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Development of a fully automated macromolecular crystallization/observation robotic system, HTS-80

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A robotic system has been developed to be used for macromolecular crystallization and observation in typical university laboratories with a research focus on protein crystallography. The system consists of three major parts: a dispenser unit, a storage unit and an observation unit. This system is designed to automatically perform all of the processes involved in crystallization and observation without requiring any manual operations. The dispenser and observation units can carry out both sitting-drop vapor-diffusion procedures and microbatch procedures. With this system, the procedures are controlled by a personal computer running GUI-based software. After the dispensing of protein solution into the crystallization plates, they are automatically transferred to the storage units, followed by automatic observation according to a required schedule with arbitrary intervals. At each stage of crystallization, droplets in the crystallization plates are examined by original image-processing software in order to evaluate the appearance of the crystals.

1. Introduction

The crystallization of macromolecules is one of the most laborious steps in protein crystallography, as it relies on a trial-and-error approach. To achieve crystallization, many time-consuming steps must be carried out, such as the dispensing of crystallization plates and the repeated observation of those plates. The automatization of both the crystallization and the observation process is therefore indispensable for the promotion of study in the fields of structural genomics and structural biology. Recent advances in the robotics technology used for visualizing enabled us to design and construct an automatized crystallization and observation system to be used for the analysis of biological macromolecules. Such technology is widely employed in high-throughput protein crystallography in structural genomics/proteomics projects conducted worldwide (Terwilliger, 2000; Yokoyama *et al.*, 2000; Kuhn *et al.*, 2002; Taylor, 2002; Luft *et al.*, 2003). The equipment used at the larger facilities and factories is not targeted to typical university laboratories, which operate on a smaller scale. Our newly developed system is suitable for achieving optimal performance in such typically smaller sized university laboratories. Although several commercial systems are available for dispensing (Rupp *et al.*, 2002), they still require manual processing. As has previously been proposed as 'middle-throughput' crystallization (Sulzenbacher *et al.*, 2002), we developed a fully automatized robotic system for use in typically sized university/institute laboratories that commonly perform crystallization studies for the investigation of biological macromolecules.

The fundamental concept of the present robotic system is that it is fully automated and can be used for all protein crystallization processes, including the following steps: the dispensing of mother liquids and protein solutions, the observation of crystal growth and the evaluation of crystals. Each stage is achieved by the use of two types of highly accurate 12-channel dispenser for the crystallization reagents and a one-channel for protein solution, sealing device for the crystallization wells, storage boxes and observation device, with an optics system for crystal growth and an observation schedule-control system. Both commercially available crystallization plates and any of the standard sparse-matrix crystallization reagents can be employed with this system. Each process is controlled by the software

and the current status and the final results are displayed on a personal computer.

2. Construction of the system

2.1. System overview

A fully automated macromolecular crystallization/observation robotic system (referred to as the 'HTS-80') is presented in Fig. 1. The details regarding its performance and specifications are shown in Table 1. This system consists of three separable units for dispensing, storage and observation. The units are then integrated on a personal computer. Both the sitting-drop vapor-diffusion method and the microbatch method can be employed for crystallization with this system. Because this system was aimed at middle-throughput operations, we developed it with the goal of enhancing the accuracy of dispensing rather than promoting dispensing speed. The system employs commercially available crystallization equipment (*e.g.* 24- or 96-well plates with an SBS standard) and also widely used sparse-matrix screening reagents such as Crystal Screen I, II and HT (Hampton Research, http://www.hamptonresearch.com) and JBScreens 1–10 (JENA Bioscience, http://www.jenabioscience.com/staticpage/index.html). The system was designed to minimize the evaporation of protein solution from crystallization wells during dispensing in order to promote crystallization reproducibility. In addition, the contamination by dust is prevented by an air-filter unit in operation during the entire experimental procedure.

The observation processes, following dispensing, are extremely time-consuming and laborious; therefore, full-automated processing is an advantage introduced into this novel system. The crystallization plates are automatically transferred to the storage units and the observation optics is placed in the storage units to prevent temperature drift in the plates. The current status of each droplet is also automatically examined by the system.

