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A manual nanoscale method for protein crystallization

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To overcome one of the major hurdles in three-dimensional crystal structure determination – the requirement for large quantities of purified material to grow crystals – crystallization methodologies have been developed that require only a total of 2–5 µl of a concentrated macromolecular solution to screen more than 100 conditions. These procedures employ a circular slide containing an array of 25 wells designed for crystallization setups in the nanolitre volume range. These 'crystallization slides' fit into the wells of standard crystallization trays. These nanoscale crystallization approaches have been used to reproducibly obtain well diffracting crystals of three proteins, two that are being actively studied (glycerol kinase and NADH peroxidase) and one test protein (lysozyme), using only 40–350 µg (0.04–0.35 mg) of proteins to screen 100 conditions. These nanolitre crystallization methods are easily adapted for the typical laboratory, without the requirement of robotics or expensive equipment.

1. Introduction

Recent developments in the miniaturization of protein crystallization are premised on the adoption of robotic technology (Snook *et al.*, 2000; Mueller *et al.*, 2001). Many of these advances are towards automation in structure determination, fueled by structural genomics initiatives and emphasizing high-throughput structural studies (Burley *et al.*, 1999; Gerstein *et al.*, 2002). However, many academic research facilities do not have the production capacity nor the economic resources for automation. Hence, other approaches need to be developed to enhance or enable efficient structural studies for standard academic laboratories.

To overcome one of the major hurdles to structure determination, the necessity for large quantities of material for crystallization, we have developed several nanoscale crystallization approaches. These methods are based on a crystallization slide format, where each slide is capable of containing between 25 and 49 samples in an array that is formed on a 1.54 cm circular footprint. These slides are easily adopted in the laboratory without expensive equipment or robotic requirements, needing a total of only 40–350 µg of protein to sample 100 conditions, and crystals from them can be directly mounted for data collection. These results demonstrate that manual crystallization at the nanoscale level is possible and practical, making it now feasible to crystallize macromolecules that can only be obtained in small quantities.

2. Materials and methods

2.1. Crystallization slides

Crystallization slides were fabricated from both glass and polycarbonate materials. Circular slides with diameters of 1.54 cm were produced so they could easily fit into standard 24-well plates. For both materials, we explored several designs for the array and currently use slides that contain five columns and five rows of sample wells with two different sample-well sizes. Sample-well dimensions of 0.33 mm deep by 1.5 mm in diameter resulted in maximum spherical volumes of 780 nl and were used for setups with total volumes of less than 150 nl. For setups larger than 150 nl, we used slides with sample-well dimensions of 2 mm diameter by 0.9 mm deep, which held maximum volumes of 3.8 µl. We tested arrays containing up to 49 wells (7×7) with spacing between wells of 0.2–0.5 mm. For ease of set-up, we currently use a 25-well format (5×5) with a distance between wells of 0.7 mm (Fig. 1).

The slides made from glass were more difficult to produce and required diamond-head drill bits. Chipping at the edge of the sample wells during fabrication occasionally created an irregular surface on some slides (Figs. 2 and 4). Surface etching with 40% hydrofluoric acid solution was only minimally successful in creating a smoother surface. However, despite these drawbacks, the glass base seemed most 'benign' for the protein samples, resulting in less surface-contact precipitation (discussed further in §3). Prior to use, these slides were siliconized to produce a hydrophobic surface that promoted beading of sample drops. This minimized the spreading of the protein and crystallization solutions, reducing the amount of surface area exposed and decreasing the rate of evaporation, a critical parameter that needed to be reduced.

The polycarbonate slides were easier to fabricate, as standard steel drill bits could be used, with the advantage that the material did not chip, and were more cost-effective to produce. A 0.16 cm diameter ball mill, plunged down to 0.033 cm deep, was used to produce the smaller well size and a 0.24 cm diameter ball mill, plunged down to 0.09 cm deep, was used to produce the larger well size. The slides were chemically polished after fabrication by heating a mixture of 50:50 tetrahydrofuran:cyclohexanone in a water bath and holding the slides in the vapors generated for 5–7 s. This chemical polishing step was crucial to obtaining a smooth surface and to produce transparency in the slides, which were opaque prior to polishing.

