The high-speed Hydra-Plus-One system for automated high-throughput protein crystallography

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An automated high-throughput dispenser has been developed for the setup of protein crystallization trials by vapor diffusion or Microbatch methods. The Hydra®-Plus-One is composed of a Hydra®-PP system equipped with a motorized XYZ-platform, 96 precision glass syringes and a single-channel microsolenoid dispenser, which transfers 100nl-50µl of protein solution with an accuracy of > 90% at a speed of 60s per 96 wells. Up to 300µl of premixed cocktails can be aspirated with the 96-syringe-assembly and dispensed into reservoir and droplet wells within 60s. The Hydra®-Plus-One combines high precision, reliability and speed in a cost-effective high-throughput system ideally suited for protein crystallization

Keywords: automation; crystallization; high-throughput; Hydra®; liquid-handling system

1. Introduction

Several large-scale structural genomic projects have been initiated to obtain three-dimensional structures of proteins that hold key information for a comprehensive understanding of the biological functions of proteins and provide a basis for rational drug design. In the United States alone the Protein Structure Initiative (PSI) of the National Institutes of Health (NIH) has funded the launch of nine structural genomics centers. The PSI has two principal goals: to demonstrate the overall feasibility of structural genomics, and to encourage the development of technology necessary for high-throughput structure determination. At the crystallization center for the *Mycobacterium tuberculosis* (TB) structural genomics consortium (Terwilliger, 2000; www.doe-mbi.ucla.edu/TB) we are addressing a major bottleneck of Structural Genomics, the rapid production of diffraction quality crystals of new proteins. Our efforts focus on adapting automated design of crystallization screens using CRYSTOOL (Segelke, 2001) to procedures for automated setup, tracking and analysis. Automated systems and procedures for high-throughput crystallization screening are needed to reach the necessary capacity for large-scale structure determination. Proteins and buffers have to be set up in crystallization plates with great speed to prevent evaporation of voiltile components in protein solution as well as crystallization cocktails. High precision for both volume and dispensing position are crucial to ensure reproducibility. As protein is often precious, recovery of material as well as the possibility of sub-µl drop volumes is desirable.

Currently only a limited number of automatic crystallization systems are commercially available: the Oryx 6/IMPAX 1-5 system sold by Douglas Instruments (Douglas Instruments, www.douglas.co.uk) for microbatch and vapor diffusion experiments; and the Gilson/Cyberlab workstations (Cyberlab, www.gilson.com) for hanging drop and sitting drop vapor diffusion in standard Linbro 24-well plates (a recent update of the Cyberlab station now allows 96 well format as well). Inclusively, these systems do not encompass a configuration suited for high-throughput crystallography, requiring user intervention for multiple tray processing and having material processing issues (Stevens, 2000). Proprietary industrial systems are capable of hundreds of thousands of crystallization set ups per day, but are prohibitively expensive for the average academic research laboratory. At least two structural genomics endeavours are using Hydra® systems for automated crystallization setup (Jurisica *et al*., 2001; Mueller *et al.*, 2001).

The Hydra®-Plus-One system, combining the proven Hydra® technology with a contact-less solenoid protein microdispenser was designed to provide an easy-to-handle and low-cost device to meet the demands of high-throughput crystallization by enabling high precision pipetting down to nanoliter volumes.

2. Materials and methods

The Hydra®-Plus-One incorporates a Hydra®-PP (Hydra® dispenser with Plate Positioner) with 96 290 µl (or optional 100 µl) stainless steel or DuraFlex™ syringes and an additional high-precision non-contact single-channel microsolenoid dispenser (the latter formed from components of Innovadyne Technologies NanoDrop™ system) developed by Robbins Scientific Corporation, California. Fluorescein was purchased from Molecular Probes, Oregon (cat. # F-1300). Tris-HCl, pH 8.0 (cat. #93378) was purchased from Sigma, Missouri. Nunc 96-well clear plates (cat. #269620) and 96W protein crystallization plates (cat. #609101) were purchased from Nunc, New York, and Greiner, Germany, respectively. Fluorescence measurements were done with a TECAN™ SPECTRAFluor® Plus fluorescence plate reader (TECAN, North Carolina) set at 485nm excitation and 530nm emission wavelength. Coulter-Clenz solution was purchased from Fisher, Illinois (cat. #23-257520). The 8-channel MultiPROBE II HT system (Packard Biosciences, Perkin Elmer Life Sciences, www.packardscience.com) was used for comparison of automated pipetting.