2.2. Dispenser unit

The layout of the dispenser unit is schematically depicted in Fig. 2(*a*). The dispenser head has a 12-channel dispenser for the crystallization reagents and a one-channel dispenser for the protein solution, as shown in Fig. 2(*b*). The pipetting range of the 12-channel dispenser is between 0.5 and 50 µl, and that of the one-channel dispenser is between 0.5 and 5.0 µl. The processing of one 96-well crystallization plate is completed within 30 min from dispensing to sealing. The detailed dispensing procedure is illustrated in Fig. 2(*d*). The accuracy of dispensing was examined by the Bradford method (Bradford, 1976) using a Bio-Rad protein quantification kit (Bio-Rad Laboratories Inc.).

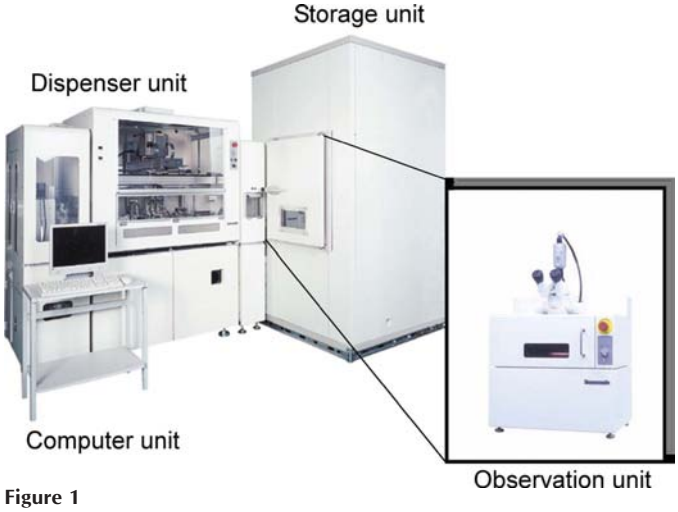


Figure 1
Overview of the HTS-80 system. The HTS-80 system consists of three separable units: a dispenser, a storage unit and an observation unit built into the storage unit. These units are all controlled by a computer unit, upon which the control GUI is installed.

Table 1
Performance specifications for the HTS-80.

External dimensions, W/D/H (mm)	
Dispenser unit	1900/930/1800
Observation unit	550/420/850
Storage unit	1100/1100/2400
Dispenser unit	
Dispenser head	Dispenser for crystallization reagents, 12-channel: 0.5–50 µl Dispenser for protein solution, one-channel: 0.5–50 µl
Plate capacity	Deepwell plate for crystallization reagents: 10 plates Crystallization plate: 10 plates (24/96-well)
Tip capacity	Five tip racks (384 tip by Panasonic)
Sealer	Apply to SBS standard 24/96-well plates
Observation unit	
Observation stage	*X*/*Y*/*Z* axes
Microscope	Upright-stereo microscope
Lighting	Halogen lamp
Application software	Auto 4-stage evaluation of crystal maturation
HDD capacity	800 GB
Storage unit	
Crystallization plate capacity	400-plate max.
Temperature range (K)	277 ± 2, 293 ± 2

The inside of the unit is kept clean by a HEPA-filter unit to prevent contamination with dust during the operation. Dispensing tip racks, crystallization reagents and crystallization plates are stored in each stacker. The stackers for the crystallization reagents can be removed from the dispenser unit for storage in a refrigerator (Fig. 2*c*). During the procedure, tip racks for micro-tips are transported to the tip-rack stage by the plate conveyor. Each crystallization reagent rack is covered with a lid when it is stored in the stacker. The deepwell plate for the crystallization reagents is carried by the plate conveyor to the crystallization reagent stage and the lid is subsequently opened by the lid-off unit. The cooling stage for the protein solution is cooled by circulating water from a chiller. Each crystallization plate is transferred to the crystallization plate stage. The sealing unit immediately seals each line of 12 crystallization wells in the crystallization plate after the dispensing of the corresponding line. Such a quick sealing procedure promotes the reproducibility of crystallization by minimizing the evaporation of the crystallization reagents from the crystallization plates. After dispensing, the crystallization plates are conveyed to the storage unit *via* a barcode reader. A barcode seal is placed on each of the crystallization plates for later identification and further sample management.

