical constituents (St John *et al.*, 2008). This bias, if significant, would lack the advantages in diversity and potential discovery anticipated from nucleating crystals under truly novel conditions. It would ultimately imply that the experiments were more or less reproducing the original condition. We devised a novel application of acoustic liquid-transfer technology to enable seed delivery in an insignificant volume of carrier solution, essentially eliminating the potential chemical bias. In this context, the importance of the seeds in inducing crystal growth is clearly indicated and we present a compelling method of matrix seeding with increased potential for discovery and improvement of crystal-growth conditions.

In an important distinction, our experiments included both a no-addition control (background) and a seedless control (blank) to provide effective benchmarks allowing validation of seed delivery. Earlier studies lacked this dual gauge of seed integrity and used disparate protein systems, which may explain some of the divergent interpretations reached in these studies (D'Arcy *et al.*, 2007; St John *et al.*, 2008). Without these controls it is hard to discern interference from chemical bias or if the seeds are perhaps simply unsuitable for delivery in the seed-carrying buffer. As explorations continue testing the suitability of generic universal nucleants or even noncrystalline materials or precipitate mixtures as seed solutions, our demonstration of this acoustic technology provides a useful tool to rule out unintentional introduction of chemical bias.

Comparison of the seeded and unseeded trials led to several interesting observations. While there were significant notable exceptions, we found that seeded crystal growth is favored in optically clear soluble protein starting points. Trials with a greater number of protein drops giving rise to non-translucent precipitates in the background control set were less likely to result in protein crystals in the seeded trial set. Conversely, proteins with a lower incidence of non-translucent precipitate

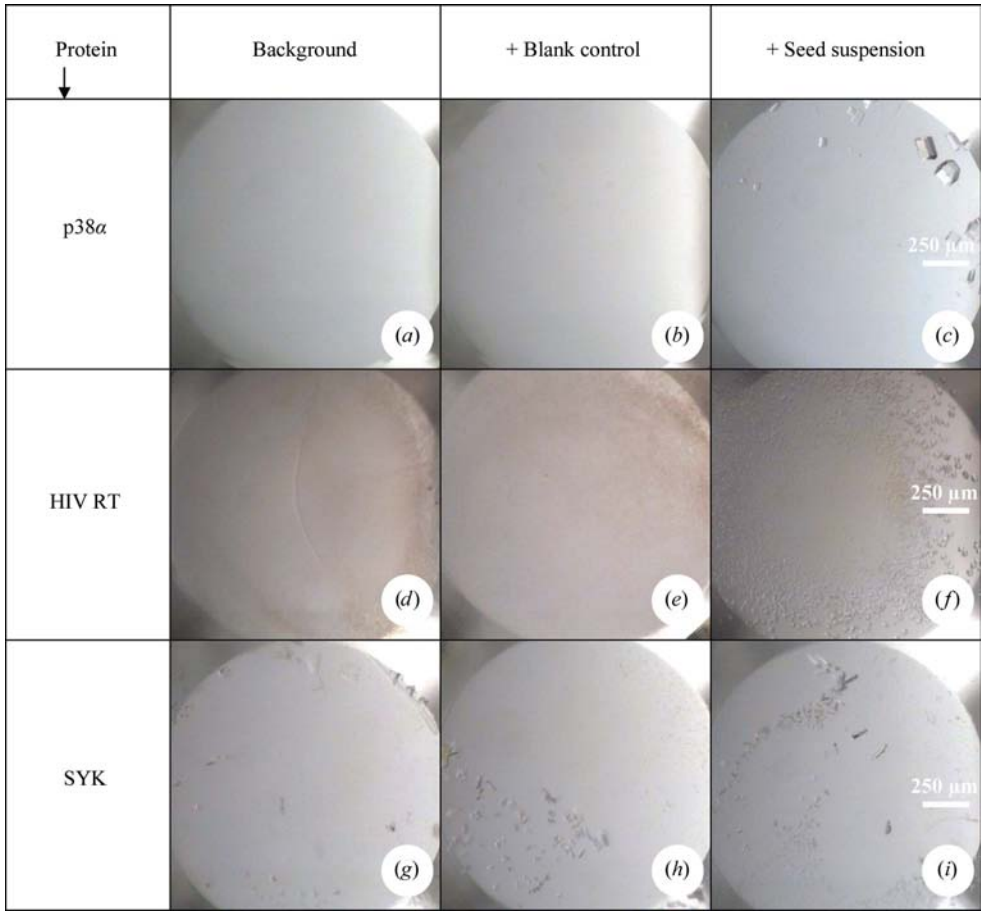

Figure 6
Background, blank-control and seeded drops of p38α, HIV RT and SYK. Crystal seeding was not essential for crystallization of SYK. However, crystal growth of (*c*) p38α and (*f*) HIV RT was only obtained with crystal seeding, as shown in these 30-day-old drops.

Table 1
Original starting conditions (highlighted in bold) are compared with well solutions supporting crystal growth in seeded drops.

hAQP4 crystal growth was observed in the pH range 5.5–8.5. The seeded trials identified three, eight, six and five new precipitants for the crystallization of DEN POL, ITK, p38α and HIV RT, respectively. The formulations shown in this table come from one of two plate duplicates of each protein.