2.1. Protein crystallization

Crystals of TB protein Rv2697c, a deoxyuridine 5'-triphosphate nucleotidohydrolase from *Mycobacterium tuberculosis*, were obtained at 22°C by sitting drop vapor diffusion (McPherson, 1982) in 4µl drops (2µl of reservoir solution and 2µl of protein stock solution at 60.1mg/ml) suspended over a 200µl reservoir solution (16.55% polyethylene-monomethyl ether (PEG-MME) 2000, 3.24% DMSO and 0.66% glycerol).

2.2. Preparation of the Hydra®-PP microdispenser

Prior to the setup of crystallization experiments, the Hydra®-PP system's syringes were washed three times with distilled water, with each wash composed of 3 wash cycles. If crystallization cocktails contained organic solutions, the syringes were washed with methanol, ethanol, or acetone for three full-syringe-volume wash cycles and then with water for a further three wash steps. After the last use of the dispenser for the day, the Hydra® syringes were washed three times with Coulter-Clenz and then the syringes were rinsed with water for an additional three wash cycles.

2.3. Preparation of the single channel microsolenoid dispenser

Before aspirating protein samples, the single channel microsolenoid dispenser was washed with water for three full-syringe-volume (500µl) washes. An air gap (adjustable volume from 1µl to 10µl) was then aspirated into the dispenser followed by the aspiration of the protein sample. The above wash step was repeated between proteins. After the last use of the system for the day, the microsolenoid dispenser was washed three times with Coulter-Clenz and then was rinsed with water for an additional six wash cycles. The outside of the microsolenoid dispenser was rinsed and wiped clean.

3. Results and discussion

3.1. System design

The Hydra®-Plus-One system (Figure 1) consists of a Hydra®-PP 96-channel contact dispenser and a single channel, non-contact microsolenoid dispenser specifically designed to facilitate high-throughput protein crystallization. With the single channel dispenser, the protein samples are aspirated from a 0.5 ml microtube and dispensed at a speed of 60s per 96 single wells. More drops per well (for example, three in a Greiner plate) can be set up in a proportionally longer time. There is no waste of protein due to rearraying - with the exception of a few µl at the air gap interface, excess material can be recovered from the dispenser tubing. Up to 300µl of premixed crystallization cocktails can be aspirated by the 96-syringe-assembly from a 96 well deep well plate and dispensed into reservoirs and into droplet wells of a 96-well crystallization plate at a dispensing speed of 60s per 96-wells.

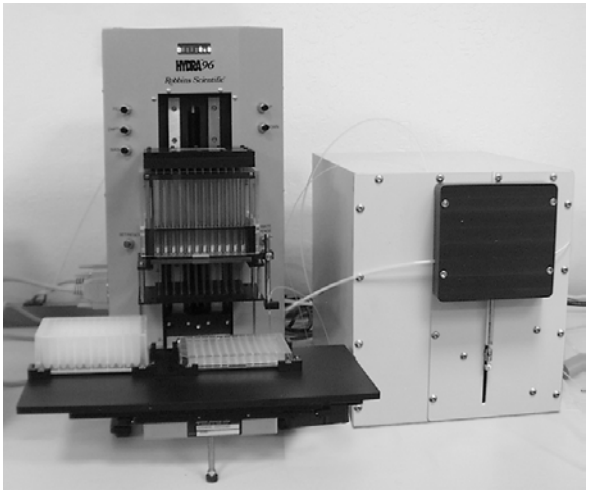

Figure 1

The Hydra®-Plus-One microdispenser, consisting of a Hydra®-PP (unit on left side) and a single channel microsolenoid dispenser (right side).

Washing the tips is a separate process that can be carried out after the crystallization plates are tape sealed, between changing proteins and crystallization screen cocktail plates. The Hydra®-PP microdispenser includes a programmable automated syringe wash system. The single channel dispenser is semiautomatically washed through the execution of wash commands from the software. Wash times for both the 96-syringe-array and the single-channel are a few minutes. One of the great advantages of the single channel dispenser is that it is based on a proprietary hybrid valve design, where the solenoid actuator is isolated from the sample flow. As a result, the microsolenoid orifice never comes in contact with the sample, and therefore does not get clogged.

3.2. Dispensing precision of the Hydra® system and the single channel microdispenser

Prior to the setup of crystallization reactions, the uniformity and consistency of the volumes dispensed across the array of 96 syringes in the Hydra®-PP systems were determined by the coefficient of variance (CV = standard deviation/mean) for specific dispensing volumes (0.1-10µl). Different volumes of a 10µg/ml fluorescein solution were dispensed into each well of a 96-well plate containing 0.1M Tris buffer. The final volume in each well after dispensing the fluorescein solution was 100µl. Each plate was read in a TECAN fluorescence plate reader and the CVs were determined across each plate for each of the dispensed volumes. A high precision in dispensing volumes as low as 0.1µl was evident with CVs of less than 8% (results shown in Table 1).

