Product Application Focus

Fast Drops: A High-Throughput Approach for Setting Up Protein Crystal Screens

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ABSTRACT

There is significant demand to rapidly obtain protein structure information for both structural genomics and drug discovery applications. To meet this demand, all steps in the process of determining protein structure by X-ray crystallography need to be optimized and streamlined with high-throughput methodologies. This communication describes a method that brings high-throughput technology to protein crystallization in both manual and automated modes, suitable for virtually every crystallography laboratory.

INTRODUCTION

A variety of methods exist for setting up protein samples in crystallization screens (2,5,6). The most widely used method is crystallization by vapor diffusion. In this method, vapor diffusion occurs in a closed system because of a difference in concentration between a droplet of protein and a larger body of liquid. The protein drop is comprised of protein diluted 2-fold,

usually, with liquid from the larger body. Therefore, the protein drop starts at half the concentration with respect to the contents in the larger body of liquid, also known as mother liquor. This concentration gradient drives vapor diffusion, resulting in the gradual concentration of protein and, if the variables are right, protein crystallization.

Vapor diffusion experiments are set up in either a hanging or sitting drop arrangement where the protein drop hangs from the top of a sealed well or sits on a supporting surface near the top of the well while the mother liquor rests below on the floor of the well. The mother liquor can contain a wide range of chemical variables, including but not limited to buffers for pH control, salts [$\text{NH}_4(\text{SO}_4)_2$, NaCl, etc.], precipitating agents (PEG, alcohols, etc.), reducing agents, and detergents. As the variability of conditions required to crystallize proteins is too large for exhaustive searches, more practical approaches are employed such as "sparse matrix" screening (3). One sparse matrix screen that has been commercially available for a decade consists of 50 mother liquors (<http://www.hamptonresearch.com>). More recently developed crystal screens include the "Wizard" screens (<http://www.emeraldbiostructures.com>), a sodium malonate screen (4), the randomly generated screens by CRYSTOOL software (8), and the clear strategy screens (1). The number of screens available has grown, as has the number of challenging crystallization problems

(i.e., membrane proteins, structure-based drug design, and structural genomics), thereby creating the need for improved efficiency in setting up protein crystallization trials.

The traditional way to set up protein crystallization trials, in either hanging or sitting drop format, uses single-channel manual pipets for liquid handling. Typical setups are performed using a 24-well plate with room for 24 independent vapor diffusion experiments and a manual throughput of 60 experiments per hour. Although widely used, the traditional method is labor intensive, time consuming, and unable to deliver high-throughput rates. Furthermore, automated systems that use single-channel liquid handling deliver relatively low throughput rates, such as 60 crystallization experiments per hour for setups with one single drop per well or 150 experiments per hour for setups with four drops per well.

Fast Drops is a new method that provides an efficient way to conduct thousands of crystallization experiments manually in a matter of days, or in hours using automation. The method uses the Corning Crystallography Plate (CCP), a 96-well microplate for sitting drop vapor diffusion. The microplate developed by Corning Life Sciences (Acton, MA, USA) meets industry standards, thereby ensuring compatibility with most existing multi-channel liquid-handling pipets and robots. We describe here the use of the CCP and the Fast Drops method to dramatically increase the ef-

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efficiency and speed of setting up protein crystallization screens in an automated or non-automated laboratory.

MATERIALS AND METHODS

Crystallization screens consisting of 384 mother liquor conditions are used in the crystallization trials [Crystal screens I & II from Hampton Research (<http://www.hamptonresearch.com>), Wizards I & II from Emerald Biostructures (<http://www.emeraldbiostructures.com>), a PEG screen (A. D'arcy, personal communication), and a sodium malonate screen (4)]. All 384 conditions are stored in 96-well storage blocks with 2 mL capacity per well (Matrix Technologies, Hudson, NH, USA). A mat applicator (Corning Life Sciences) and sealing mats (Matrix Technologies) are used to seal the storage block when not in use. All crystallization screens are set up in the CCP. The physical layout of this plate is shown in Figure 1. Manual liquid handling is done using EDP3 electronic pipets (Rainin, Emeryville, CA, USA). An eight-channel pipet (Rainin) is used for the task of dispensing mother liquor from storage blocks into reservoirs in the CCP. A single-channel pipet (Rainin) is used for aliquoting protein drops into the protein wells of the CCP. A second eight-chan-

