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The open-access high-throughput crystallization facility at EMBL Hamburg

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Here, the establishment of Europe's largest high-throughput crystallization facility with open access to the general user community is reported. The facility covers every step in the crystallization process from the preparation of crystallization cocktails for initial or customized screens to the setup of hanging-drop vapour-diffusion experiments and their automatic imaging. In its first year of operation, 43 internal and 40 external users submitted over 500 samples for a total of 2985 crystallization plates. An electronic booking system for registration, the selection of experimental parameters (*e.g.* drop size, sample-to-reservoir ratio) and the reservation of time slots was developed. External users can choose from more than 1000 initial crystallization conditions. By default, experiments are kept for six months and are imaged 15 times during this time period. A remote viewing system is available to inspect experiments *via* the internet. Over 100 stock solutions are available for users wishing to design customized screens.

1. Introduction

Worldwide efforts to sequence the complete genomes of organisms from all kingdoms of life have created an enormous wealth of information over the past decade. However, it has been realised that this information is in no way sufficient to explain the complex biological processes which form the basis for the functioning and inner workings of the cell and therefore of life itself. In order to further our understanding of these processes and to deliver substantial improvements in human health, the one-dimensional information derived from genome sequences has to be translated into the three-dimensional structures of all molecules in the cell in atomic detail. In this context, X-ray crystallography has emerged as the most widely applied technique in structural biology, providing about 85% of all structures deposited in the PDB (Berman *et al.*, 2002; http://www.rcsb.org), a repository for structural information. The pre-eminence of this technology is a consequence of the fact that targets can be resolved at atomic resolution without limitation on their size, often within days of data collection. Current bottlenecks are the necessity of obtaining relatively large amounts (>5 mg) of chemically and structurally homogenous sample, in a soluble state and crystallizing them. Crystallization is an empirical process in which hundreds of solutions have to be scrutinized for their ability to convert soluble biological macromolecules (often dozens of constructs per target molecule) into well ordered crystals which diffract X-rays to high resolution. This procedure is repetitive and very time-consuming.

As a consequence, the process of biological crystallization has been heavily automated in order to increase both its success rate by minimizing the amount of sample per experiment (Chayen *et al.*, 1994; DeLucas *et al.*, 2003) and its time efficiency by parallelizing the process of experiment setup (Krupka *et al.*, 2002; Hazes & Price, 2005). These efforts have resulted in the establishment of several academic (Heinemann *et al.*, 2000; Watanabe *et al.*, 2002; Albeck *et al.*, 2005) and industrial high-throughput facilities (Peat *et al.*, 2002; Hosfield *et al.*, 2003). However, there are only a few academic laboratories worldwide that offer access to such a facility to the general user community (Luft *et al.*, 2003). Here, we report the establishment of Europe's largest high-throughput crystallization facility at the EMBL Hamburg Outstation, which is open to the general user community.

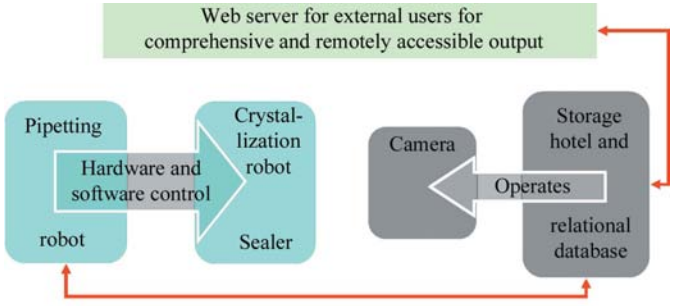


Figure 1
Schematic representation of the two robotic modules (blue and grey, respectively) constituting the high-throughput crystallization platform at EMBL Hamburg. All relevant experimental data with the exception of images are stored in a relational database which is part of the storage hotel. This database serves as the entry point for internal users. External users access the system *via* a secured web server. Links for data exchange are indicated by the red double-headed arrows.