Table 1

Dispensing precision of a Hydra®-PP System equipped with 96 290µl syringes.

Volume of fluorescein dispensed (µl)	Relative fluorescence units	%CV
10.0	44481	1.62
5.0	23649	2.81
1.0	4805	3.21
0.5	2499	6.13
0.1	619	7.45

Uniformity and consistency of the volumes dispensed by the microsolenoid dispenser were also determined for volumes of 0.1-2µl. The CVs obtained were less than 7%. The high uniformity for the volumes dispensed is shown in Figure 2, where 1µl of a 100mg/ml BSA solution was dispensed onto a glass slide.

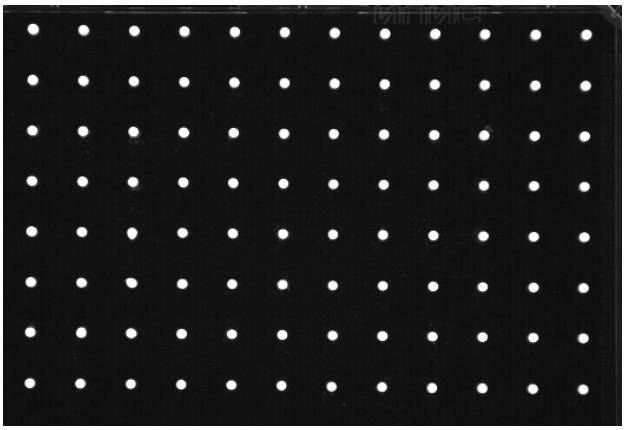

Figure 2

Uniformity and consistency of dispensing 1µl of a 100mg/ml BSA solution with the single channel microsolenoid dispenser. Bright spots are fluorescence signal of the BSA-fluorescence conjugate in drops on a glass slide (CVs $\leq$ 7%).

3.3. Accuracy of single channel dispenser

Dispensing accuracy of the single channel unit was evaluated by dispensing 1µl of 100% DMSO into a microplate and measuring the weight of the liquid dispensed. DMSO was chosen as the dispense medium to minimize measurement inaccuracies incurred by evaporation. The single channel dispenser transferred a 1µl solution with a dispense accuracy of greater than 90%.

3.4. Removal of residual protein contamination in single channel dispenser

A BSA-fluorescein conjugate solution (100mg/ml BSA) was used to test the effect of residual protein contamination in the single channel dispenser (needle and tubing). After dispensing of 100 µl of BSA-fluorescein solution, the dispenser was washed with multiple full-syringe-volumes (500µl) of water. The results (Figure 3) indicate that the dispenser can be washed out efficiently with three full-syringe-volumes (1500µl in total) water washes.

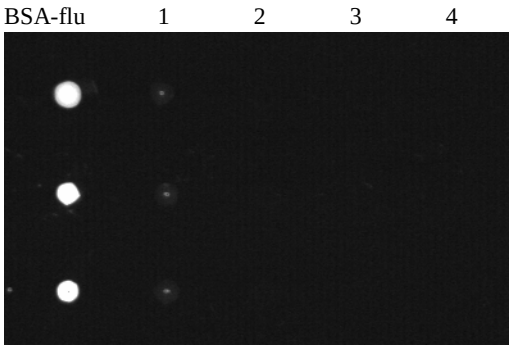

Figure 3

Removal of residual protein contamination from the single channel dispenser. A BSA-fluorescein solution (100mg/ml) was dispensed and the dispenser was washed with multiple full-syringe-volumes of water (500µl each). Numbers refer to the number of washes performed. Bright spots are fluorescence of BSA-fluorescein conjugate.

3.5. Dispensing speed of Hydra®-Plus-One system

Dispensing speed for the protein samples by the single channel microdispenser was 60s per 96 wells. With the Hydra®-PP 96-channel dispenser, the premixed crystallization cocktails were aspirated from a deep well plate and dispensed into the reservoir and into the droplet wells of a 96-well plate at a dispensing speed of 60s, resulting in a total setup time of two minutes per plate (90 seconds with integrated software). Allowing a generous time of four minutes for protein aspiration and washes per dispensing cycle (plate), the throughput of the system should easily exceed 10 96-well crystallization plates per hour.

3.5.1. Comparison of setup time between Hydra®-Plus-One and 8-channel MultiPROBE II HT. Initially the TB crystallization facility proposed automated pipetting and set up of crystallization plates with an 8-channel MultiPROBE II HT system (MP2) from Packard Biosciences, typical for many general-purpose liquid handling stations. Time for setup of one 96-well crystallization plate was directly compared between the MP2 and the Hydra®-Plus-One at each stage of pipetting action. By setting up a 96-well crystallization plate within 4 min 40s the Hydra®-Plus-One clearly outperforms the MP2 which requires 19 min for the same task (including 12 flush/wash cycles). Comparing each step of pipetting action the Hydra-Plus-One completes each task 3 to 20 times faster than the MP2. The greatest drawback of the MP2 are the time-consuming flush and wash cycles that have to be carried out after every group of 8 pipetting actions. Furthermore the MP2 system requires some manual intervention (e.g. putting tip ejection sleeves on the multi-purpose pipetting tips for use with disposable tips).

