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Lessons from high-throughput protein crystallization screening: 10 years of practical experience

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Introduction: X-ray crystallography provides the majority of our structural biological knowledge at a molecular level and, in terms of pharmaceutical design, is a valuable tool to accelerate discovery. It is the premier technique in the field, but its usefulness is significantly limited by the need to grow well-diffracting crystals. It is for this reason that high-throughput crystallization has become a key technology that has matured over the past 10 years through the field of structural genomics.

***Areas covered*:** The authors describe their experiences in high-throughput crystallization screening in the context of structural genomics and the general biomedical community. They focus on the lessons learnt from the operation of a high-throughput crystallization-screening laboratory, which to date has screened over 12,500 biological macromolecules. They also describe the approaches taken to maximize the success while minimizing the effort. Through this, the authors hope that the reader will gain an insight into the efficient design of a laboratory and protocols to accomplish high-throughput crystallization on a single-, multiuser laboratory or industrial scale.

***Expert opinion*:** High-throughput crystallization screening is readily available but, despite the power of the crystallographic technique, getting crystals is still not a solved problem. High-throughput approaches can help when used skillfully; however, they still require human input in the detailed analysis and interpretation of results to be more successful.

Keywords: crystallization, high-throughput, structural biology, X-ray crystallography

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1. Introduction

The language of life is represented by the genome but the alphabet of the primary sequence reveals only part of the story. Structural biology provides the dictionary and grammar, the tertiary and quaternary structure which in turn provides clues to function and mechanism. Three principal techniques are used to gain this structural knowledge, X-ray crystallography, NMR and cryo-electron microscopy (cryo-EM). X-ray crystallography is the predominant technique accounting for 86% of our structural knowledge, with NMR and cryo-EM providing 14% and 0.4%, respectively [1]. The importance of structure in the pharmaceutical field comes from the ability to accelerate discovery; to date, there are more than 200 pharmaceuticals produced from structure-based studies [2-5].

Outside major pharmaceutical industry research, the efforts of structural genomics (SG) have been a major driver for technological advances in high-throughput crystallographic methods. These advances have been substantial, including developments both upstream of crystallization (molecular biology, cloning, protein expression and purification) and downstream (beamline robotics, software developments, etc.). Protein structure initiative (PSI) efforts, the NIH supported SG program, have produced over 36,000 different purified, soluble proteins and have led

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Article highlights.

- Automation makes it possible to set up experiments faster; faster does not imply better.
- Automation does not replace, or eliminate humans from the crystallization laboratory; if properly applied automation simply improves the laboratory's efficiency.
- High-throughput crystallization screening technologies are mature; high-throughput optimization technologies require further development.
- High-throughput crystallization works best when applied to laboratory settings that have the capacity to adequately feed samples and analyze the data that will be generated; shared resources are one way to achieve high input and the ability to process the high output of data.
- Automation is well suited for fundamental research into the crystallization problem, increasing protein and chemical diversities and providing statistically meaningful numbers of reproducible experimental results for analysis.

This box summarizes key points contained in the article.

to ~ 5,000 protein structures, more than 7% of our public structural knowledge to date. [6] While these numbers are impressive, of immediate note is the loss associated with the process. Only 13% of the purified, soluble proteins result in structural depositions to the Protein Data Bank (PDB); the vast majority of the proteins remains structurally uncharacterized [6]. Crystallization is a significant problem.

Structural genomics relies upon well-defined protocols to achieve high structural output. These are codified in a process termed the pipeline. Oftentimes, once standardized, these protocols are applied uniformly to process every protein. High output is accomplished by increasing the pipeline's capacity. Crystallization screening is one aspect of this pipeline that is readily adapted to increase throughput using repetitive robotic methods and standardized protocols. A "standardized" set of screening cocktails is applied to many proteins. This is an approach enabled by technology and governed by resources. This approach is very efficient in terms of throughput; it is an effective means to process large numbers of samples. It will by default neglect the more intense efforts required for samples recalcitrant to the "standard pipeline" that may be of significant biological and pharmaceutical importance. On the other hand, this pipeline will capture structural information for many important, biologically significant samples using standardized methods.

For more than 10 years we have been operating a high-throughput crystallization-screening service at the Hauptman-Woodward Medical Research Institute. This service is open to the SG community, the general biomedical community and industry. In this review we detail specific aspects of crystallization, automation of those aspects and our experiences operating a high-throughput laboratory. We compare our approaches with other high-throughput crystallization efforts and describe anticipated technological developments that could improve the success rate of a structural pipeline. We focus on soluble proteins and plate-based screening to complement a previous review describing structure-based pharmaceutical design and alternative technologies and topics [5].

2. Crystallization in the laboratory setting

Numerous methods of crystallization have been developed and utilized over the years [7]. These can be classified into the three basic categories of batch diffusion, vapor diffusion and liquid diffusion. Whatever the method used, identifying the crystallization conditions for macromolecules is a formidable, multi-parametric problem [8]. Exhaustive searches through physical and chemical parameter space are simply not an option, even with high-throughput technologies. Two main approaches have been applied to resolve this problem. Chemical sampling where there has been historical success, or statistically designed sampling strategies to cover larger areas of chemical space. The first is exemplified by the work of Jancarik and Kim [9], and often referred to as the "sparse matrix screen". Commercialization of this screen and others [10-12] has led to the popularity of this approach and it is often the first strategy used in a crystal growth laboratory. The second, a statistical sampling approach, was pioneered by Carter and co-workers [13] who applied principally incomplete factorial designs to crystallization. Relative levels of important chemical factors are sampled to achieve good coverage and good balance in the sampling. Both approaches are complementary and frequently combined, providing scaffolds upon which further experiments are built. In each case screening is a first step, with the results guiding subsequent rounds of optimization. The crystal grower must decide upon both the primary variables to test and the range of those variables (including chemical species and concentrations) to sample. The end product of this step is a set of cocktails; solutions that provide coverage of chemical space relevant to crystallization.

