Short Technical Report

Ink-Jet Printer Heads for Ultra-Small-Drop Protein Crystallography

BioTechniques 33:1302-1306 (December 2002)

**E.I. Howard^{1,2} and
R.E. Cachau³**

¹Institute de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France, ²Instituto de Fisica de Liquidos y Sistemas Biologicos, La Plata, Argentina, and ³National Cancer Institute, SAIC, MD, USA

ABSTRACT

Mass-produced automated piezoelectric driven picoliter delivery systems (printer heads) are fast, inexpensive, and reliable devices that are capable of delivering a very large range of volumes and are ideally suited for high-throughput protein crystallography studies. We used this technology to set up under-oil crystallization experiments with drop sizes from the 200-nL to 3- μ L volume range, commonly used in protein crystallography, and show its application in setting ultra-small (2 nL) drops, the smallest drop volume reported to date for this type of assay.

INTRODUCTION

X-ray crystallography is the technique most frequently employed to provide structural information in high-throughput structural proteomics and genomics studies (12). Advances in experimental techniques allow the automation of most of the steps required by high-throughput crystallography experiments (6). A remaining challenge is the application of fully automated approaches to protein crystallization and crystal manipulation (6,14). Crystallization conditions depend on a large number of parameters (e.g., protein concentration, pH, temperature, and ionic strength). Experimental screens, such as those based on sparse matrix and incomplete factorial designs, can be used to find initial conditions ([www-structure.llnl.gov/crystool/crystool.htm](http://www.structure.llnl.gov/crystool/crystool.htm)) (1,2,7,8). Additional experiments are required to optimize the crystallization conditions (13). Large amounts of highly purified, highly homogeneous protein solutions are required to scan and zero-in on the crystallization conditions. Using the robotic controlled setting of small-volume assays is the simplest way to reduce the amount of material required and to accelerate crystallization assays (9). Automated piezoelectric driven picoliter delivery systems (PDPDSs) are capable of delivering sub-picoliter size drops at a very high speed and with great volume accuracy (15). Commercially available piezoelectric fluid-dispensing devices are used in many different applications

to dispense drops in a wide range of volumes. However, the most common use of PDPDSs is for drop-on-demand ink-jet printing. Ink-jet printer heads have a fluid-filled chamber with an inlet at one end and a nozzle at the other (11,15,16). A piezoelectric element induces an acoustic wave in the fluid, causing a drop to be ejected from the nozzle. No mechanical parts are involved in the fluid handling, which makes the printer heads very reliable and accurate devices. In this work, we use printer heads to deliver protein and precipitant solutions to set the crystallization assays in a range of volumes extending to 2 nL per drop.

MATERIALS AND METHODS

Lysozyme crystallization assays were carried using an Epson 480SX printer (Epson, Long Beach, CA, USA) (Figure 1). The printer paper carrier mechanism was dismantled to gain access to the printer head, but no modifications were made to the printer head. Solutions were dispensed through the standard tubing using empty (i.e., not previously filled) cartridges. Crystallization solutions were adjusted with small amounts of glycerin and Tween[®] 20 to a final surface tension of approximately 42 dyn/cm. All solutions were prepared from chemicals of the purest quality available and dissolved in Milli-Q[®] quality water. All solutions were filtered through 0.22 μ m filters before the addition of Tween 20. A light min-

eral oil or a very low-density siliconized oil (polydimethylsiloxane)(3) was placed in the wells of standard NUNC™ microplates (Nalge Nunc International, Rochester, NY, USA) before the delivery of the solution components. After the individual solution components were dispensed, the microplate was centrifuged at 3000–7000 rpm using a modified basket with an approximate 5-cm rotor radius corresponding to relative *g* values between 500 and 2700. We were concerned about the actual accuracy of ink-jet printer heads when used in the context of protein crystallography. The mean variation of the ejected drop size in standard factory tests is rated at $\pm 10\%$. It is important to know whether this error is constant when larger accrued drops (delivered by repeated injection of picoliter drops) are delivered. We measured drops of different sizes, ranging from 100 nL to 40 μL , using 5% glycerin solutions including dye reactants or lysozyme. Drop size was measured in several ways. Drops were collected either in mineral oil-filled microplates or in Whatman® paper sandwiched in cellophane to avoid evaporation. The later experiments were used for the direct gravimetric analysis of the drop size. The analysis of these samples indicates that the rela-

tive error versus drop size is largely constant with size (all drops measured fall within $\pm 3\%$ relative error from the average value). This suggests that the error is mostly randomly distributed, as claimed in the literature (15,16). It was also important to know the variability among the nozzles. We used the three-color nozzle arrays to test this variability. Empty color ink cartridges were filled with glycerin solution (5% w/w). A total of 27 tests were done, including all possible combinations among the three nozzles (from 100% + 0% + 0% ... to 0% + 0% + 100%), scanned in thirds. The variability observed is similar to that observed in single nozzle experiments ($\pm 4\%$). The variability of drops set under oil was tested by preparing drops composed of a pH indicator (either Bromphenol Blue or Phloxine B) and preparing series of drops of variable size with increasing additions of a neutralizing agent. This test, equivalent to a microtitration experiment, can be calibrated to 5% error. No significant differences with drop size were observed in these experiments either. The overall average error of the colorimetric assay (estimated in $\pm 5\%$) limited our ability to discern smaller variations among the assays.