2. System overview

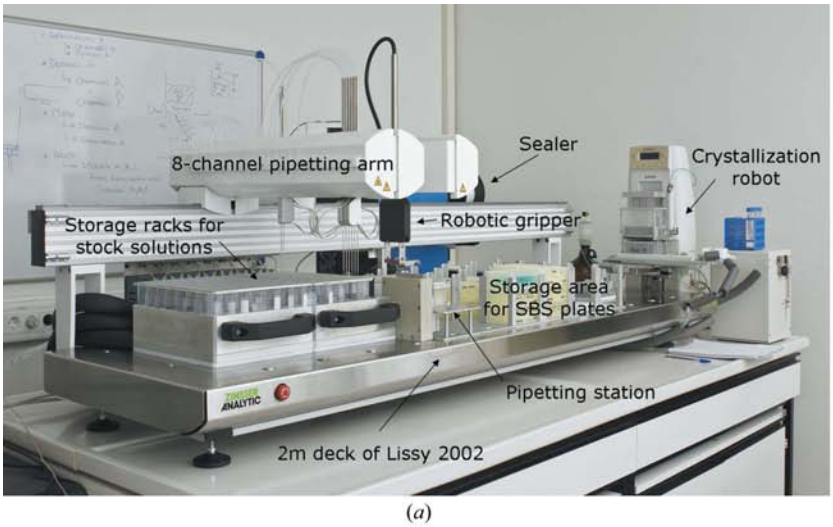
The high-throughput crystallization facility at the EMBL Hamburg Outstation was designed with the following objectives in mind.

(i) The ability to cover the entire crystallization process: the generation of initial and optimization cocktails from stock solutions, the production of crystallization plates and their storage and automatic imaging.

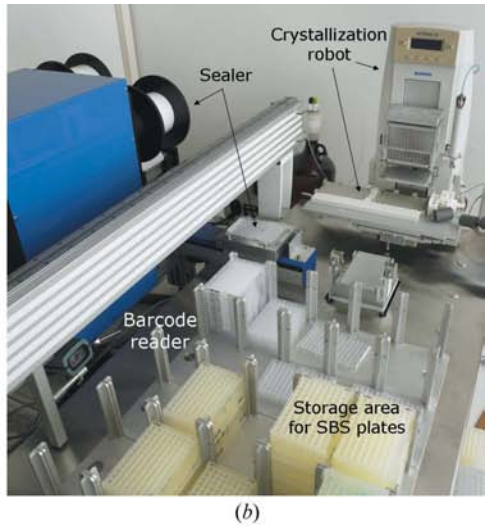
(ii) A high degree of automation to minimize manual intervention.

(iii) A large storage and throughput capability to provide sufficient capacity for a large international user community.

In order to fulfill the first objective, the platform had to consist of at least one liquid-handling robot, a crystallization robot and a storage and retrieval unit with a computer-controlled imaging system. To achieve the goal of high automation with minimal manual intervention, the individual devices had to be integrated to the largest possible degree and their software-control systems had to be interconnected to facilitate a centralized management structure with a seamless data flow between the components. Additionally, the throughput capacities of the individual components had to be in tune in order to avoid system-related bottlenecks and to permit a smooth production rate throughout the entire platform. During the design deliberations of this facility in 2004, there were a few commercial products available that already combined individual components into integrated crystallization platforms. Each of them entailed a variety of disadvantages (*e.g.* high running costs through heavy use of consumables or a degree of integration that would prevent easy upgrades of components in the future). We decided therefore to select equipment that was consistent with our objectives from individual manufacturers and to have the components integrated according to our specifications.



(*a*)



(*b*)

Figure 2
Liquid-handling unit of the EMBL high-throughput crystallization facility. (*a*) View of the entire module with the 2 m deck of the Lissy 2002 pipetting robot in the centre, the Hydra II Plus One crystallization robot to the far right and the RoboSeal in blue towards the back. (*b*) Close-up look at the attached crystallization and sealing robots, which were placed adjacent to each other for fast sealing of crystallization plates.

The automated crystallization platform consists of two separate modules (Fig. 1). A liquid-handling module covers all steps of the crystallization process from the preparation of crystallization cocktails to the generation of sealed crystallization plates. The second module consists of a fully automated storage and retrieval system with an integrated imaging unit. The latter has the capacity to store and image 10 000 96-well crystallization plates. The former has a maximum throughput of about 120 96-well crystallization plates per 8 h working day. Both modules are described in more detail in the following sections.