2.2. Proteins

Three soluble proteins were used to test this method; glycerol kinase (GK) at 15–20 mg ml^{-1}, NADH peroxidase (Npx) at 8–10 mg ml^{-1} and lysozyme at 75 mg ml^{-1}. GK and Npx were expressed and purified in this laboratory according to published protocols (Ross & Claiborne, 1992; Charrier *et al.*, 1997) and lysozyme was purchased from Sigma. All three soluble proteins have been crystallized *via* traditional approaches, although our nanoscale-screening procedure identified a new condition for GK (Yeh *et al.*, 2003). All proteins were concentrated to their respective working concentrations using Centricons and maintained in 50 m*M* Tris–HCl buffer pH 7.5 with 1 m*M* DTT.

2.3. Nanolitre injector

To dispense nanolitre volumes, a handheld nanoject pipettor (Drummond Co.) with a silanized glass capillary was used for sample transfer. Depending on the setup, the glass

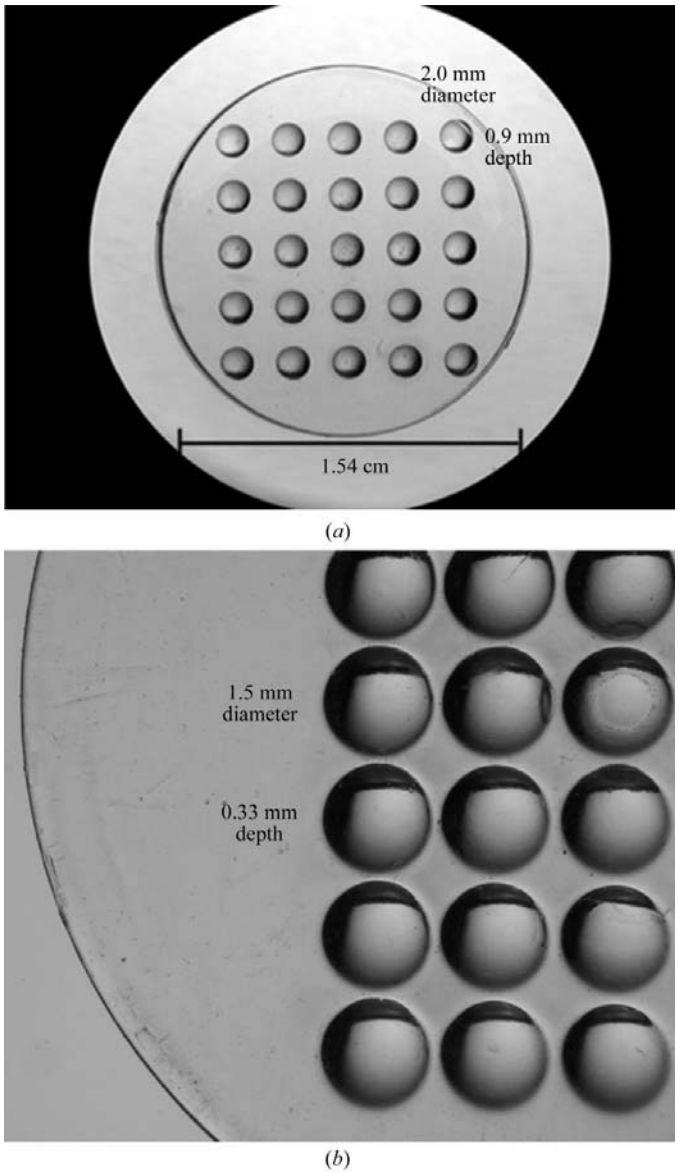

Figure 1
Polycarbonate was used for fabricating the crystallization slides and several array designs were tested, with two shown. For drop volumes of over 150 nl, the sample wells were fabricated with dimensions of 2.0 mm diameter by 0.9 mm depth with 0.7 mm distance separating the sample wells. These held a total spherical volume of 3.8 µl, with a total of 25 sample wells (*a*) fitting within the 1.54 cm diameter slide that easily fits into the wells of standard 24-well crystallization plates. For drop volumes of less than 150 nl, the sample-well dimensions were 1.5 mm diameter and 0.3 mm depth with 0.2 mm separation distance between wells, accommodating up to 49 sample wells on a slide (*b*). Chemical polishing in THF:cyclohexanone vapor was critical to creating transparency in the slides and helped to smooth out the surface.

capillary was either rinsed between setups with distilled water or the capillary was changed. Silanization of the glass capillary made sample adherence to the glass surface negligible, so that rinsing by pipeting between samples was sufficient to minimize carry-over contamination. A foot-pump model was also tested and both models dispensed drop volumes of equivalent accuracy, as determined by weighing drops of 100–500 nl of water on an analytical balance accurate to 0.0001 g. For drops of 25 and 50 nl, 5–10 drops were dispensed and an average taken. We found that for drops above 100 nl volume the error was of the order of 5–9%, while for drops smaller than 100 nl volume errors were about 7–12%.