DISCUSSION

The primary aim of this work was to make it possible to use mass-produced ink-jet printer heads for the automated screening of protein crystallization conditions. Ink-jet printer heads allow the exploration of crystallization conditions at a very low cost, while extending the technique of high-throughput crystallography to volume regimes previously unexplored. Ink-jet printer heads are obtained by sintering multiple layers of patterned clays that, upon sintering, solidify in a single body. Thus, these devices do not contain mobile parts and are highly resistant to corrosion. The parameters controlling drop ejection are related by the Weber number (W_e) (10). W_e is proportional to the square of the oscillation frequency of the piezo element and to the inverse of the fluid surface tension; thus, a wide variety of fluid compositions can be handled by tuning the oscillation frequency. The acoustic propulsion of the drop from a printer head nozzle does not require the assistance of gravity or capillarity (i.e., drops can be ejected in any direction including sideways and upwards), allowing for great flexibility in the equip-

Figure 1. Epson Stylus 480SX printer and placement of NUNC microplate.

Figure 2. Protein crystallization assay, using ultra-small drops. (A–C) A screen using 160 nL volume drops to mix NaCl solutions (100, 70, and 60 mg/mL, respectively) and lysozyme (32 mg/mL) in 1:1 ratios. Note the systematic growth of the crystals from the surface of the drop inwards when very small drops are used. This may suggest an active role of the water-oil surface in the nucleation process. (D) A 2-nL drop. This drop was set using the black ink reservoir only by pre-mixing NaCl (45 mg/mL) with lysozyme solution (36 mg/mL). The crystal size is approximately $20 \times 30 \times 50 \mu\text{m}$. This crystal grew in less than 8 h. All drops were incubated at 4°C .

Proteomic Technologies

ment design. Printer head nozzles are arranged in arrays that reach densities two orders of magnitude higher than customary in fluid-dispensing robots used in crystallography assays. The fluid pass-through mechanism in these devices allows for repeated dispensing without reloading, which drastically reduces the setting time of experiments. The total flux in ink-jet printers can be estimated from the frequency of oscillation and the number of nozzles in the printer head. At oscillation frequencies of 10^{-4} s, a printer head can deliver 10^8 ultra-small drops per minute. Total fluid flux of milliliters per minute can be obtained with a typical number of nozzles in the printer heads (30–600). Mass-produced printer heads can deliver variable size drops (0.3–40 pL), thus adding flexibility to the experimental design. Plate density and other experimental parameters obtained using these devices can be estimated from the im-

age resolution of ink-jet printers (Epson). Ink-jet printers can reach a resolution of 2880 dots per inch (3 μ m separation between dots) and can print several pages (8.5×11 in) per minute. Coalescing matrices of 100×100 ejected microdrops per experiment to form the crystallization drops would result in approximately 5000 settings per minute. This rate is faster than that of commercially available crystallization robots. Using the smallest drops attainable with ink-jet printer heads [approximately 0.3 pL (4)], a full page (2×10^8 drops) can be filled with approximately 0.055 mL of material, resulting in great savings in the amount of protein required to screen a very large number of conditions. This super-fast setting of ultra-small samples can be used to obtain a near continuum screening of conditions. A nearly continuous sampling of conditions should help in the identification of narrow regions of metastability (5). This may be

especially helpful in the crystallization of complex multimers (e.g., RNA or DNA protein complexes) or membrane proteins, in which the metastable regime is characteristically narrow. The smaller drop size attainable with printer heads will contribute to the faster analysis of conditions. The shorter time required for crystal formation in smaller drops results in a reduction in the degree of protein decomposition caused by aggregation, denaturation, oxidation, and deamidation, resulting in cleaner, more reproducible experiments. Ultra-small drops may also result in simplifications in the automated scanning of the settings because depth focusing during drop scanning may not be required.

Although technology is frequently transferred from the research bench to the mass market, the reverse process has been a common occurrence in recent years. Clusters of personal computers (Beowulf Computers; www.beowulfcomputers.com).