2.3. Storage unit

The conveyor belt transports the dispensed crystallization plates from the dispenser unit to a plate stage external to the apparatus. The plate-handling arm grasps the crystallization plates and then places them into each partition on a shelf in the storage unit (Fig. 3). The storage unit can hold a maximum of 400 crystallization plates. The temperature in the storage unit is controlled at 277 or 293 K.

2.4. Observation unit with image-analysis software

The observation unit is placed in the storage unit to prevent temperature changes during the observation procedure. The obser-

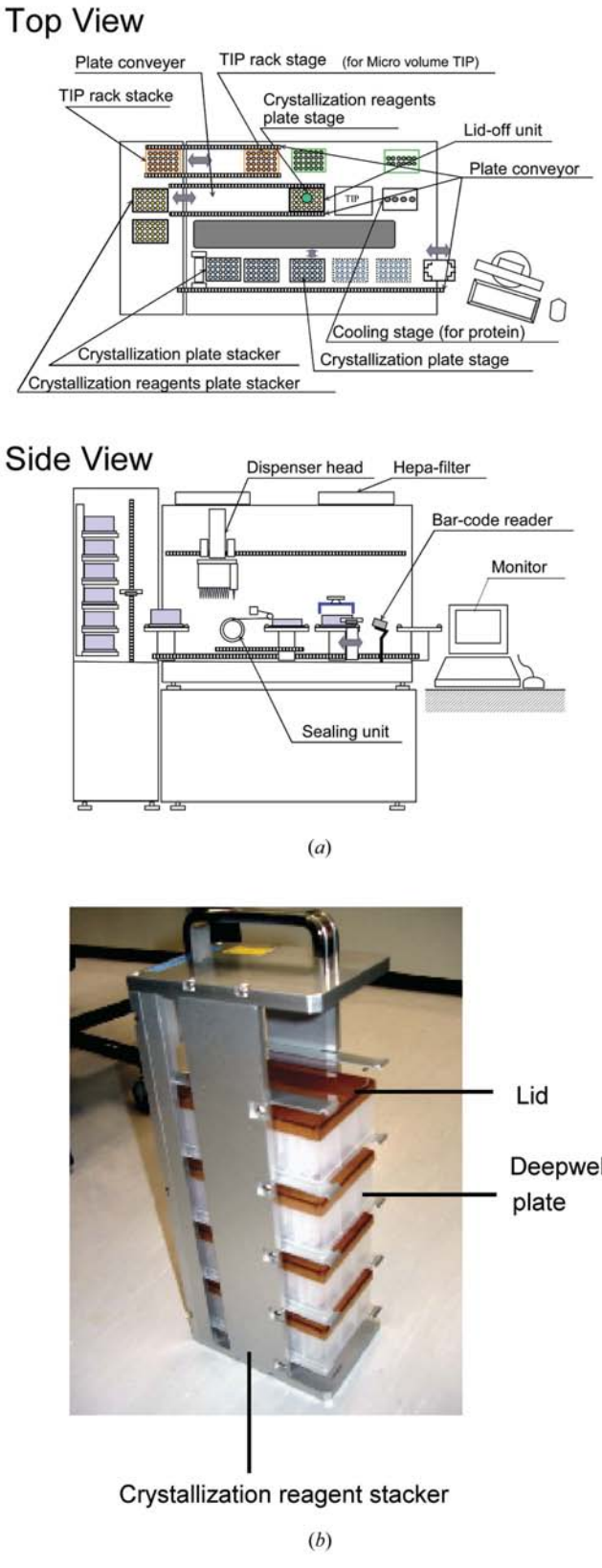


Figure 2
(*a*) Schematic drawing of the top and side views of the dispenser unit. The stackers for the tip racks, the deepwell plates for the crystallization reagents and the crystallization plates are carried by a plate conveyor belt to the tip rack