Crystallization methods are used to apply these cocktails to a protein. The combination of a cocktail and a method defines a specific location on a physicochemical map. Two experiments with the same starting chemical conditions but using different methods can have very different final chemical concentrations. Besides having a unique, end point, path and rate to equilibration, each method will have other distinct advantages and disadvantages. For the majority of crystallization-screening experiments, there is little foreknowledge of the specific protein and cocktail solution thermodynamics; the process is empirical.

Batch methods take a protein solution and combine it with a cocktail solution. The initial chemical conditions are essentially the final chemical conditions unless there is a phase change in the solution. Batch experiments require high levels of supersaturation from the onset and ideally place the macromolecule at labile supersaturation, required for

nucleation. Because batch experiments do not use a dynamic process to increase the level of supersaturation, the initial chemical conditions are well defined; experimental outcomes can be readily interpreted. Batch experiments are very efficient and have minimal manipulation during their set up. Vapor-diffusion methods co-dilute a protein and cocktail drop on a surface and then seal the drop in a container with a larger volume of cocktail solution. The reservoir dehydrates the drop until the vapor pressure of water over the drop and reservoir is equivalent. The drop begins at a lower concentration of precipitating agent and during equilibration approaches the end point at a higher concentration of precipitating agent. Crystals can form, and the drop will still continue to dehydrate until the vapor pressures of volatile species (typically water) over the drop and reservoir are equivalent. The rate of equilibration is partially dependent upon the solutions' colligative properties [14]. One benefit of this approach is that as sampling takes place along the path toward the end point, there is a potential to identify chemical conditions that support crystallization. Although the exact chemical conditions at the time of crystallization are generally unknown, reproducing the experimental conditions is still readily accomplished. According to the RCSB PDB [15] (www.pdb.org), the vapor-diffusion technique is the most common crystallization method. Liquid-diffusion methods such as counterdiffusion [16] are less common but are analogous to the dynamic process of vapor diffusion, sampling a region of chemical space along a path to a set end point.

In each case crystallization cocktails are prepared, the crystallization experiments set up, observed over time and the results noted. Automating this process has the benefit of increasing speed (a great number of experiments can be set up in a given amount of time) and decreasing sample volume (the average automated system can deliver smaller solution volumes than the average manual pipette). Potential pharmaceutical targets and complexes of potential pharmaceuticals with those targets can be screened rapidly with minimal sample. In translating this to high throughput, any automated process has to duplicate cocktail preparation, set up, imaging and notation.

3. Automating laboratory crystallization processes

Complicated, advanced automation techniques rely upon the same methods, thermodynamics and kinetics as manual crystallization experiments. The seeds of the necessary technologies for automation can be traced back to the 1980s.

In 1987, syringe pumps were used to deliver reservoir and experiment drop solutions to two separate plates, the plates were then adjoined using foil with pinholes to facilitate slow equilibration [17]. The combined preparation of solutions and set up of experiments provided two of the four key ingredients for successful automation. Another early automation system used two syringe pumps coupled with a valve control box to dispense hanging drop grid screen experiments into a 4×4 array [18]. This system introduced an information tracking system developed for solution preparation, the third key ingredient in automating the process. Parallel to these studies the APOCALPYSE system was constructed by Jones and co-workers [19], leading to a robot friendly American Crystallography Association (ACA) plate to set up hanging, sitting or sandwich drop crystallization experiments [20]. This evolved into a system including image analysis software to detect crystals (REVELATIONS) [21], the fourth and final key ingredient for a fully automated system.

A complete automated crystallization laboratory, combining these key ingredients, was developed by Ward and co-workers [22]. The system used syringe pumps to deliver solutions to a specially designed plate [20] with image analysis of the results. These early developments focused mainly on the most common crystallization method in the laboratory, vapor diffusion. Chayen *et al.* [23] developed a system for automated microbatch-under-oil crystallization. Douglass Instruments, Ltd (Berkshire, UK) sells updated versions of one of the first commercially available robotic systems for the home laboratory.

Enhancing the process by reducing human intervention was one of the goals set for a system developed by Rubin *et al.* [24]. This system used a single glass plate to hold the experiment drops and had the capacity, without human intervention, to set up 51 standard 24-well hanging drop experiment plates in 40 h. On a smaller scale, a partially automated crystallization system, the PIPEX, was developed around a computer-controlled, motorized pipette [25]. The system prepared crystallization solutions interactively with the investigator, using information entered into a spreadsheet; an example of a semi-automated method that could aid in the laboratory's overall throughput, without a major financial investment. At the other extreme, a system capable of setting up as many as ten 24-well crystallization plates without operator intervention was developed using a five-axis robot [26].

There are important liquid-handling considerations for implementing any crystallization strategy in an automated manner. Most if not all of the automated formulation of cocktail solutions is achieved through volumetric co-dilution of a set of concentrated stock solutions. These solutions typically consist of a range of chemicals for example, buffers, polyethylene glycols (PEGs), salts, cryoprotectants and water, covering a wide range of physicochemical properties. Particularly relevant with respect to liquid handling are viscosity and surface tension. Concentrated PEG solutions, especially those of high M_r, have a high viscosity. The solutions can have a consistency similar to that of room-temperature honey. A system has to have exceptional precision and accuracy across the whole range of stock solutions that are used in the typical protein crystallization laboratory to have reproducible results. Commercial liquid-handling robots, capable of dispensing these solutions for crystallization screens, made their way

into the laboratory. Two contemporaneous efforts used the Robbins Scientific Hydra, a 96-syringe bank liquid-handling system but took very different approaches. The Protein Structure Factory [27] used the Hydra robot to deliver reservoir solutions to 96-well vapor-diffusion experiment plates, while an approach at the Hauptman-Woodward Institute used the same robot to set up microbatch-under-oil crystallization experiments in 1536-well microassay plates [28]. Both approaches are valid and offer advantages that fit the particular goals of the groups using them. There have been many developments since then, including a proliferation of robots available commercially. We will focus on our own efforts and results, and on where we are heading to use them, as practical advice in implementing high-throughput crystallization screening.