2.4. Crystallization procedure

Two plate types were used to obtain sitting-drop and sandwich-drop setups: Q-plates and VDX plates, respectively. Using the Q-plates, the crystallization slides were placed on a ridge in the well, suspending the slide over the crystallization solution, resulting in a sitting-drop setup which was sealed by applying clear tape over the tray. In the VDX plate, two microbridges form the platform for the crystallization slides, with the top microbridge perpendicular to the bottom. This resulted in a setup whose height allowed the formation of a sandwich drop between the oil overlayer and a glass cover slip, which sealed the well when depressed onto vacuum grease placed at the periphery of the well. Both of these setups were used for vapor diffusion, while the microbatch approach used the sitting-drop format with Q-plates.

2.4.1. Vapor diffusion. Two different approaches were used for crystallization screening, both based on vapor diffusion. The first allowed up to 25 different proteins to be screened on one slide and equilibrated against one solution condition using the vapor-diffusion method for producing supersaturation (Fig. 2), while the second screens one protein using up to 25 different conditions.

To screen up to 25 different proteins with one solution condition, the solution was first dispensed into the reservoir of the plate and the crystallization slide was placed either on microbridges in VDX plates or on the ridge of Q-plates, as described above. Oil was first added on top of the slide to minimize evaporation, followed by the dispensing of protein into a sample well using the nanolitre injector. The crystallization solution was then added to the drop. Although care was taken to add the second drop into the first, this was not such a critical factor as the droplet eventually sinks to the bottom of the oil-filled well and merges with the protein droplet. We have also centrifuged the plates at very low speeds, which ensures that the two drops coalesce.

The second approach screens one protein using 25 different conditions. This approach required the well solutions to be formulated to allow vapor diffusion but using a 'general' precipitant solution rather than independently equilibrating against 25 different well solutions. The specific reservoir solution formulated for a particular group of crystallization solutions, described below, was first dispensed into the well of the plate and the crystallization slide placed on microbridges in VDX plates or on the ridge of Q-plates, as described earlier. To set up the crystallization slides, oil was first added on top of the slide, followed by dispensing of the protein and crystallization solutions using the nanolitre injector. In this setup, a different solution was added to each drop so that 25 different conditions were tested on one slide; the glass sample-transfer capillary was rinsed by pipetting water between solutions. The six screens encompassed 150 conditions, which were formulated to sample a large variety of chemical classes but remained amenable to accurate nanolitre dispensing by maintaining low to medium viscosity in all the solutions.

The screens were divided into six groups, based on the following precipitant classes: (1) low-molecular-weight (MW) organic precipitants (glycerol, PEGs of MW 100 and below, MPD, alcohols and ethylene glycol) between 10 and 20% concentration, (2) low-MW organic precipitants (glycerol, PEGs of MW 100 and below, MPD and ethylene glycol) between 20 and 30% concentration, (3) high-MW organic precipitants (PEGs above MW 1000) between 10 and 20% concentration, (4) high-MW organic precipitants (PEGs above MW 1000) between 20 and 30% concentration, (5) low salt

Figure 2
Glycerol kinase crystallized with a glass crystallization slide containing a 4 × 4 array using final drop volumes of 100 nl (50 nl protein:50 nl crystallization solution). These glass slides have some surface imperfections owing to the drilling procedure, but nonetheless yielded data-collection quality crystals. From left to right, the images are at 20×, 40× and 70× magnification and only part of the crystallization slide is shown. The single crystal of GK (right panel) is about 0.1 mm in all dimensions and gave 2.4 Å resolution diffraction data using our in-house Cu rotating-anode source. Note that only the peripheral sample wells were used for setups and each of these produced crystals.

concentration (under 0.4 *M)* and (6) high salt concentrations (above 0.4 *M*). These crystallization screens contained, in addition to the major precipitant, buffers at 0.1 *M* concentration and varying pH values and additives, including low concentrations of various salts at or below 0.05 *M*.