stage, the deepwell plate stage and the crystallization plate stage, respectively. (*b*) The dispenser head equipped with the 12- and one-channel dispensers. (*c*) A stacker for the deepwell plates of the crystallization reagents. This stacker can be removed from the dispenser unit for refrigeration and storage. (*d*) Schematic illustrations of the dispensing. In the procedure, (1) each tip of the 12-channel dispenser takes up the crystallization reagent from a 96-deepwell plate and then ejects it onto the crystallization stage, (2) the tip of the one-channel dispenser leaves individual droplets of protein solution on each stage of a single sequence of 12 wells, (3) the 12-channel dispenser dispenses each crystallization reagent onto the stages and (4) the sealing unit seals one sequence of 12 dispensed wells.

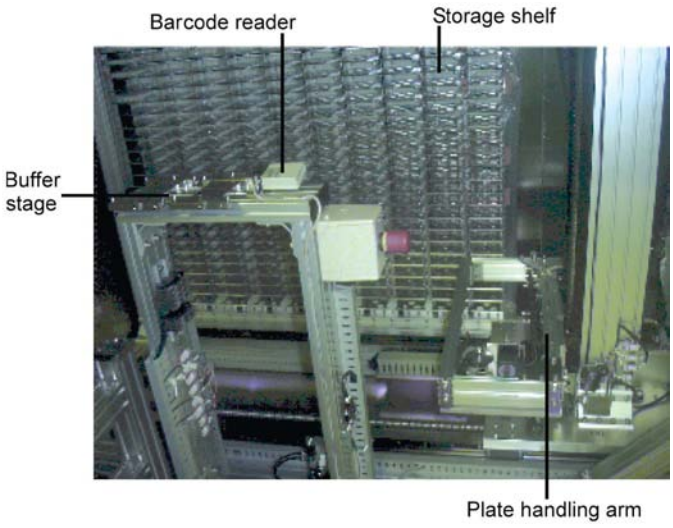
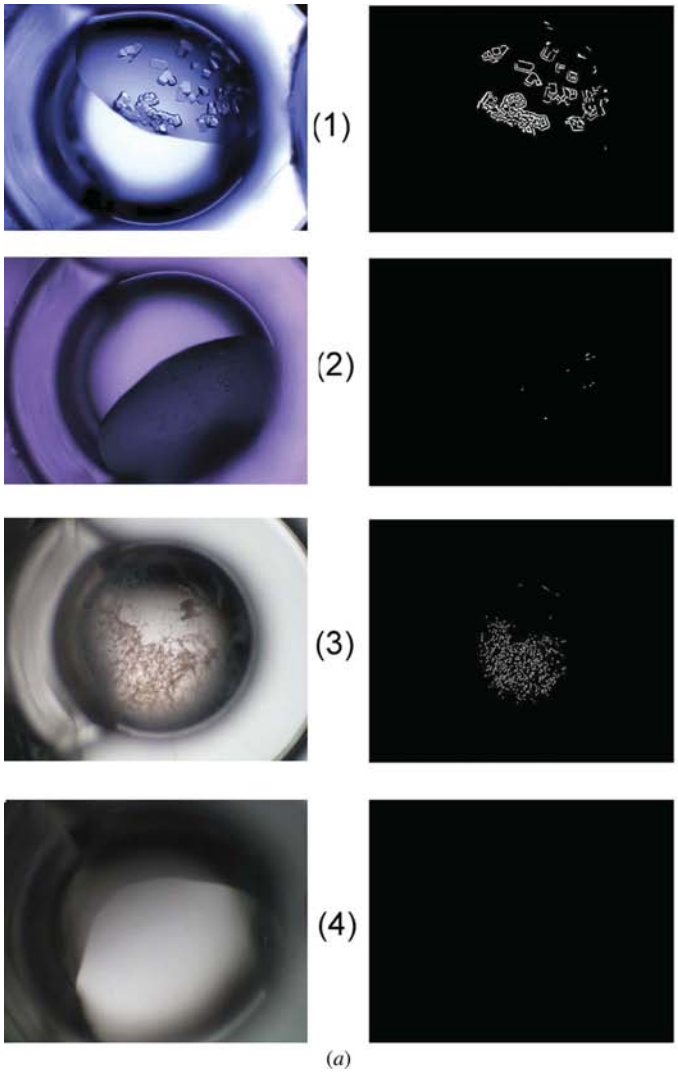