4. The HWI high-throughput crystallization screening experience

Our experiences in automated crystallization screening, from the perspective of an HTS laboratory that started operations in early 2000, were built upon the application of commercial liquid-handling robots to the microbatch-under-oil technique. To date, we have conducted crystallization trials on over 12,500 macromolecules, ~ 20 million experiments, which have generated over 120 million imaged outcomes at regularized time points. While vapor diffusion is the most popular technique for structural crystallography, we have adopted the batch method due to the small volume requirements for both macromolecule and cocktails, and relative thermal and vibrational stability. The batch method provides easily traceable chemical parameters; the solution concentration of chemicals in the crystallization experiment remains constant until there is a phase change. Batch under oil offers practical advantages for automation. The experiments do not require additional sealing processes to prevent experiments from evaporating to dryness, there are no coverslips to manipulate and there are no additional reservoir solutions required for controlled dehydration of the experiment drop. Our history, operation and developments are summarized in **Figure 1**. Two things are immediately obvious in this figure, the first being the steady progression in samples and the second being the development and implementation of improved technologies and techniques. We describe the process and outcomes, and then touch on the developments that have helped us to continuously improve our operations.

4.1 The process

Screening plates are prepared with paraffin oil and cocktails in advance of the crystallization experiments. The plates are centrifuged and stored at 4°C for up to 2 weeks. The 1536 different cocktails cover a range of chemical space that spans properties thought to be important determinants of crystallogenesis including pH; Hofmeister series; surface tension of water; viscosity; activity of water; excluded volume; density; osmolality and ionic strength. Rather than focusing strictly on minimizing the sample volume, we took the approach of maximizing the information obtained from initial screening while keeping reasonable sample requirements. The cocktails are formulated on an annual basis with formulations changing significantly during the first three generations (learning with experience), then less dramatic changes during subsequent years. The refractive index of the stock solutions and final cocktails is measured to fingerprint the solutions. This technique requires small amounts of sample and is a sensitive, rapid and reliable technique to identify changes in chemical concentrations for example, as little as 1% (v/v) in the case of PEG [29]. This quality-control step ensures cocktails are accurately prepared to maintain generational consistency. Periodic checks of the cocktails' refractive indices ensure consistency during the course of the screen's lifetime. The current generation of cocktails contains three groups: Group 1, salt/buffers; Group 2, PEG/salt (at low concentrations)/buffers are both constructed using an incomplete factorial design [27] and Group 3 commercial screens. Groups 1 and 2 sample 8 buffers at 100 mM concentrations; CAPS (10.0), TAPS (9.0), Tris (8.0), HEPES (7.5), Bis-Tris propane (7.0), MES (6.0), sodium acetate (5.0) and sodium citrate (4.0). The 229 salt cocktails of Group 1 contain 33 different, highly soluble salts at ~ 30, 60, and 90% of room temperature saturation in water. The 687 PEG cocktails of Group 2 include 5 different molecular weights of PEG, 20, 8, 4, 1 KDa and 400 Da combined with 36 salts at 100 mM concentration. The 620 commercial conditions of Group 3 are from Hampton Research (Aliso Viejo, CA) and include Silver Bullets™, Silver Bullets Bio™, PEG/Ion HT™, Crystal Screen HT™, Index™, Crystal Screen Cryo™ and Grid Screen Salt HT™. The combination of the incomplete factorial sampling of chemical space coupled with commercial screens allows the results to be related to the wider universe of proteins crystallized from these commercial screens and those results to be related to data from our high-throughput laboratory.

Our sample flow starts with a monthly call for applications sent to all the laboratories that have previously used our service; there is no general solicitation to groups that have not used the service but groups can be placed on the mailing list by request. Samples are not judged on merit; we do not have the resources to accomplish this but are accepted on a first-come first-serve basis. Basic information is collected on samples to be sent to the laboratory to ensure that they are nonhazardous and help us improve the crystallization-screening process. Samples are shipped to the laboratory by commercial shipping companies, usually for overnight delivery on dry ice, wet ice or at room temperature. If the sample was received frozen, it is thawed as directed by the investigator who supplied the sample. The sample (in a 1.5-ml microcentrifuge tube) is centrifuged to pellet any precipitate that may have formed during transit. Notes are taken on the condition of the sample, and then the sample is manually loaded into