2.1. Liquid-handling unit

2.1.1. Crystallization robot. Commercial crystallization robots differ in their ability to use different crystallization methods, their general flexibility and overall setup times. For example, the Oryx system from Douglas Instruments Ltd (http://www.douglas.co.uk) is capable of setting up sitting-drop as well as microbatch experiments with a variety of drop volumes, while the Mosquito from Molecular Dimensions Ltd (http://www.moleculardimensions.com) can generate crystallization plates as sitting and hanging drops and under oil up to 100 nl. To the best of our knowledge, there were only two crystallization robots in 2004 [Hydra II-Plus-One from Matrix Technologies (http://www.matrixtechcorp.com) and Screenmaker 96+8 from Innovadyne Technologies (http://www.innovadyne.com)] with nanolitre dispensation capabilities while also offering the possibility of dispensing volumes above ∼20 µl with the same set of needles. Consequently, most crystallization robots only worked with crystallization plates that were preloaded with reservoir solution. Their production signifies an additional step in the crystallization process and adds a considerable overhead. We selected the Hydra II-Plus-One crystallization robot for our platform.

The dispensation of crystallization solutions is handled by a positive-displacement unit which transfers 96 solutions in parallel and is therefore very fast. Depending on the task at hand, the syringes of the Hydra II-Plus-One multi-dispenser can range in volume from 100 to 1000 µl. Our 96-channel dispenser is equipped with 100 µl syringes, which increases the accuracy during nanolitre dispensations. This restricts the reservoir volume to 100 µl, but this is sufficient for the equilibration of crystallization drops up to a total volume of at least 5 µl (Forsythe *et al.*, 2002). Through the use of positive-displacement syringes, reservoir solutions can be aspirated and dispensed accurately independently of their viscosities as long as the 96-channel unit and the working deck are precisely aligned (see below). The same set of syringes is used to transfer up to 100 µl reservoir solution to the reservoir well and to dispense as little as 200 nl for use as precipitant in the crystallization droplet. In order to avoid contamination of the needles with biological sample, reservoir solutions are always dispensed first.

The solutions containing the purified biological sample are dispensed with a single-channel non-contact nanoneedle which is based on the microsolenoid technology of Innovadyne Inc. (http://www.innovadyne.com/). They are added to the reservoir droplet in the crystallization well. Our setup permits the aspiration of 250 µl sample solution before flushing the microsolenoid valve, which enables us to fill a 96-well plate with 100–2500 nl droplets in one step. Biological sample and system water are separated by a 1.25 µl air gap which prevents sample dilution and results in less than 1 µl of dead volume for the sample.

A variety of protocols can be generated with the user-friendly *Hydra II-Plus-One* software. This enables us to operate very flexibly and accommodate unusual user requests [*e.g.* combination of different screens in the same reservoir well of a microtitre plate (MTP) or the use of solutions in the reservoir different from that added to the crystallization cocktail]. Typical setup times per crystallization plate are between 4 and 5.5 min. This includes the solution transfer between the deep-well plate (DWP) and MTP, the addition of protein and the washing of both the 96-needle head and the nanoneedle of the Hydra II-Plus-One. The amount of time between dispensing reservoir solution into the crystallization well, adding sample and sealing the MTP is less than 90 s. We therefore do not apply any evaporation-control mechanism (*e.g.* sliding cover or humidity chamber) during crystallization-plate setup. Neither would have been compatible with automation or our desired throughput capacity.

In contrast to the pipetting robot (see below), the crystallization robot is never left unsupervised in order to ensure the integrity of precious sample material, even though the Hydra II-Plus-One has now been operating without major failure for more than a year.

2.1.2. Pipetting robot. The centrepiece of the liquid-handling unit is the Lissy 2002 from Zinsser Analytic (http://www.zinsseranalytic.com), a 2 m wide eight-channel pipetting robot with a robotic gripper (Fig. 2). Both the Hydra II-Plus-One crystallization robot and an automatic sealer from HJ Bioanalytik (RoboSeal; http://www.hj-bioanalytik.de) are attached to the pipetting robot's periphery such that they can be served by the robotic gripper. All devices can be operated individually or from within the *WinLISSY* software package which has been installed on the Lissy 2002 under *Windows XP*. Thus, the *WinLISSY* software package enables us to create working protocols that can operate the entire liquid-handling unit without manual intervention.