nel pipet (Rainin) is employed for adding mother liquor onto protein drops. Crystallization experiments are sealed using ClearSeal Film™ for microplates (Hampton Research).

Fast Drops Method

Manual setups are done as follows. (i) Mother liquors from each of the crystallization screens are transferred from the storage block into reservoirs in the CCP using an eight-channel pipet (50 μ L/reservoir). (ii) A single-channel pipet, on multiple dispense mode, is used to dispense a 1 μ L protein drop into all 96 CCP protein wells. (iii) One microliter of mother liquor from each CCP reservoir is transferred onto the corresponding drop using an eight-channel pipet. (iv) The CCP is sealed with ClearSeal Film.

Automated crystal screen setups are done using the Cybi-Well robot (<http://www.cybio-ag.com>). The CCP and crystal screen storage blocks are placed on the horizontal platform of the Cybi-Well along with a V-bottom, 96-well plate containing pure protein in all 96 wells. The robot's horizontal platform moves plates and positions them directly below the 96-tip head. The tip head in turn aspirates and dispenses liquid as follows. (i) All 96 mother liquids are transferred from the storage block into the large CCP reservoirs (50 μ L each). The robot then washes all 96 tips extensively with distilled water. (ii) The

96-tip head picks up protein from the protein plate and dispenses all 96 protein drops simultaneously to the middle of each individual protein well (1 μ L protein). (iii) One microliter of mother liquor is delivered to each protein drop using the 96-tip head. (iv) The plate is sealed manually with ClearSeal Film. The complete set up of 96 experiments, including the tip washing step, takes 2 min to accomplish.

Currently, protein drop observations are done manually using an inexpensive plexiglass platform (described below), mounted on the table of a light microscope, to guide the CCP and keep track of drop location at all times.

RESULTS AND DISCUSSION

There are several critical features of the CCP (Figure 1) that allow efficient, high-throughput protein crystallization setups by virtually any crystallography laboratory. First, the standard microplate platform design makes it possible for manual and robotic users alike to take advantage of multi-channel liquid handling and therefore capitalize on speed and efficiency. Second, the protein well with round inner-bottom edges confines the drops to the middle of the well, thereby simplifying the task of finding the drops when making observations. The CCP is made from a proprietary polymer that is 100 times less permeable to aqueous solutions

Figure 1. The Corning Crystallography Plate has room for 96 vapor diffusion crystallization experiments. Each well consists of a large reservoir to hold mother liquor and a protein well to hold a protein drop.

Figure 2. Large-scale crystal screens. The traditional manual method, using 24-well plates and single-channel pipets, requires 130 working hours or months depending on individual pace to set up 10 distinct protein entities against 800 crystallization conditions, a total of 8000 drops. The manual Fast Drops method requires 20 working hours or less than a week. The automated Fast Drops method requires 3 h.

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Figure 3. The dual plexiglass platform mounted on a light microscope. The plexiglass assembly simplifies manual observation of protein drops. Simply slide crystallization plate from drop to drop on the top plexiglass platform. Then, lift the top platform and reseat it into metal pins, on the bottom platform, to select subsequent rows.

than polystyrene and therefore can keep drops hydrated for longer periods of time. The new polymer allows for relatively low birefringence when viewing drops through polarized light.