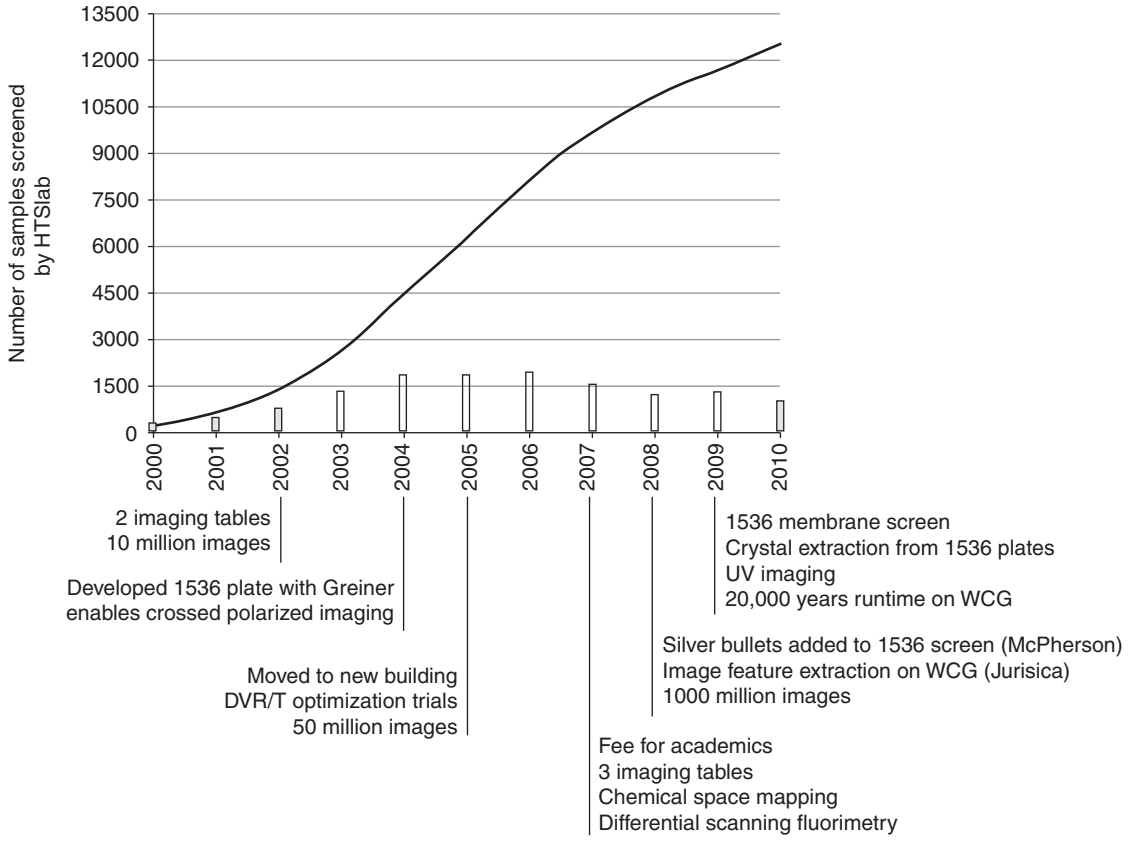


Figure 1. A historical perspective of the Hauptman-Woodward high-throughput crystallization screening laboratory, illustrating the cumulative and yearly number of macromolecules screened against time and notable events in the laboratory. Detail is given in the text.

12 wells (one row) of a 96-well source plate. A liquid-handling system (equipped with a 12-syringe bank head to minimize dead volume loss) is used to aspirate protein from the source plate and deliver 200 nl to each well of the 1536-well experiment plate (already containing oil and cocktail). The experiment plate is centrifuged at low speed to ensure that the aqueous cocktail and protein solutions merge under the oil. The plate is then ready for imaging. The majority of samples are kept at 22°C, although special cases that require other temperatures can be accommodated. Temperature is an important variable affecting solubility [30] and therefore crystallization, but adds to the number of experiments. In the majority of cases we explore temperature during the optimization, rather than the initial screening stage.

Imaging takes place on one of three automated digital photomicrography systems designed in-house. Each accommodates up to 28 experimental plates (43,008 experimental conditions). Each individual experiment is imaged by translating the well of interest under a digital camera with a medium magnification zoom lens and illumination from below. Each image is saved as a grayscale image in compressed TIFF format (lossless compression). The full complement of 1536 images are packaged (WinRAR compressed) with an XML file that describes the chemical cocktail used to formulate the experiment drop (i.e., image) and converted to a JPEG format for distribution to collaborators through a secure FTP server. Multiple backup systems are incorporated. When a plate reaches the end of its imaging cycle, TIFF images are written to DVD-R and JPEG images are written to CD-R. Optical discs and tapes are archived. Our current imaging schedule is one image prior to the addition of protein (cocktail control), additional images 1 day after adding protein, then at 1, 2, 3, 4 and 6 weeks. This has been revised from the original schedule based on the performance of the cocktails used in the screen. Plates are sent to the investigators on request but typically discarded due to the difficulty of harvesting crystals.

4.2 Results

For the general biomedical community, we do not track results after images have been recorded, with the notable exception of a set of manually classified images from a study of 96 proteins that underwent HTS [31,32]. We ask that investigators reference a paper describing the early HTS laboratory and track citations to this. For our SG collaborators, the status of each target is tracked in detail. A summary of the number

of samples we see and the corresponding number of structures resulting in the SG case is shown in **Table 1**. Not shown are those from industry which are dealt with separately.

Selected structures from the general biomedical community are shown in **Table 2**. These demonstrate the diverse range of samples where initial conditions for crystallization have been successfully determined. It is important to note that although initial screening was performed using the HTS laboratory, the vast majority of the work required to produce these structures occurred outside the HTS laboratory, with our crystallization lead providing only the initial starting point for optimization.

We have been analyzing the SG data in detail to develop automated image classification techniques (described later). As part of this analysis for 269 (32.7%) macromolecules out of a selection of 823 targets where crystals occur, the average percentage of conditions that show hits in the screen is 3.58%, or ~ 55 hits per sample; these can be crystals that visually appear to require little optimization, or those that may require significant effort to optimize. **Figure 2** shows an example of results that we currently classify as a hit compared to ones that are ignored.