The deck space of the pipetting robot is used for the preparation of crystallization cocktails and as accessible storage space for SBS-format MTPs and DWPs (*e.g.* to load or unload the pipetting station or the crystallization robot during automatic mode). The deck space also contains removable and temperature-controlled racks for stock solutions, two different washing stations, a pipetting station for up to three DWPs and a barcode reader (Fig. 2). The crystallization robot and the stock-solution racks are positioned on the opposing small ends of the deck, which enables their simultaneous operation and manipulation through the robotic gripper. The stock-solution racks have a total of 132 available positions which consist of 96 positions for 50 ml conical tubes, 20 positions for 15 ml tubes and 16 positions for 2 ml Eppendorf tubes. The 96 50 ml tube

Table 1
Comparison of requested [first column in (*a*), top row in (*b*)] *versus* actual volumes (µl), which were determined gravimetrically.

All solutions were dispensed with a standard stainless-steel pipetting needle. High-molecular-weight PEGs (PEG 8000 and larger) are dispensed by a slurry needle, which enables faster dispensation compared with the standard pipetting needles. The tables show the average volumes and coefficients of variance (CV, in parentheses).

(*a*) Single pipetting of PEG 8000, 10 000 and 20 000. Each measurement was repeated five times.

	50%(*w*/*v*) PEG 8000	50%(*w*/*v*) PEG 10 000	40%(*w*/*v*) PEG 20 000
40	41.2 (1.0%)	39.7 (0.3%)	40.2 (1.2%)
100	103.8 (0.4%)	99.2 (0.2%)	100.4 (0.2%)
300	306.7 (0.1%)	296.1 (0.1%)	299.7 (0.1%)
800	814.1 (0.1%)	789.3 (0.4%)	798.6 (0.03%)
1200	1219.5 (0.1%)	1179.0 (0.3%)	1197.2 (0.3%)

(*b*) Multi-pipetting of 100% ethanol. For this measurement, 1600 µl of ethanol was aspirated by each channel and then successively dispensed as 100, 700, 300, 150, 250 and 100 µl. The volumes were determined after each dispensation step and the run was repeated three times.

	100	700	300	150	250	100
Channel 1	100.9 (0.2%)	696.2 (0.03%)	295.7 (0.1%)	148.6 (0.1%)	249.2 (0.4%)	99.1 (0.5%)
Channel 2	100.0 (0.3%)	695.6 (0.1%)	295.2 (0.1%)	148.1 (0.1%)	247.1 (0.1%)	97.4 (0.1%)
Channel 3	99.9 (0.4%)	694.6 (0.1%)	294.9 (0.1%)	147.7 (0.2%)	246.7 (0.2%)	97.4 (0.1%)
Channel 4	99.7 (0.4%)	692.7 (0.1%)	294.2 (0.1%)	146.8 (0.04%)	245.9 (0.1%)	97.5 (0.1%)
Channel 5	99.8 (0.3%)	695.3 (0.1%)	294.9 (0.1%)	148.2 (0.1%)	247.4 (0.1%)	97.1 (0.2%)
Channel 6	99.1 (0.5%)	691.9 (0.01%)	294.9 (0.1%)	147.9 (0.2%)	245.8 (0.1%)	98.6 (0.6%)
Channel 7	100.3 (0.3%)	696.1 (0.04%)	295.1 (0.2%)	148.1 (0.1%)	247.7 (0.04%)	97.3 (0.4%)
Channel 8	100.8 (0.3%)	694.9 (0.01%)	295.2 (0.04%)	148.4 (0.3%)	246.9 (0.1%)	97.7 (0.2%)

positions can also be loaded with 15 ml tubes for the simple reformatting of less frequently used commercial screens into DWPs. Since each of the rack positions is accessible by each of the eight needles of the pipetting arm, it is possible to use up to 116 different stock solutions and 16 additives (Eppendorf tubes) at any time. If more solutions were needed, the system could be expanded through the use of multiple racks.