The Fast Drops method described here allows protein crystallization screens to be performed with dramatic improvement in speed and efficiency. Figure 2 provides a comparison of the obtainable rates for crystallization setups using the traditional manual method and the Fast Drop methods (manual and automated). While the traditional manual method averages 60 drops/h, the manual Fast Drops method achieves 400 drops/h, and the automated Fast Drops method delivers 2880 drops/h (i.e., 96 drops every 2 min). Figure 2 shows a further side-by-side comparison of the methods. For example, the complete setup of 10 distinct protein entities against 800 conditions (a total of 8000 crystallization experiments) takes a minimum of 130 working hours using the traditional method or up to several months depending on individual pace. Using the manual Fast Drops method, however, the same number of experiments can be done in a week, or in 3 h with the automated Fast Drops method using the Cybi-Well robot. Clearly, the rates provided by the Fast Drops methods are impressive, whether compared to the traditional methods or specialized protein crystal-

lization systems (9).

Employing alternative robotic systems can further optimize the automated Fast Drops method. The Cybi-Well requires that the protein be provided in 96 individual protein wells, which accounts for some protein sample loss. Possible alternatives that can minimize protein loss are the Cyberlab C-400 (<http://www.gilson.com>), the Biomek FX (<http://www.beckman.com>), and the Cybi-Disk (<http://www.cybio-ag.com>). All three commercially available robots can be equipped with two tip heads where an eight-channel tip head aspirates and delivers protein drops and a 96-tip head achieves delivery of mother liquors. Since the protein sample solution is aspirated via the eight-channel tip head, only eight individual protein solution wells need to be provided (as opposed to 96), thereby minimizing protein sample loss.

Further modifications to the method may include setting up sub-microliter protein drops (the Cybi-disk and Biomek FX robots can deliver 250-nL volumes with the purchase of appropriate accessories). Prior work done by others has demonstrated that crystallization of proteins is possible and reproducible in sub-microliter drops (7).

Protein Drop Observations

The Fast Drops method allows the

generation of thousands of crystallization experiments in a short period of time, thus creating the need for improved efficiency in the monitoring of protein drops. Automated imaging systems that record drop images are commercially available; however, none are currently equipped for the task of recognizing protein crystals. Therefore, the researcher must retrieve images recorded by the imaging system and search image by image for protein crystals. In other words, the current imaging systems provide a semi-automated solution that may not be suitable for every crystallographer.

For those of you who would rather not purchase the imaging systems currently available, you may consider a simpler solution. Figure 3 shows a low-cost dual plexiglass platform that can greatly simplify the task of observing drops manually. The dual platform guides the CCP from drop to drop with maneuverability in two directions. The CCP is simply pushed across from drop to drop on the first row. Then, the top plexiglass platform is lifted and seated to the next row by simply aligning it onto fixed metal pins in the bottom platform. This simple, low-tech solution allows the observation of 96 drops in 7 min when recording drop observations numerically (1, heavy precipitate; 2, moderate precipitate; 3, aggregated precipitate; 4, phase separation; 5, light precipitate; 6, clear drop; 7, grainy precipitate; 8, clear amorphous particles; 9, micro-crystals and needles; and 10, medium-to-large crystals).

Using the Fast Drops method described here, we have improved the efficiency and success rate of our crystallization experiments. Crystallization screens of 12 protein entities (distinct proteins or protein-ligand complexes), not previously crystallized, provided the following results: nine out of 12 entities crystallized after one week of incubation at 20°C, some in more than one condition. Seven entities crystallized in the sodium malonate screen. Four crystallized in the Wizard screens. Two crystallized in the PEG screen, and one crystallized in the sparse matrix screen. Drop observations for all 12 entities will continue as long as the drops remain hydrated. After three

months of incubation, our 2 μ L protein drops, with a mother liquor volume of 50 μ L, remain well hydrated.

With the Fast Drops method, we have devised a highly efficient manual or automated method for high-throughput protein crystallization that does not require any specialized equipment or expertise. In the future, we plan to further improve the efficiency of this method by developing approaches for the automated observation of protein drops.

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