4.3 Practical implementation of HTS

In implementing the screening laboratory perhaps the most significant development effort has been made in the cocktail development. These are formulated on a yearly basis with the first generation not designed for crystallization screening but as a means to fingerprint macromolecules based upon their precipitation behavior. The cocktails were initially divided into five groups: simple inorganic salts of high solubility, PEG/salt combinations, PEG/buffer combinations, fine screens for three highly soluble salts and Hampton Research Crystal Screens I and II to relate the results to standard commercially available screens. The concentrations were chosen based on crystallization results available in the Biological Macromolecular Crystallization Database [33-35]. We used the software SAmBA [36] to formulate an optimal set of cocktails that tested all variables and pairwise interactions between them symmetrically. In the first group are 46 distinct, highly soluble (> 1 M) simple salts, each at 10 concentrations surveying 11 distinct cations and 14 distinct anions. In the second group are PEG/salt combinations. Three distinct PEGs (1, 8 and 20 kDa) at 5 concentrations were combined with each of the 46 salts from the first group. The salts were uniformly at 200 mM concentration. In the third group were PEG/buffer combinations. Eight different PEGs, ranging in molecular weight from 200 Da to 35 kDa, at 5 concentrations, were combined with 100 mM buffers ranging in pH from 4.8 to 10.4. In the fourth group were the 20-step (0.2 M interval) fine screens for ammonium sulfate (0.2 – 3.8 M), lithium chloride (0.2 – 10.0 M) and potassium thiocyanate (0.2 – 9.7 M).

The first generation of cocktails was successful in producing precipitation profiles for biological samples. The second generation of cocktails was specifically designed for crystallization screening. The overall screen was significantly re-structured and the majority of the cocktails buffered. Ten salts were eliminated from those used in the first generation due to poor solubility. The fine screens of ammonium sulfate, lithium chloride and potassium thiocyanate were replaced with a buffered salt screen sampling 3 concentrations of the 36 salts based on 90, 60 and 30% of their maximum solubility in water at room temperature. SAmBA was used to reformulate the cocktails. The second generation, and all subsequent generations of cocktail including the latest are grouped into three major categories: buffered salt, buffered PEG/salt and commercial screens. Buffering of the cocktails for Generation 2 led to precipitation in a number of conditions with or without the macromolecular sample. These cocktails were removed in formulating the Generation 3 cocktails. We did not re-implement the incomplete factorial for this generation, as this would have simply re-populated regions of chemical space that produced precipitated cocktails. The selected cocktails were simply eliminated from the screen and replaced with further commercial cocktails from Hampton Research, adding the grid screen MPD, PEG-6000, ammonium sulfate, sodium malonate and PEG 6000/lithium chloride. Minor changes have been implemented in cocktails from Generation 3 onward. Of the largest changes, for Generation 5 HEPES buffer was used at pH 7.0 rather than 7.5 to compare buffer influence on the crystallization result. In Generation 8, the Hampton Research Quik Screen and PEG Ion 2 cocktails were duplicated. This allowed us to monitor reproducibility in preparation for replacement of these duplicates by a set of 96 novel cocktails (in Generation 8A) that use small molecules to promote macromolecular contacts [37].

We maintain a deliberate balance between the incomplete factorial-derived portion of the screen and the sampling of commercial screens. This provides a means to derive chemical space information while the commercial screens allow us to relate our screening results to a much larger population of samples that makes use of those screens. The commercial screens themselves operate on several different principles. The conditions from Hampton Research include Silver Bullets™ and Silver Bullets Bio™ (use libraries of small molecules to establish stabilizing, intermolecular, hydrogen bonding, hydrophobic and electrostatic interactions to promote lattice formation and crystallization), PEG/Ion HT™(PEG produced 60% of published crystals, uses purified PEG 3350 as a precipitating agent with salts, pH, neutralized organic acids and multivalent ions to promote crystallization) Crystal Screen HT™ (a sparse matrix sampling approach, 48 of the conditions coming from Jancarik and Kim [38] and 48 from Cudney *et al.* [39]), Index™ (targets zones of different chemical space and pH to identify what specific chemical space is effective for crystallization or reducing solubility), Crystal Screen Cryo™ (a selection of Crystal Screen HT™ conditions with the proper amount of glycerol added for cryoprotection) and Grid Screen Salt HT™ (a 96-condition

Table 1. Samples submitted to the high-throughput screening (HTS) laboratory and the number of structural depositions in the Protein Data Bank (PDB) by year divided into those from the general biomedical community and those from our structural genomics (SG) collaborators.

Year	Total samples	General biomedical community samples	Structural genomics		
			Samples	Structures	%
2010	910	406	504	75	14.8
2009	748	345	403	96	23.8
2008	1,185	617	568	86	15.1
2007	1,505	868	637	82	12.8
2006	1,886	1,468	418	68	16.2
2005	1,784	1,008	776	86	11.1
2004	1,833	1,130	703	59	8.4
2003	1,268	1,043	225	20	8.9
2002	749	644	105	12	11.4
2001	426	398	28	1	3.6
2000	227	214	13	0	0
Total	11,581	7,735	3876	485	12.5

Note that structures are deposited some time after the sample has been through the laboratory and trends in that column will lag trends in the sample column.

grid of salt concentrations and pH for ammonium sulfate, sodium malonate, sodium potassium phosphate and sodium chloride). The commercial screens provide a complementary set of cocktails to the incomplete factorial sampling of chemical space.

Practically, we ensure that the effort involved in formulation is as minimal as possible. We have several quality-control steps in the process that have maintained consistency over the 10 years of operation including measuring the refractive index of the cocktail solutions and a set of standard proteins to test reproducibility of screening experiments. Preparation uses stock solutions either made up and tested in-house or purchased commercially from Hampton Research.

Contamination and the use of washing or disposable items was a practical consideration. Disposable tips are a viable option for many laboratories. They can save a significant amount of time, as they do not require wash cycles but increase supply costs and typically require additional manual intervention. Systems using syringes with nondisposable tips require thorough washing to prevent cross-contamination. Chemicals such as PEGs require significant numbers of wash cycles to thoroughly clean the capillary-like needles of a syringe. We chose to use nondisposable syringes. They are well suited for dispensing into the narrow diameter, high-density 1536-well plates, estimations on throughput were within our target range, and it made financial sense as our protocols would have required thousands of disposable tips. This cycle does not affect productivity as the most time-consuming task, waiting on crystallization, is not affected by this.