Every stock solution is defined by a unique chemical name, a concentration, a unit of concentration [*e.g. M* or %(*v*/*v*)] and a barcode. A growing list of stock solutions is stored on our PC and updated versions are imported into the Lissy 2002 database as needed. A separate database entry contains the associations of stock solution and rack position. It is generated by reading the barcodes of the stock solutions with a handheld barcode reader in order of their rack position. Once a rack is loaded with stock solutions they are secured with a rubber-coated plate (see below) and there is no danger of inadvertently swapping solutions. All of our stock solutions have been prepared according to standard operating procedures (SOPs, which can be made available upon request) and a complete list of stock solutions is posted on our web page (http://www.embl-hamburg.de/services/crystallisation). This information is for users that want to design customized screens. The instructions for customized screens have to adhere strictly to the format given on our web page and are restricted to the use of already existing stock solutions. There are currently 115 stock solutions in use, which is sufficient to prepare 816 non-redundant crystallization solutions (8.5 96-well DWPs) for initial screens. This covers about 75% of the initial screens used for the 2985 crystallization plates (∼290 000 experiments) that were set up in 2005.

Before the preparation of a crystallization screen, the *WinLISSY* software optimizes the pipetting run by minimizing the number of movements through a sorting algorithm and the number of dispensation steps through multi-pipetting. Multi-pipetting refers to the system's ability to aspirate the volumes of several pipetting steps at once and dispense them into different wells. Single pipetting and multi-pipetting are executed with excellent accuracies and precisions independent of the solution's viscosities or surface tension. Table 1 lists the accuracies and coefficients of variance for three different PEG solutions and ethanol at different volumes. The PEG solutions exemplify high-viscosity solutions and ethanol was chosen for its low surface tension.

During operation, the stock solutions are covered with a rubber-coated metal plate with 3 mm holes which permits access to the solutions while minimizing evaporation. A similar plate without holes is used when the solutions are not used. Similarly, after positioning the desired number of DWPs (between one and three) on the pipetting station, the robotic gripper places a metal lid with 96 septa onto each DWP. Each lid is fastened to the DWP by pneumatic rods. The preparation time per screen depends on the kind and the number of screens prepared and is between 60 and 120 min. This time includes the mixing of solutions (∼20 min), which is achieved through the aspiration and rapid dispensation of ∼80% of the well content with eight needles in parallel. Through the use of food dyes, we determined that this way of mixing homogenizes even the most viscous solutions. During these tests, it was also discovered that certain viscous solutions were not completely removed from the tubing when the standard washing procedures was applied. Therefore, an additional wash step was introduced with a 5%(*v*/*v*) detergent solution at 323 K, which is followed by the standard washing procedure.

Our typical working day begins with the preparation of crystallization cocktails either for initial screens or based on customized protocols. The recording of each action by the Lissy 2002 and a sophisticated error-detection system allows us to run this process unsupervised.

The third device that is integrated in the liquid-handling unit is an automatic plate sealer (RoboSeal from HJ Bioanalytik) which uses pressure during the sealing step. Any SBS footprint plate can be sealed with this robot. Different plate types are defined in the software and their dissimilar heights are automatically accommodated by an adjustable stage. This mechanism facilitates the fast sealing of a plate immediately (<3 s) after setup, which is particularly important for nanolitre crystallization plates.