For each of the six groups, where each group contained 25 conditions, a specific solution was used for the reservoir to initiate vapor diffusion so that these were formulated as a general precipitant solution. These contained the following: for group (1), 20% ethylene glycol, 0.1 *M* Tris–HCl pH 7 and 0.05 *M* NaCl; group (2), 30% ethylene glycol, 0.1 *M* Tris–HCl pH 7, 0.05 *M* NaCl; group (3), 20% PEG 3000, 0.1 *M* Tris–HCl pH 7 and 0.2 *M* NaCl; group (4), 30% PEG 3000, 0.1 *M* Tris–HCl pH 7 and 0.2 *M* NaCl; group (5), 0.4–0.8 *M* NaCl and 0.1 *M* Tris–HCl pH 7; group (6), 0.8–3 *M* NaCl and 0.1 *M* Tris–HCl pH 7. After several days, additional vapor diffusion could be further induced by increasing the precipitant concentration in the reservoir either by increasing the concentration of the major organic or salt component in the well or by transferring the slide to another well containing the precipitant at a higher concentration.

2.4.2. Microbatch. Microbatch achieves supersaturation of the protein directly and unique crystallization conditions have been found using a batch-crystallization approach that were not obtained using vapor diffusion (Baldock *et al.*, 1996). We adapted the microbatch approach so that up to 25 different conditions can be sampled, as described above for the vapor-diffusion formulations, but at higher precipitant concentrations so that supersaturation is achieved more immediately. The screening solutions were formulated so that the concentration of the major precipitant was increased to between 10 and 40%, depending on the nature of the precipitant. For viscous reagents (*e.g.* PEGs, glycerol), we increased the concentration by 10–15% from that used in the vapor-diffusion screens, so that the final solutions' viscosity would not significantly adversely affect the accuracy of nanolitre dispensing. For salts, we were able to increase the concentrations up to an additional 40%. The setup was very similar to that described for vapor-diffusion screening of 25 different solutions, but the slides were placed in wells of Q-plates that contained some moistened filter paper to prevent complete dehydration and were sealed with tape.

For all of these nanoscale setups, oil was essential for minimizing evaporation effects, which can be extensive owing to the small sample volumes. Although the sample wells accommodated considerably larger volumes than the volumes occupied by the protein/crystallization solution drop (or the 'working volume'), this extra 'head volume' allowed the placement of a layer of oil to reduce evaporation during setups. Formulation of oils with different water-permeation properties modifies and controls the rate of vapor diffusion between the sample drop and well solution (Chayen, 1996). In this study, we used a mixture of 1:1 silicon oil and paraffin oil. Additionally, we found it helpful to centrifuge the trays at very low speeds of 300–500 rev min^{-1} to ensure mixing of the protein and solution drops under oil, which can be difficult to visualize.

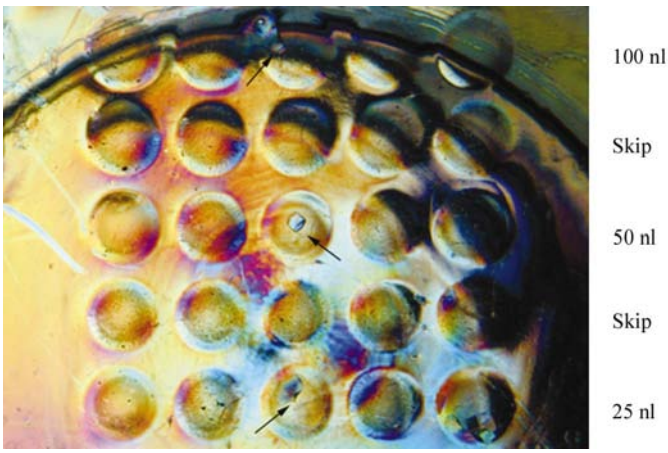

Figure 3
Lysozyme crystallized using polycarbonate slides with a 5 × 5 array at three different volumes; only part of the slide is shown. Crystals were obtained even with 25 nl volumes and were not significantly reduced in size when compared with crystals obtained at 100 nl volume (crystals indicated by arrows; color owing to polarizers). The polycarbonate surface appeared to result in greater surface precipitation, as shown by the brownish precipitate in the sample wells, although this may be attributed to the extremely high concentrations of lysozyme used in the crystallization protocol (see text).