Protein	Well	Buffer	Precipitant	Other
hAQP4		**0.05 *M* sodium citrate pH 6.0**	**25%(*w*/*v*) PEG MME 2000**	**5.00% glycerol**
	H06		20%(*w*/*v*) PEG 3350	0.20 *M* sodium formate
	G06	0.10 *M* bis-tris pH 5.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G10	0.10 *M* bis-tris pH 5.5	25%(*w*/*v*) PEG 3350	0.20 *M* magnesium chloride
	F10	0.10 *M* bis-tris pH 5.5	25%(*w*/*v*) PEG 3350	0.20 *M* sodium chloride
	G07	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G11	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* magnesium chloride
	F12	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* sodium chloride
	G08	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G12	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* magnesium chloride
	G09	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
DEN POL		**0.10 *M* imidazole pH 7.0**	**1.1 *M* K/Na tartrate**	**0.10 *M* Mg_2SO_4, 0.5% PEG 5K MME**
	H02		20%(*w*/*v*) PEG 3350	0.20 *M* K/Na tartrate
	H05	pH 7.0	15%(*w*/*v*) PEG 3350	0.10 *M* succinic acid
	H08		15%(*w*/*v*) PEG 3350	0.10 *M* magnesium formate
	F02	0.10 *M* Tris pH 8.5	20%(*w*/*v*) PEG MME 2000	0.20 *M* trimethylamine *N*-oxide
	C07	0.10 *M* Tris pH 8.5	0.5%(*w*/*v*) PEG MME 5000	0.80 *M* K/Na tartrate
ITK		**0.10 *M* HEPES pH 7.5**	**2.5 *M* sodium chloride**	
	C06	0.10 *M* bis-tris pH 6.5	1.5 *M* ammonium sulfate	0.10 *M* sodium chloride
	C02	pH 7.0	1.1 *M* ammonium tartrate	
	H05	pH 7.0	15%(*w*/*v*) PEG 3350	0.10 *M* succinic acid
	H02	20%(*w*/*v*) PEG 3350	0.20 *M* K/Na tartrate	
	H03	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* sodium malonate
	H04	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* triammonium citrate
	H06		20%(*w*/*v*) PEG 3350	0.20 *M* sodium formate
	H07	pH 7.0	20%(*w*/*v*) PEG 3350	0.15 *M* DL-malic acid
	H10		20%(*w*/*v*) PEG 3350	0.20 *M* trisodium citrate
	D09	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	
	G07	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G08	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G09	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium acetate
	G01	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* sodium chloride
	C10	0.10 *M* HEPES pH 7.0	1%(*w*/*v*) PEG MME 2000	1.00 *M* succinic acid
	B12	pH 7.0	2.8 *M* sodium acetate	
	C01	pH 7.0	3.5 *M* sodium formate	
	E11	0.10 *M* HEPES pH 7.5	22%(*w*/*v*) sodium polyacrylate	0.02 *M* magnesium chloride
	B10	pH 7.0	0.8 *M* succinic acid	
	C04	pH 7.0	35%(*v*/*v*) tacsimate	
p38α		**0.05 *M* HEPES pH 7.6**	**17%(*w*/*v*) PEG 3350**	**0.02 *M* calcium chloride**
	C06	0.10 *M* bis-tris pH 6.5	1.5 *M* ammonium sulfate	0.10 *M* sodium chloride
	A04	0.10 *M* bis-tris pH 6.5	2 *M* ammonium sulfate	
	B06		0.49 *M* NaH_2PO_4, 0.91 *M* K_2HPO_4	
	F01	0.10 *M* HEPES pH 7.5	10%(*w*/*v*) PEG 3350	0.20 *M* proline
	H05	pH 7.0	15%(*w*/*v*) PEG 3350	0.10 *M* succinic acid
	H08		15%(*w*/*v*) PEG 3350	0.10 *M* magnesium formate
	H03	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* sodium malonate
	H04	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* triammonium citrate
	H06		20%(*w*/*v*) PEG 3350	0.20 *M* sodium formate
	H07	pH 7.0	20%(*w*/*v*) PEG 3350	0.15 *M* DL-malic acid
	F07	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium sulfate
	F09	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium sulfate
	G01	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* sodium chloride
	G03	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* lithium sulfate
	G04	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* lithium sulfate
	G12	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* magnesium chloride
	F12	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* sodium chloride
	G11	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* magnesium chloride
	F03	0.10 *M* HEPES pH 7.0	10%(*w*/*v*) PEG MME 5000	5.00%(*w*/*v*) tacsimate
	E11	0.10 *M* HEPES pH 7.5	22%(*w*/*v*) sodium polyacrylate	0.02 *M* magnesium chloride
	C10	0.10 *M* HEPES pH 7.0	1 *M* succinic acid	1.00%(*w*/*v*) PEG MME 2000
	B10	pH 7.0	0.8 *M* succinic acid	
	C04	pH 7.0	35%(*v*/*v*) tacsimate	

in the background set resulted in a greater number of conditions that produced crystals upon seeding. This trend agrees in concept with the phase diagram of protein crystallography, which places crystal growth in the saturation but optically