2.2. Storage and imaging

The second module of the high-throughput crystallization facility at EMBL Hamburg consists of an integrated storage (Odyssey SRS) and imaging system (Minstrel IV) from RoboDesign (http://www.robodesign.com), which is kept at a constant temperature of 292 ± 1 K (Fig. 3). The storage system has a capacity for up to 10 000 MTPs of any SBS format. Different plate types are identified by their barcodes, which facilitates their proper handling. During their residence in the storage vault, crystallization plates are kept in trays which can hold up to six MTPs. For an inspection, the entire tray is loaded into the Minstrel IV camera box, which has the advantage of excluding additional manipulations of the MTP itself (*e.g.* transfer from a shuttle arm to a camera deck and back). Since we only combine same-day productions in a tray (which leaves the occasional empty slot), all plates in a tray have to be imaged at the same time, preventing unnecessary movements and maintaining a high efficiency for the entire system. The imaging time per crystallization plate depends on the number of images that are recorded per crystallization well. The minimum time required is 4 min (one image per well). Since the Minstrel IV contains two cameras, it is possible to image two crystallization plates in 4 min. Once the SRS approaches ∼70% capacity, a second Minstrel IV with another two cameras becomes necessary. In a setting with two Minstrel IV boxes with two cameras each, four crystallization plates can be imaged in parallel, reducing the average imaging time per crystallization plate to 1 min.

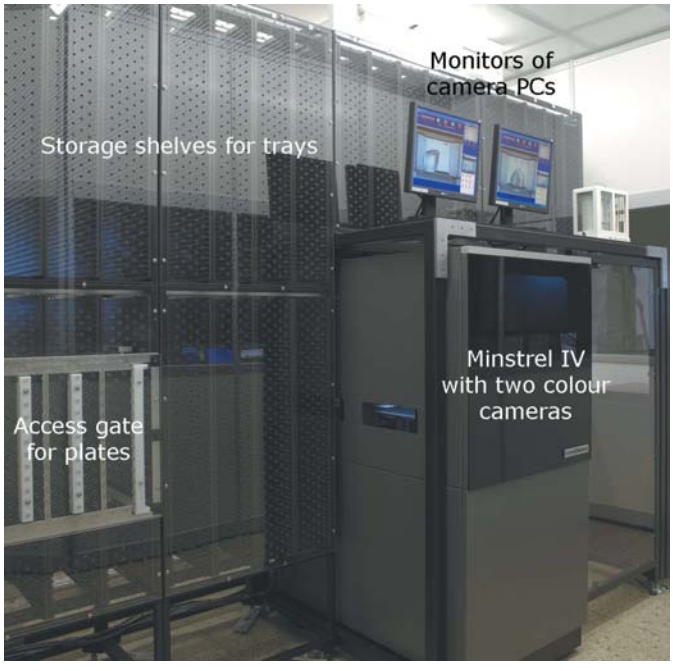


Figure 3
Storage and retrieval unit for up to 10 000 crystallization plates with one Minstrel IV housing two colour cameras. The empty slot for a second Minstrel IV camera box can be seen to the right of the current camera box. Each camera is controlled by a separate PC. Trays with crystallization plates are entered or retrieved manually through the access gate. The typical time for the retrieval of a crystallization tray is under 1 min.

The two 5 Mb colour cameras of a Minstrel IV work in parallel, independently imaging two crystallization plates per tray. The plates are illuminated from the bottom by an arrangement of white-light LEDs which travel with the camera, thereby exposing only the relevant parts of the MTP. Various predefined illumination patterns of the LEDs are chosen for different plate types or crystallization setups (*e.g.* hanging or sitting drop or batch crystallization) to obtain appropriately exposed images without shaded areas. Different crystallization plates are identified by their barcodes, which upload the corresponding lists with *xy* offsets (to find crystallization wells) and *z* offsets (for focusing). Several magnification levels ranging from 0.7-fold to 4.5-fold are available. Our standard setting is at a magnification level of twofold, which gives a field of view of 4.3 × 3.2 mm and a field depth of 800 µm. This setting captures the entire crystallization well of our crystallization plate of choice (Greiner CQ low profile) and renders >95% of all drops in focus. In order to overcome the occasional focusing problem, we take two images, one at the base level and another 800 µm (the field depth) above the