Table 2

Comparison of the setup speeds between the Hydra®-Plus-One and the Packard Instruments 8-channel MultiPROBE II HT system. Setting up a 96-well crystallization plate within 4 min and 40 seconds, the Hydra®-Plus-One clearly outperforms the MultiPROBE II HT which requires 18 min 52s for the same task (including 12 flush/wash cycles).

Pipetting Action	Robbins Scientific Hydra®-Plus-One in s / [min]	Packard Biosciences MultiPROBE II HT in s / [min]
Aspirate and dispense of 2µl protein	90 [1.50]	296 [4.93]
Aspirate and dispense of 200µl buffer to reservoir	60 [1.00]	200 [3.33]
Aspirate and dispense of 2µl buffer to drop	10 [0.17]	236 [3.93]
Intermediate wash cycles	-- --	360 [6.00]
Final wash cycle	120 [2.00]	40 [0.67]
Total time for setup of one complete 96-well plate	280 [4.67]	1132 [18.86]

3.6. Reproducibility of crystallization experiments

The first crystals of Rv2697c from *Mycobacterium tuberculosis* were grown *de novo* in CRYSTOOL screens set up in Greiner crystallization plates using the Hydra®-Plus-One system. Results were highly reproducible (Figure 4). By setting up crystallization plates with high speed, evaporation of protein solution and volatile crystallization buffers is minimized, therefore ensuring consistency of the experimental conditions. Either dispensing mode (protein first or cocktail first) yielded the same results.

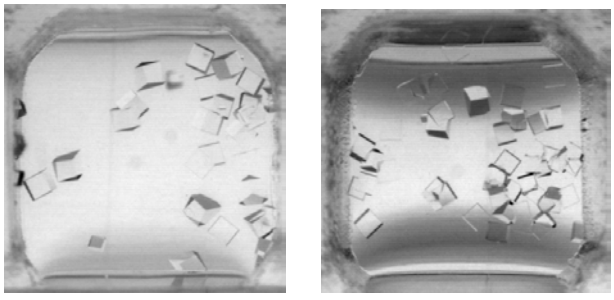

Figure 4

Crystals of Rv2697c from Mycobacterium tuberculosis, grown *de novo* in two different wells of a 96 condition CRYSTOOL screen using the Hydra®-Plus-One system. Diffraction limit 1.95Å.

4. Conclusions

The Hydra®-Plus-One system, consisting of a Hydra®-PP 96-channel contact dispenser and a single channel non-contact microsolenoid dispenser was specifically designed to facilitate high-throughput protein crystallization. The speed of plate setup (2 min 40 s) using 96-well Greiner plates clearly exceeds the performance of a typical general-purpose liquid handling system (MultiPROBE II HT, 18 min 52 s) about seven-fold. Protein loss is minimal due to absence of rearraying. Removal of protein and buffer cross-contamination from both dispensing systems is efficient with optimized volumes of washing liquid, as shown by testing with BSA-fluorescein solution. High volume and positional precision are key features of the device and have lead to *de novo* crystallization of deoxyuridine 5'-triphosphate nucleotidohydrolase from *Mycobacterium tuberculosis* with high reproducibility. The most outstanding feature of the Hydra®-Plus-One is the system's high precision of low-volume dispensing and positioning of the protein droplets. This capability ensures reproducibility - a major factor for successful automated optimization of crystallization conditions. Possibility of further miniaturization of crystallization screening down to nanoliter volumes is a major, material saving advantage for high-throughput efforts.

The Hydra®Plus-One System's modular design allows for easy integration with various lab automation components for liquid handling and plate transport available from a variety of vendors. Embedded in a fully automated process, the throughput of the system is projected to exceed 10 96-well crystallization plates per hour. Combining highly reproducible, cost-effective and fast technologies, the new Hydra®-Plus-One System appears to be a valuable contribution to efficient high-throughput protein crystallization.

The TB consortium cloning and protein production facilities under J. Perry, C. Goulding, D. Eisenberg (UCLA) and M. Park and G. Waldo (LANL) have supplied the Rv2697c TB protein crystallized in this study. LLNL is operated by University of California for the US DOE under contract W-7405-ENG-48. This work was funded in part by NIH P50 GM62410 (TB Structural Genomics) center grant.

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