We use volumes of 200 nl of cocktail and 200 nl of sample solution. This is an important consideration. There is some debate in the literature over the best sample volume to use for crystallization screening. Sample volumes as low as 20 nL are used for automated vapor-diffusion experiments; up to a two-order magnitude reduction in sample volume and a 10-fold reduction in the time required for crystal formation was reported [40]. Low-volume drops have to be accommodated by not only the liquid-handling systems but also the imaging/crystal detection and if desired the crystal-retrieval steps. The 200 nl + 200 nl drops are readily dispensed by our liquid-handling systems and are well accommodated by our imaging systems.

The data acquisition was an important design consideration from the start for data mining. Information is captured in a plate-centric manner. Each plate is associated with an investigator, protein sample(s), cocktails and a setup date. Currently, we have 1,815 investigator accounts in our database; these accounts capture who should have access and be notified of any changes in the experiment's status, the preferred method of delivering information to the user alone and the confidentiality status of the sample. There are three categories of confidentiality, fully open access for those samples associated with the PSI efforts or where an investigator tells us details can be released, those samples from individual investigators where we protect the sample details (the majority of our cases) and those from industrial collaborators where often we do not know the identity of the sample. Each sample receives a consecutive P-code for identification (to maintain confidentiality of the sample) and information about the sample and the crystallization-screening conditions is recorded.

Dozens of scripts run in the background to generate imaging schedules, data archiving and distribution and notifications to users and staff. A plate imaging schedule is scripted, along with software to control archiving. This scripting that runs behind the database is invisible but critical for successful operation of the laboratory.

Table 2. Examples of structures from individual laboratories where the initial crystallization lead was obtained in the high-throughput screening (HTS) laboratory.

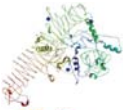
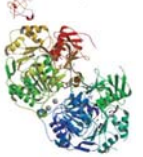
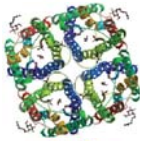
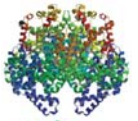
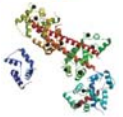
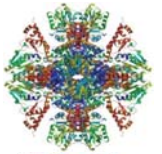
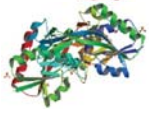
Name	PDB	Ref.		Name	PDB	Ref.	
Toxoplasma gondii Cathepsin L	3F75	[68]		*Haemophilus influenzae* immunoglobulin A1 Protease	3H09	[69]	
N-domain of Wilson disease-associated protein	N/A	[70]	N/A	acyl-CoA synthetase from *Methanosarcina acetivorans*	3ETC	[71]	
Precursor Form of Human Tripeptidyl-peptidase 1	3EDY	[72]		Human aquaporin 4*	3GD8	[73]	
T. gondii cysteine protease cathepsin L (TgCPL)	3F75	[74]		*Thermus thermophilus* and *Bacillus subtilis* M32 carboxypeptidases	3HQ2 3HOA	[75]	
Inhibitor-bound Hsp90 and GRP94	2FXS 2GFD 2EXL	[76]		Rad60 SLD2	3GOE	[77]	
Dimeric cardiac L-type calcium channel regulatory domains	3G43	[78]		*Pseudomonas dacunhae* aspartate-β-decarboxylase dodecamer	3FDD	[79]	
VH1, a dual-specificity phosphatase	3CM3	[80]		Hemophore HasAp from *Pseudomonas aeruginosa*	1B2V	[81]	
NS1A effector domain from the influenza A/Udorn/72 virus	3EE9	[82]		BenM, a LysR-type transcriptional regulator from *Acinetobacter baylyi* ADP1	2H99	[83]	

Table 2. Examples of structures from individual laboratories where the initial crystallization lead was obtained in the high-throughput screening (HTS) laboratory (continued).

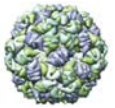
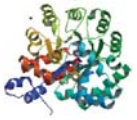
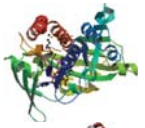
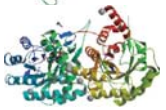
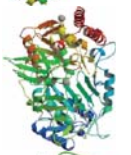
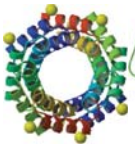
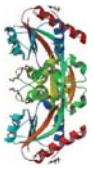
Name	PDB	Ref.		Name	PDB	Ref.	
Pseudomonas aeruginosa PvcA and PvcB	3E59 3EAT	[84]		Small RNA Phage PRR1 capsid	2VF9	[85]	
Adenosine deaminase from a Human Malaria Parasite	2PGF 2PGR 2QVN	[86]		CoA ligase characterization	3CW8 3CW9	[87]	
Trypanosoma brucei α/β-hydrolase fold	2Q0X	[88]		MACPF domain of human complement protein C8 complex	2RD7	[89]	
N-terminal domain of *Mycobacterium tuberculosis* DnaB helicase	2R5U	[90]		GspK-GspI-GspJ complex from the enterotoxigenic *Escherichia coli* type 2 secretion system	3CI0	[91]	
Pyocyanin biosynthetic protein PhzS	2RGJ	[92]		Virulence-associated cathepsin L proteases *of Fasciola hepatica*	2O6X	[93]	
Trypanosoma brucei dihydroorotate dehydrogenase	2B4G	[94]		Pseudomonas aeruginosa bfd and FPR	2QDX	[95]	
Escherichia coli protein YmgB	2OXL	[96]		Rat cytosolic PEPCK	2QEW 2QEY 2QF1 2QF2	[97]	
Homo sapiens kynureninase	2HZP	[98]		p120 RasGAP SH3 domain	2J05 2J06	[99]	

Table 2. Examples of structures from individual laboratories where the initial crystallization lead was obtained in the high-throughput screening (HTS) laboratory (continued).