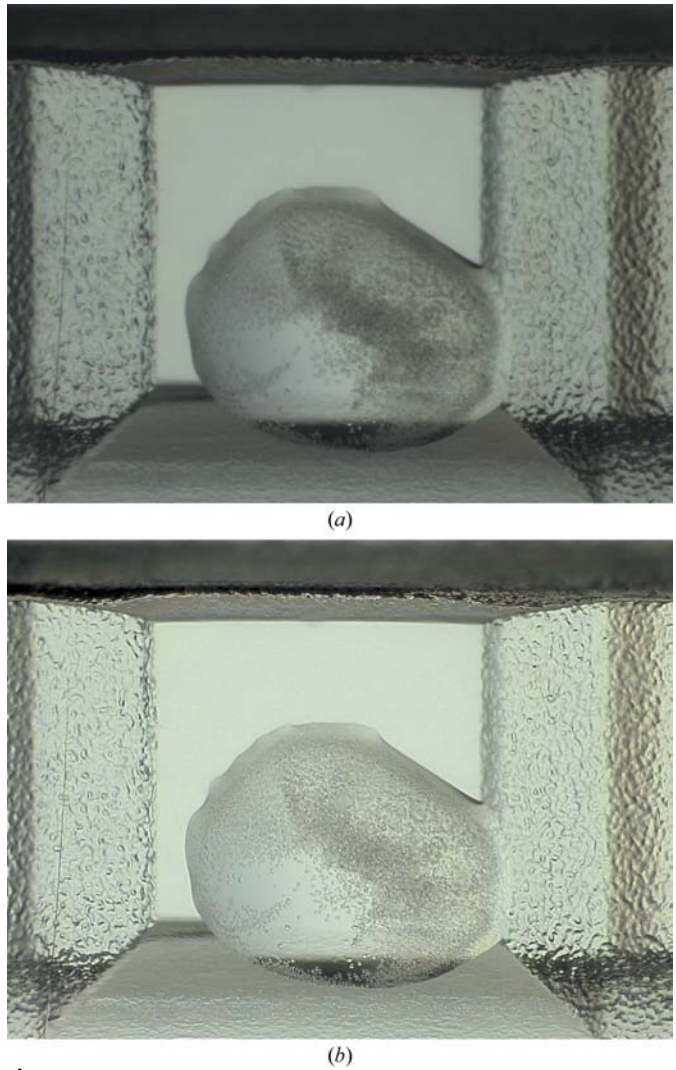


Figure 4
Side-by-side comparison of a high-resolution crystallization image before (*a*) and after (*b*) enhancement by the sweet-image algorithm.

base level. In multi-slice mode the software retains low-resolution images (640 × 480 pixels) of both slices and a high-resolution image (2560 × 1920 pixels) of the better focused image which is automatically detected. The best image is subsequently processed by the sweet-image algorithm, which increases contrast and sharpness (Fig. 4). Image processing is executed on a server which is part of the computational network of the storage and imaging system. Eventually, all images are transferred to our storage server, which has an expandable storage capacity of currently 3.5 Tb.

In-house users of the storage and imaging system can browse their images and associated data through the *CMView* software package from their desktop computer. This provides access to all personal crystallization plates and all image types and enables the manual scoring of images. A separate program provides information on the crystallization conditions, details of construct design and the possibility of designing optimization screens. An integration of the two programs is currently under way.

External users have remote access to their images and crystallization data *via* the internet (http://icarus.embl-hamburg.de:2000). This system is under constant development to add further functionality.

For uniformity and consistency, all barcodes are generated within the SRS data-management system. The generation of barcodes within *CMView* primes the SRS for the input of crystallization plates. Every crystallization plate is associated with one barcode and through that with a variety of relevant information, such as a user name, a sample name, a viewing schedule *etc.*

3. Validation of the system

Both modules of the high-throughput facility were installed on-site in early 2005 with factory alignments. Until the end of 2005 the alignments were tested and improved, working protocols were generated and the platform was validated in a gradual process which initially included 43 in-house users (∼290 samples) and, towards the end of 2005, 40 external users (∼250 samples) in addition. In 2005, we produced a total of 2985 96-well crystallization plates (∼290 000 experiments).