2.5. Data analysis

Crystals grown from the slides were transferred with a Pipetman to a siliconized glass base or depression well for mounting in a loop and flash-frozen in the nitrogen stream at 100 K. For GK and lysozyme, the crystals were grown from solutions containing 30% PEG 400 and 25% ethylene glycol, respectively, which also served as cryoprotectants. For Npx, an additional step of flash-soaking in its mother liquor with a further 25% ethylene glycol added as a cryoprotectant allowed the crystals to be frozen at 100 K. All crystals, whether grown on slides using nanolitre volumes or grown traditionally using microlitre volumes, were manipulated similarly during the mounting and flash-freezing procedures. Data collection took place using an R-AXIS II detector system with a copper rotating-anode generator and Yale mirrors with a 0.3 mm collimator. All data were processed and analyzed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

We first developed our manual nanoscale crystallization methods to target proteins that are difficult to express abundantly in their active state as a means of obtaining preliminary crystallization conditions. Our initial intention was to scale up successful results by traditional approaches. However, using these crystallization slides and the three test proteins, we have now grown data-quality crystals that can be well over 100 µm in size in drops as small as 25 nl (but more routinely using 50–100 nl as our smallest volumes). Crystals with these dimensions occupy a calculated spherical volume of about 4 nl, illustrating that even using nanolitre volumes for setups,

crystals can be grown directly for data collection. These results indicate that these nanoscale methods can be generalized for growing single diffraction-quality crystals of practically any macromolecule using only microgram quantities of material.

Crystallization slides are easily fabricated from both polycarbonate and glass substrates and both yielded well diffracting crystals with all three test proteins. However, although polycarbonate has been widely used as a material for biological applications, it caused substantial surface-contact denaturation with the test protein lysozyme. This phenomenon appeared to be related to the extremely high concentrations of lysozyme (75 mg ml^{-1}) used, since neither GK nor Npx exhibited as significant contact denaturation.

There was little correlation between sample-drop volumes and the size of the crystals obtained. For two of the three proteins used in this study, GK and lysozyme, the final drop volumes tested were from 50 to 300 nl in 50 nl increments and crystals of at least 0.1 mm dimensions were obtained with all volumes (Figs. 2, 3 and 4). For Npx, crystals with dimensions of over 0.6 mm in the longest edge were routinely grown using final volumes of 2–4 µl. The smaller drop sizes used in this study may have yielded correspondingly smaller crystals, although these were still of the order of 0.04–0.05 mm for the smallest and larger crystals over 0.1 mm were also obtained (Fig. 4). The generality of this correlation of drop size to crystal size is limited by this small study, although reports have suggested that such a relationship may exist (Stevens, 2000). However, even in cases where miniaturization results in microcrystals, nanoscale crystallization can be a quick and efficient means of finding preliminary crystallization conditions for larger-scale setups. Once a condition is identified using the nanoscale procedure, more traditional approaches using more typical volumes can be used to optimize the crystal-growth conditions.

The amount of time needed for nucleation and crystal growth was perhaps 10–35% less than that using traditional volumes and none of the crystals took longer times to nucleate or grow. Based on previously determined crystallization conditions for GK and lysozyme, using final drop volumes of 2–6 µl in sitting-drop and hanging-drop setups, nucleation was observed 24–48 h after setup at room temperature, with continued crystal growth for another 7–10 d. For Npx, traditional crystallization volumes resulted in crystals after about one week, with continued growth for another 2–3 weeks. Using volumes of 100 nl, nucleation was observed within 24 h for GK and lysozyme, and crystals reached their final sizes within 3 d. All three of these proteins exhibited similar nucleation and crystal-growth behavior with final drop volumes of 50–300 nl. The crystal morphology, the timeline for growth and even the crystal size were largely comparable between crystals grown using different nanolitre volumes. It should be stressed that these results are from our study on three proteins and while we found some differences in the amount of time needed for crystal growth, theoretical models predict that vapor-diffusion rates may increase dramatically with smaller drop volumes, leading to faster equilibration times than observed in this study (Barid, 1999; Santarsiero *et al.*, 2002).