Name	PDB	Ref.		Name	PDB	Ref.	
TPR protein superhelice	2HYZ	[100]		Effector binding domain of BenM	2F97 2F8D	[101]	
P120 Rasgap SH3 domain	2IO5	[99]		2Fe 2S outer mitochondrial membrane protein	2QH7	[102]	

The key aspects of our operation are instrumentation, scale, staff and expertise. We have invested significant resources in dedicated robotics for many steps of the process. These are in constant use due to the number of samples we receive from the external community. A dedicated staff supports these robotic systems interfacing between the different stages where manual steps are more efficient than a completely automated process. For example, our current maximum throughput is some 200 individual proteins per month. Cocktail plates are manually positioned and removed after filling. This is not an onerous task and for the cost of an automated system to help in this we have a full-time technical staff member who can help with other aspects of the process when the cocktail filling is not taking place. Automation has its place but to be fully cost effective it should be applied in all stages that need it but not every stage that could use it. Robots are not best at everything; we do not assume robots will always outperform humans. Talented, conscientious, intelligent and observant people are the most important ingredient for a successful crystallization laboratory, not the robotics.

5. Optimization

We have a successful system to perform crystallization screening and record results. Others have similar capabilities [41-43], and commercial instruments are available to replicate this process on a slightly smaller scale in the home laboratory. The HTS process has reached a stage of maturity in that we can replicate the set up and imaging of crystallization trials set up by hand but in a massive way. Where we have data on failure as well as success, we advance from a purified, soluble protein to a crystal for 21% of samples and advance from a purified soluble protein to a structure for only 8% of samples [6]. Optimization of initial crystallization hits to produce crystals suitable for structural determination is a significant bottleneck.

What is currently lacking is the ability to go from the initial screening results to a well-diffracting crystal in an automated manner. A good deal of manual effort is needed after the screening stage to interpret the results and proceed to data collection. Individually, many of the processes are there, but they are not linked into a pipeline. For example, automated image classification shows promise in identifying defined regions of the crystallization phase diagram, even if crystals are not seen in the screening [44]. Chemical space mapping can use that information together with a knowledge of theoretical phase diagrams to identify the key trends promoting crystallization and thereby rationally design the cocktails needed to sample the most likely regions of chemical space for crystallization [45,46]. By X-raying lead conditions in situ [47-50] or harvesting them directly from the screening plates (currently in development) we can use diffraction quality rather than visual quality as a quantitative metric for optimization. By the use of

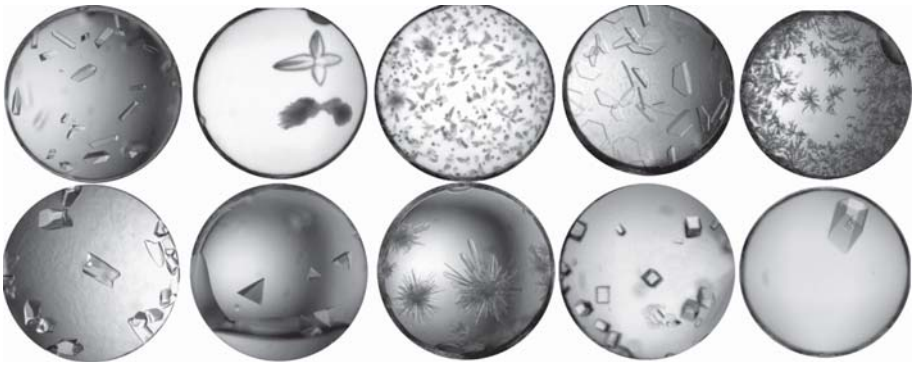


Figure 2. High-throughput microbatch-under-oil screening outcomes from our laboratory that show examples of crystal leads for several different proteins. Each image displays a well of 0.9 mm diameter. Some hits are obvious crystals, others need closer examination and are more difficult to discern on the scale of the figure.

concentration variation and temperature in a technique termed drop volume ratio and temperature (DVR/T) [51], we can rapidly automate the optimization process.

Despite significant improvements, optimization remains a significant technical challenge for high-throughput methods. The optimization steps tend to be less standardized and often rely upon protein-specific strategies to improve the crystals' diffraction properties.

6. Conclusions

There are no fundamental limits to producing a completely automated pipeline where a purified protein sample enters and, if it can exist, a well-diffracting crystal results. High-throughput screening techniques work well. We have described those based on plate technologies but others exist, in particular exploiting the use of microfluidic devices [47,52-54]. Practically, the number of conditions that can be sampled increases and/or the volume of sample required decreases. We have focused on soluble samples but similar approaches can be applied to membrane proteins [55]. In terms of pharmaceutical discovery, efficient crystallization-screening strategies exist, but the results require interpretation to be utilized most effectively.

High-throughput optimization will be the key development that shifts the paradigm of structural biology. In screening for the SG program, a standard set of screens is used and lead conditions are optimized manually. The manual step imposes a finite number on the leads that can be followed up, frequently only a fraction of the most promising cocktails are chosen. If crystals are not observed with different constructs or species, we can make use of solubility information provided by the clear and precipitated experiments to refine our search [45]. If the same automation technologies can be applied to optimization, coupled with computational classification and analysis, we may be able to considerably increase the success rate of going from a new protein to a structure. While we do not yet know what this increase would be, our own small angle X-ray scattering studies on several hundred SG targets (complementary to X-ray crystallographic and NMR studies) [56] characterized the majority of the samples as globular and well folded. More excitingly from the pharmaceutical view is the increase in number of lead compounds we could incorporate at the screening stage. High-throughput crystallization screening has an important role in structure-based pharmaceutical design.