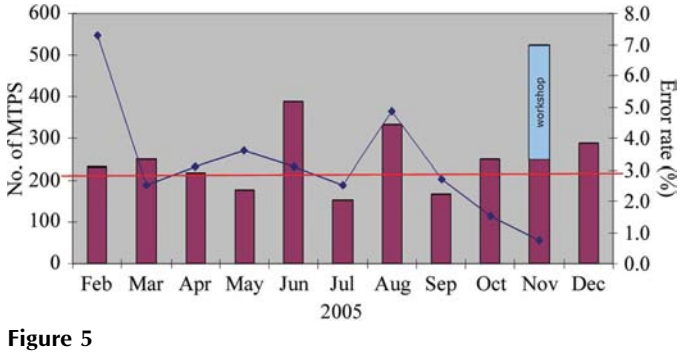


Figure 5
Plot of monthly production numbers (red bars, left *y* axis) and error rates (blue line, right *y* axis) of crystallization plates for 2005. The red line indicates the average error rate for all plates until the end of November and without the production for the workshop (light blue bar).

Table 2
Success rates of the high-throughput crystallization facility at the EMBL Hamburg Outstation based on a workshop given in November 2005.

The first two rows state the response rate to this experiment based on the total number of three criteria: users, plates and samples. The second part of the table shows the success rates of the three criteria based on the available information.

	Users	Plates	Samples
Total	26 (100%)	276 (100%)	37 (100%)
Response rate	17 (65.4%)	196 (71.0%)	27 (73.0%)
	17 (100%)	196 (100%)	27 (100%)
Crystals reported by/for	10 (58.8%)	56 (28.6%)	13 (48.1%)
Excluding salt crystals (at least 1)	9 (52.9%)	55 (28.1%)	12 (44.4%)
Total No. of crystals	197	197	197
Average No. of crystals (excluding salt)	11.6	1.0	7.3

3.1. Validation of the crystallization robot

The accuracy and reliability of the Hydra II-Plus-One crystallization robot required a careful alignment. The alignment was critical with respect to both the distance and the relative orientation of the platform supporting the crystallization plate and the 96-channel unit. Both parameters were refined until systematic errors were no longer detectable. We defined the error rate as the sum of incorrectly dispensed volumes (too much, too little or no solution, judged visually) and the failure to combine reservoir and sample solution in a single droplet. The average error rate for all plates produced in 2005 until the end of November (excluding 276 plates set up during a workshop) was 2.9% (Fig. 5).

3.2. Validation of the pipetting robot

The preparation of crystallization cocktails requires the pipetting of solutions spanning almost three orders of magnitude in dynamic viscosities and with very dissimilar surface tensions. A variety of parameters such as pipetting speed, delay times and air gaps, to name a few, are combined into liquid classes to which the different solutions are assigned. This process is repeated and refined until some defined criteria (usually accuracy and precision) have been achieved. Examples for the accuracy and precision of the Lissy 2002 pipetting robot for four solutions and liquid classes in two pipetting modes are listed in Table 1.

4. Results

During a workshop in November 2005 at the EMBL Hamburg Outstation, participants were given the opportunity to submit samples to the crystallization facility with the request to make the results of their experiments available. 70 participants from 16 nations joined the workshop and 26 individuals submitted 37 samples with a total of 276 crystallization plates (Table 2). On average, every target was screened in 7.5 plates or 720 conditions. All 276 plates were setup on three consecutive 8 h shifts, which amounts to 77% of the theoretical maximum throughput capacity.

The total number of crystals reported was 197, excluding one inorganic salt crystal which was exposed at beamline X13 in Hamburg. It cannot be excluded that there are additional salt crystals among the reported 197 crystals. On average, every user observed 11.6 hits or one hit in every crystallization plate (96 experiments). The average number of hits per sample was 7.3.

Unfortunately, we are not able to correlate these numbers with manual crystallization setups owing to a lack of data.

5. Access to the facility

An electronic booking system has been designed for remote reservations and experiment design. Users are prompted for administrative information and can then enter personal preferences on a variety of crystallization parameters such as drop volume, type of screen, reservoir-to-sample ratio *etc*. This information must be repeated for each biological sample and can be edited. Before final submission, users book time slots in 30 min increments with the request to reserve one such time slot for every seven 96-well crystallization plates. The earliest booking possible is for the following day. First-time users will be given a username and password to access their results at http://icarus.embl-hamburg.de:2000. The remote access pages are under constant development, taking into account requests from users. More information (including prices) can be found at http://www.embl-hamburg.de/services/crystallisation.

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