Figure 3
The inside of the storage unit. The storage shelves can accommodate a maximum of 400 crystallization plates. The plate-handling arm carries each plate from a partition on the shelf to the observation unit inside. The barcodes placed on the plates are read on the buffer stage.

vation unit consists of an upright stereo microscope (4× or 10× object lens magnitude), a digital camera (800 000 pixels) and an *XYZ* stage for the crystallization plates. In order to increase the depth of field, multiple images can be taken for each well by moving the *Z* (perpendicular) axis according to the input pitches. The image data are immediately transferred to the computer unit and examined by the image-analysis software in order to categorize the current status of the droplet based upon a variety of features corresponding to crystals. In contrast, most other observation systems evaluate the crystals by the template-matching technique. However, they have to beforehand memorize the various patterns of crystal images. Thus, they may not recognize the crystals with unknown patterns. Our system, on the other hand, extracts the features common in all of crystals, such as shapes and continuousness of the edges, without any templates for the pattern matching. It can distinguish crystals in the droplets and other noises arising from the crystallization plates or the precipitants in the droplets (Fig. 4*a*). Our current software categorizes the droplet status into four classes by corresponding colors: red, green, yellow and no color, as shown in Fig. 4(*b*). The red frames in the photographs represent droplets with status 'crystal'; those in green, 'pre-crystalline'; yellow, 'non-crystalline'; and no color, 'clear'. By this four-stage categorization, the labor of the operators to judge the crystallization with their own eyes is greatly reduced, because the



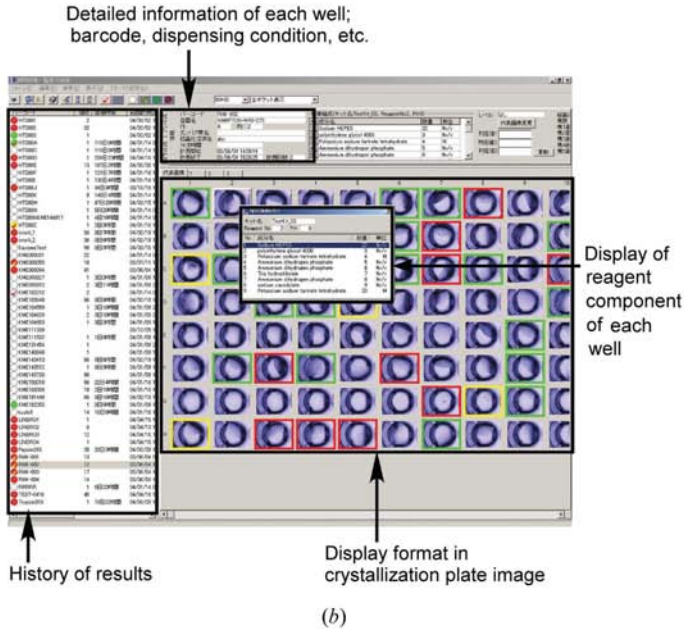


Figure 4
(*a*) Example photographs of the image-processing analysis. The image-processing software assesses each state in the droplet according to an algorithm developed by our group. The photographs in the left row were taken by the observation unit. The images in the right row were obtained by the image-processing software with regard to differentiation, binary coding, thinning and noise removal. As a result, the condition of each droplet can be automatically recognized and classified as follows: (1) crystal, (2) pre-crystalline, (3) non-crystalline (precipitation) and (4) clear. (*b*) An example image of the control GUI window with droplet photographs. We can intuitively recognize these conditions at one view of the GUI window, because frame colors of the photographs arrayed in the GUI correspond to the droplet statuses as mentioned above: red represents the condition of 'crystal', green, 'pre-crystalline', yellow, 'non-crystalline' and no color, 'clear'. The observation history of each droplet in the control GUI window can be displayed by double-clicking on a photograph of interest. A colour version of this figure is available in the online edition of the journal.

droplets evaluated as 'pre-crystalline' or 'clear' can be excluded from the examination targets.