Diffraction qualities were more significantly affected by this nanoscale approach and, somewhat unexpectedly, in a positive manner. Resolution limits were minimally affected: crystals of GK, Npx and lysozyme grown using nanolitre volumes diffracted to resolutions of 2.4, 2.8 and 2.2 Å, respectively, using our in-house Cu rotating-anode source. These limits of diffraction were similar to those obtained from data collected from crystals grown using more typical volumes. This was anticipated as the sizes of the crystals (*i.e.* total diffraction volume) grown using nanolitre volumes were not significantly smaller except for Npx, which diffracted to 2.3 Å in-house when crystals of 0.3–0.4 mm were used. In contrast, mosaicity values were considerably lowered for both GK and lysozyme compared with crystals grown from microlitre volumes. Average overall values were approximately 0.3° rather than 0.6° for GK and 0.4° instead of 0.8° for lysozyme. The decrease in mosaicity values in crystals grown using this nanoscale approach compared with crystals grown from microlitre volumes may be a consequence of the reduction in time (10–40% decrease) needed for initial nucleation and growth, which may concomitantly reduce degradative events such as aggregation, denaturation *etc*. that can adversely affect the homo-

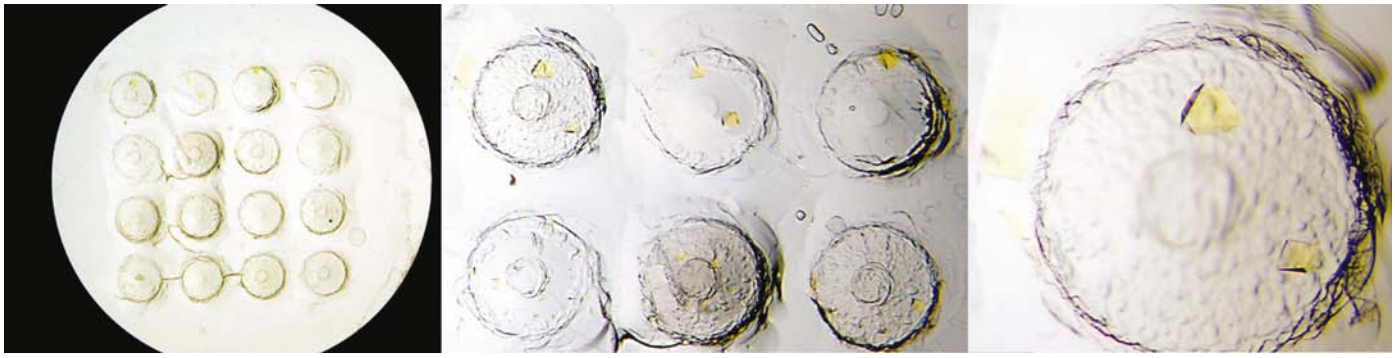

Figure 4
Npx crystals obtained at 100 nl final volumes in a 4 × 4 array on a glass slide. The yellow color is from the flavin cofactor bound to the enzyme. Using the same crystallization condition, crystals as large as 0.6 mm on an edge have previously been grown when volumes of 2–4 µl are used for the sample drops. These crystals vary from 0.04 mm to well over 0.1 mm in their longest dimension and can be easily mounted for data collection.

geneity of packing. No special limitations on the apparatus beam divergence and spectral spread were taken; consequently, the smallest value of mosaicity reported here (0.3°) is dominated by the apparatus and not the crystal.

An advantage of the approaches described here is that they do not require sophisticated or expensive setups. Our nanocrystallization methods simply require crystallization slides, a nanolitre injector capable of accurately dispensing nanolitre volumes and a microscope. Currently published crystallization methods using nanoscale volumes rely on automation in conjunction with high-throughput robotics utilized in structural genomics projects. Several robotic systems have been evaluated for dispensing nanolitre volumes of between 20 and 500 nl with favorable results (Stevens, 2000; Mueller *et al.*, 2001; Santarsiero *et al.*, 2002). However, automation and robotics are outside the financial realm of most academic laboratories. A more economical approach utilized ink-jet printer heads for delivering ultrasmall drop sizes (Howard & Cachau, 2002) and although promising, this method needs to be developed further to easily allow screening of a large number of different conditions.

The nanoscale crystallization methods described in this report are easily adopted, economical and have produced very promising results that utilize current crystallization methodologies. These miniaturization approaches should be amenable to crystallization of any biological molecules and potentially reduce not only the amount of material but the time needed for crystal structure studies. The ability to crystallize biological molecules requiring only a fraction of the amount of material typically needed will be very likely to broaden the scope of biological materials that can be crystallized and studied structurally.

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