7. Expert opinion

High-throughput crystallization screening is a mature technology. Thousands of proteins per year can be screened at large facilities such as ours. Commercially available systems allow smaller, but still significant, efforts to take place in individual laboratories. There are some practical aspects that when taken into account will make it possible to maximize the research advantages that a high-throughput approach has to offer. High throughput means high input and produces high output. To clarify this important statement, unless you can produce sufficient samples or experiments to feed robotic systems and have the capacity to collect and then interpret the results produced, you should give serious consideration and ask yourself why automation will be beneficial. High-throughput crystallization is well suited for drug discovery; large numbers of identical crystals can be prepared for ligand-soaking, or co-crystallization experiments [57]. Automation is best suited for building core facilities, linking several laboratories or operating as a large facility serving many investigators. This achieves the high sample input needed, provides expertise in the operation and maintenance of the systems and allows the analysis of results to be carried out in more detail by each individual. Idle automated systems, especially those designed for liquid handling, often require the operator to re-learn the software and operation. The instrument

itself will likely require significant preparation if not in use continuously.

While high-throughput crystallization screening is mature, the vast majority of biological macromolecules are recalcitrant to crystallization. Consider that for the "easy" cases of soluble proteins the odds of structural success are roughly 1:5. This ignores the truly challenging classes of membrane proteins and protein complexes. The *rate of structural success* for samples recalcitrant to crystallization would not increase by simply increasing the number or the rate of experimental set ups. In the initial SG studies, a fold library was created where it did not matter if 4 out of 5 samples failed; there were multiple targets to build this library. As the technology turns to biological pathways, the high failure rate of crystallization will leave important gaps in our knowledge. Studies have identified physical properties of the protein that affect crystallization [58]. We can approach this problem from the perspective of modifying the protein targets to improve crystallization success. Methods such as truncation [59], reductive methylation [60], surface entropy reduction [61], limited and *in situ* proteolysis [62] and small molecule additives that can act as freezers to reduce conformational flexibility [37] have all proven effective means to promote crystallization. These are just a few of the salvage pathways for recalcitrant samples. We can push the envelopes of other techniques such as NMR and Cryo-EM. We can also aim at the optimization process, developing more suitable protocols for automation rather than trying to adapt manual methods.

Optimization is a skilled aspect of the crystallization process. The initial information from the crystallization screening is multi-parametric and has to be digested and used to guide the next experiments. Initial analysis predominantly relies on a trained observer characterizing crystals visually. Robotic crystal mounting which is being actively developed [63,64], promises to apply reproducible mounting protocols such that initial leads can be examined with an X-ray beam. Similarly *in situ* diffraction can enhance the process considerably with less sample manipulation. Both provide a quantitative metric that can be interpreted algorithmically and used in automated optimization of the sample [65]. Developments outside crystallography in detector, beamline and X-ray sources are pushing the definition of what we consider a suitable crystal for structural determination to the extremes. Smaller and smaller crystals are being used for structural determination. It is technically feasible to collect structural data from a crystal 1 – 2 μm in size [66]. Intense fourth-generation X-ray sources such as the Linac Coherant Light Source (LCLS) collected femtosecond microdiffraction patterns from nanocrystals of Photosystem 1 that were used for structural determination [67]. These advances could in many cases actually eliminate the need for optimization. One path for the future of structural crystallography seems clear; we will use smaller crystals to produce structures of larger and more complex biological macromolecules. It is unclear how much these developments will improve the overall success rate, going from a soluble protein to a crystallographic structure. However, it is clear that scientific and technological developments in the steps that proceed and follow crystallization will enable X-ray crystallographic techniques to be applied to a broader range of targets for a broader sector of the biological community. These advances will provide structural information to those who are experts in the biological process rather than specialists in structure. This will lead to an improved understanding of biology and will specifically benefit research in novel drug targets.

While setting up the required crystallization experiments, it is possible to simultaneously use the high-throughput technologies to conduct basic crystallization research. From their very nature, high-throughput automated technologies can produce statistically meaningful data on crystallization, with precisely controlled conditions, reproducible volumes and outcomes recorded in detail. The crystal growth literature is full of relevant research on the fundamentals of crystallization. However, most of these studies used a very narrow subset of soluble proteins to establish these fundamentals. With many parameters to be studied, the high-throughput approaches also offer the most efficient means to truly gain a better understanding of the multi-parametric crystallization problem. These approaches have the capacity to rapidly duplicate all the previous studies and extend them to multiple proteins and conditions, building up a more complete picture of the entire range of outcomes seen in crystallization trials. The demands placed upon most high-throughput laboratories for required crystallization efforts often leave little time to initiate such fundamental studies. With an increase in both availability and the number of automated systems for crystallization, and appropriate support for these efforts, the resulting knowledge could significantly increase the success rate in going from a purified protein to structural knowledge of that protein.

In summary, a dedicated facility can perform high-throughput crystallization screening in an efficient manner. The tools used, staff to keep them running and the computational infrastructure are all key aspects of success. High-throughput optimization of crystals is not yet a mature, fully automated method. Humans remain the cornerstone of any laboratory efforts. Technology does not remove people from the pipeline; it will focus their strengths, which cannot be duplicated by robotics, to tasks where their talents are better applied. High-throughput approaches achieve results most effectively when they are used in a high-input manner. They are not the panacea for every crystallization problem. Automation can be used to guide crystallization efforts toward samples that are more likely to provide structures. This provides a means to obtain crystallographic structures faster and can help understand and address fundamental crystallization challenges. High-throughput techniques

can provide both applied and fundamental crystallization knowledge in an efficient, cost-effective manner. Ultimately, humans define the experiments and interpret the outcomes Crystallization robotics are tools to improve human efficiency.

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Declaration of interest

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