Proteomic Technologies

Figure 3. Crystallization optimization using large (3 µL) drops. The printer cartridges were filled as follows: cyan, 50 mM sodium acetate, pH 4.5; magenta, 40 mg/mL lysozyme (in buffer, pH 4.5); and yellow, 3 M NaCl (in buffer, pH 4.5). The concentration of NaCl decreases from left to right and top to bottom (top three rows). Bottom: a close-up view of the 11th well. The plates were incubated at 24°C. The pictures were taken after 24 h of incubation.

beowulf.org) built with commodity parts epitomize this trend. Beowulf machines revolutionized the use of supercomputers by introducing low cost, high-performance devices that expanded the use of massive computing to new areas of research. Similarly, mass-produced picoliter-dispensing technologies (ink-jet printer heads) may revolutionize the use of ultra-small samples in the laboratory environment. High throughput can be achieved by clustering these devices because of their very low cost, opening the possibility of using high-throughput crystallography in non-specialized laboratories.

ACKNOWLEDGMENTS

We thank Pascal Marie (Institut Charles Sadron-Strasbourg), Federico Martin-Ruiz, Valerie Lamour, and Raul Grigera for help with surface tension measurements and helpful discussions. We thank Dino Moras for their support during our stay at the IGBMC and Agnetha Hansgrdh for proofreading the manuscript. E.I.H. is a member of the "Carrera de Investigador," CONICET. This work was funded by the National Cancer Institute-National Institutes of Health (contract no. NO1-CO-12400). The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. **Baldock, P., V. Mills, and P.D. Shaw Stewart.** 1996. A comparison of microbatch and vapor diffusion for initial screening of crystallization conditions. *J. Cryst. Growth* 168:170-174.
2. **Carter, C.W.J.** 1990. Efficient factorial designs and the analysis of macromolecular crystal growth conditions. *Methods* 1:12-24.
3. **Chayen, N.E.** 1996. Crystallization with oils: a new dimension in macromolecular crystal growth. *Protein Eng.* 9:927-929.
4. **Chen, A.U. and O.A. Basaran.** 2002. A new method for significantly reducing drop radius without reducing nozzle radius in drop-on-demand drop production. *Phys. Fluids* 14:L1-L4.
5. **Haas, C., J. Drenth, and W.W. Wilson.** 1999. Relation between the solubility of proteins in aqueous solutions and the second virial coefficient of the solution. *J. Phys. Chem.* 103:2808-2811.
6. **Heinemann, U., G. Illing, and H. Oschkinat.** 2001. High-throughput three-dimensional protein structure determination. *Curr. Opin. Biotechnol.* 12:348-354.
7. **Hol, W.G.J., S.H. Sarfaty, L.J. Stewart, and H. Kim, inventors; University of Washington, assignee.** Crystallization media. US patent 6 267 935. 2001 July 31.
8. **Jankaric, J. and S.H. Kim.** 1991. Sparse matrix sampling: a screening method for crystallization of proteins. *J. Appl. Crystallograph.* 24:409-411.
9. **Mueller, U., L. Nyarsik, M. Horn, H. Rauth, T. Przewieslik, W. Saenger, H. Lehrach, and H. Eickhoff.** 2001. Development of a technology for automation and miniaturization of protein crystallization. *J. Biotechnol.* 85:7-14.
10. **Peakall, J. and J. Warburton.** 1996. Surface tension in small hydraulic river models—the significance of the Weber number. *J. Hydrol.* 35:199-212.
11. **Sano, T. and K. Takemoto, inventors; Seiko Epson Corporation, assignee.** Yellow ink realizing image having excellent lightfastness and color development. US patent 6 284 029. 2001 Sept 4.
12. **Schulz, W.G.** 2001. Determining structure. *Chem. Eng. News* 79:23-26.
13. **Shaw Stewart, P.D. and P.F.M. Baldock.** 1999. Practical experimental design techniques for automatic and manual protein crystallization. *J. Cryst. Growth* 196:665-673.
14. **Stevens, R.C.** 2000. High-throughput protein crystallization. *Curr. Opin. Struct. Biol.* 10:558-563.
15. **Usui, M., K. Hara, Y. Miyazawa, Y. Tanaka, F. Akahane, T. Katakura, S. Sakai, Y. Kishida, T. Abe, T. Usui, T. Seino, and Y. Yamamoto, inventors; Seiko Epson Corporation, assignee.** Ink jet recording head. US patent 5 748 214. 1998 May 5.
16. **Usui, T., T. Katakura, and T. Abe, inventors; Seiko Epson Corporation, assignee.** Ink jet recording apparatus. US patent 5 818 472. 1998 Oct 8.

Received 14 March 2002; accepted 17 June 2002.

Address correspondence to:

Dr. R.E. Cachau
Advanced Biomedical Computing Center
NCI-SAIC
430 Miller Drive
Frederick, MD 21702-1201, USA
e-mail: cachau@ncifcrf.gov

Dr. E.I. Howard
IFLYSIB
Calle 59 No. 789
1900, La Plata, Argentina
e-mail: howard@iflysib.unlp.edu.ar

**For reprints of this or
any other article, contact
Reprints@BioTechniques.com**