Table 1 (continued)

Protein	Well	Buffer	Precipitant	Other
HIV RT		**0.05 *M* potassium phosphate pH 7.2**	**1.15 *M* sodium malonate**	**5.00%(*w*/*v*) ethylene glycol**
	A04	0.10 *M* bis-tris pH 6.5	2 *M* ammonium sulfate	
	A05	0.10 *M* HEPES pH 7.5	2 *M* ammonium sulfate	
	A06	0.10 *M* Tris pH 8.5	2 *M* ammonium sulfate	
	C06	0.10 *M* bis-tris pH 6.5	1.5 *M* ammonium sulfate	0.10 *M* sodium chloride
	B06		0.49 *M* NaH_2PO_4, 0.91 *M* K_2HPO_4	
	B07		0.056 *M* NaH_2PO_4, 1.34 *M* K_2HPO_4	
	H03	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* sodium malonate
	H04	pH 7.0	20%(*w*/*v*) PEG 3350	0.20 *M* triammonium citrate
	H02		20%(*w*/*v*) PEG 3350	0.20 *M* K/Na tartrate
	H06		20%(*w*/*v*) PEG 3350	0.20 *M* sodium formate
	H10		20%(*w*/*v*) PEG 3350	0.20 *M* trisodium citrate
	F07	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium sulfate
	G03	0.10 *M* bis-tris pH 6.5	25%(*w*/*v*) PEG 3350	0.20 *M* lithium sulfate
	F08	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium sulfate
	G04	0.10 *M* HEPES pH 7.5	25%(*w*/*v*) PEG 3350	0.20 *M* lithium sulfate
	F09	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* ammonium sulfate
	G05	0.10 *M* Tris pH 8.5	25%(*w*/*v*) PEG 3350	0.20 *M* lithium sulfate
	F05	0.1 *M* bis-tris pH 5.5	17%(*w*/*v*) PEG 10 000	0.10 *M* ammonium acetate
	C09	0.10 *M* HEPES pH 7.0	1.1 *M* sodium malonate	0.50%(*v*/*v*) Jeffamine ED-2001
	C04	pH 7.0	35%(*v*/*v*) tacsimate	
	C05	pH 7.0	60%(*v*/*v*) tacsimate	

clear range of protein solubility (Saridakis *et al.*, 1994; McPherson, 1999). It also implies the importance of calibrating the protein concentration to ensure that one is in a regime poised to benefit most from the seeding protocol.

However, in a number of cases seed-induced crystallization occurred in conditions that without seed had been deemed to belong to 'unpromising' result categories. This raises the troubling implication that the determination of when one is 'close' to a successful crystallization condition may be fraught. Indeed, at least some examples of nucleation through seeding and subsequent crystal growth were demonstrated in each of the defined result categories. Of course, this challenge has been recognized: implicitly in the typical difficulties in finding good crystallization conditions and explicitly in the described challenges and efforts to extract meaningful information from the numerous non-crystal-bearing wells in novel campaigns (DeLucas *et al.*, 2005). Thus, while the aforementioned trend exists between clearer drops and a good seeding environment, our results indicate that this is not a very robust correlation. The types of drop categorization typically employed by crystallographers and the ability of a drop to support crystal growth are somewhat decoupled, so mining these data sets to predict crystallization has understandably been challenging. Further efforts have been made to find parameters (*e.g.* second virial coefficients, in-drop light scattering *etc.*) that better match the crystallization behavior and it would be interesting to explore how the greater number of positive results from large-scale seeding might be more fertile grounds for predictive analyses where applicable.

Addition of exogenous seeds has broad potential advantages beyond the distinct benefits of higher frequency of crystallization. Remarkable improvements of known crystal habits by achieving more robust or better diffracting crystals have been demonstrated (Ireton & Stoddard, 2004). One particular example with the most dramatic impact observed in our study comes from seeded DEN POL experiments. The starting crystallization conditions containing sodium/potassium tartrate prompted the growth of crystals with a diffraction limit of 4.5 Å. On the other hand, crystals from seeded conditions containing 20% PEG MME 2000 (Table 1) reproducibly delivered crystals that diffracted to 2.7 Å resolution (data not shown). Finally, using seeds may allow one the flexibility to produce crystals under conditions that are more amenable for other purposes such as simpler cryoproctection or better alignment with biochemical assay conditions.

Given the long-standing knowledge of nucleation as a key limitation in the formation of crystals and the growing practical demonstration of this in matrix-seeding scenarios, we and others have amassed compelling data on the efficacy of this approach in discovering and improving suitable crystal conditions. Acoustic liquid dispensing is an effective way to supplement hundreds of protein drops with single-digit nanolitre volumes of seed, thereby enabling efficient use of seed stocks and dispelling concerns regarding chemical bias; in essence this is a high-throughput way to capture the advantage of whisker seeding. The method opens new opportunities for researchers in search of improved X-ray diffraction and novel crystal-growth conditions.

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