3. Validation of the system

3.1. Measurement of dispensing accuracy (CV values)

In order to assess the dispensing accuracy of the 12-channel dispenser, three solvents were used as representatives of crystallization reagents and protein solutions. 20%(*w*/*v*) polyethylene glycol 8000 solution, water and 30% ethanol were employed, as they are solutions with different viscosity, *i.e.*, solutions with high, medium and low viscosity, respectively, were tested. Each solvent contained 20 mg ml^{-1} tartrazine ($C_{16}H_9N_4Na_3O_9S_2$) as a yellow dye marker. Each droplet of dispensed solvent on the crystallization stage was washed off with a small volume of water and the washed-out solvents were then transferred to each well of a 96-well plate in order to measure the optical density. The samples were then filled to a total volume of 100 µl with water in order to measure the concentration of the dye marker at 405 nm. The quantitative investigation was performed using the standard curve of each solution. Statistical analysis was carried out by computing the coefficient of variation (CV) of 96 wells. In order to evaluate the dispensing ability of the one-channel dispenser, 10 mg ml^{-1} lyophilized lysozyme (Worthington Biochemical Corporation) in 0.06 *M* sodium acetate pH 4.8 was examined. The whole volume of lysozyme solution dispensed on the crystallization stages was washed with a small volume of water and was transferred to a 96-well plate for measurement by filling water to a total volume of 100 µl. The quantitative investigation was performed using a standard curve of the lysozyme solution. The obtained CV values, including the most viscous solvent of 30% PEG 8000, converged below 10%, as shown in Table 2. The dispensing accuracy thus appeared to be comparable with that of manual dispensing.

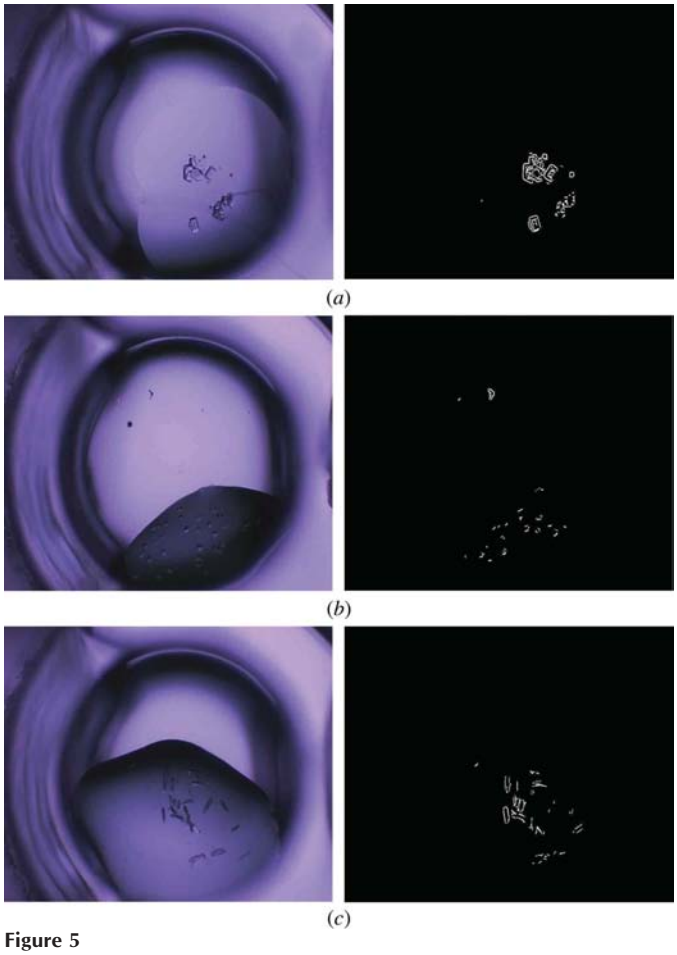


Figure 5
Examples of crystallization using the HTS-80 system. Original photographs of (*a*) DNase I, (*b*) glucose isomerase and (*c*) xylanase (left row). The processed images in the right row were obtained by the image-analyzing software to access the droplet status. In the images, only the edges corresponding to the crystal outlines are successfully extracted.

Table 2
Dispensing accuracy assessed by coefficient of variation (CV) values (%).

	12-channel dispenser for crystallization reagents			One-channel dispenser for protein solution
	Water	30% EtOH	30% PEG 8000	10 mg ml^{-1} lysozyme
0.5 µl	4.9	4.3	7.6	6.3
1.0 µl	2.9	5.1	9.8	3.5
5.0 µl	7.7	2.6	1.8	3.7

3.2. Crystallization using HTS-80

Protein samples of DNase I, catalase, glucose isomerase and xylanase were crystallized on the HTS-80 system in order to examine its performance. 20 mg of lyophilized DNase I (Sigma) and catalase (Sigma) were dissolved in 1 ml of water. 500 µl of liquid glucose isomerase (Hampton Research) and xylanase (Hampton Research) were centrifuged and the supernatant of each sample was obtained. Crystal Screen Cryo (except Nos. 49 and 50) and Crystal Screen II (Hampton Research) were stored in each well of a deepwell plate (Corning). Wizard Screens I and II (Emerald BioStructures), used as reagents, were also stored in another deepwell plate. The 96-well crystallization plates were then placed into the stacker. Tubes of protein solution were placed into the cooling stage, which was maintained at 277 K. 50 µl of crystallization reagent were dispensed into each well of the crystallization plates by the 12-channel dispenser. 0.5 µl of protein solution and the same volume of crystallization reagent were then mixed together on the crystallization stages in the crystallization plates. The dispensed plates were then incubated at 293 K in the storage unit. Each plate was periodically observed under the control of the scheduling software.

3.3. Automatic crystal detection in the droplets

After 6 d, crystals of DNase I, glucose isomerase and xylanase were successfully detected by the image-analysis software, as shown in Fig. 5. The software did not detect crystals of catalase. Manual inspection of the crystal plates revealed no catalase crystals, which demonstrated that the software was able to successfully determine the initial state of crystal growth and correctly distinguish between the presence and absence of the crystals.

In summary, we have developed and implemented a fully automated crystallization/observation robotic system, the HTS-80. This robotic system can automatically perform each step of the protein crystallization process, including the observation and evaluation of crystallization plates.

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References

Bradford, M. (1976). *Anal. Biochem.* **72**, 248–254.
Kuhn, P., Wilson, K., Patch, M. G. & Stevens, R. C. (2002). *Curr. Opin. Chem. Biol.* **6**, 704–710.
Luft, J. R., Collins, R. J., Fehrman, N. A., Lauricella, A. M., Veatch, C. K. & DeTitta, G. T. (2003). *J. Struct. Biol.* **142**, 170–179.
Rupp, B., Segelke, B. W., Krupka, H. I., Lekin, T., Schäfer, J., Zemla, A., Toppani, D., Snell, G. & Earnest, T. (2002). *Acta Cryst.* D**58**, 1514–1518.
Sulzenbacher, G. *et al.* (2002). *Acta Cryst.* D**58**, 2109–2115.
Taylor, W. R. (2002). *Nature (London)*, **416**, 657–660.
Terwilliger, T. (2000). *Nature Struct. Biol.* **7**, 935–939.
Yokoyama, S., Hirota, H., Kigawa, T., Yabuki, T., Shirouzu, M., Terada, T., Ito, Y., Matsuo, Y., Kuroda, Y., Nishimura, Y., Kyogoku, Y., Miki, K., Masui, R. & Kuramitsu, S. (2000). *Nature Struct. Biol.* **7**, 943–945.