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Approaches to automated protein crystal harvesting

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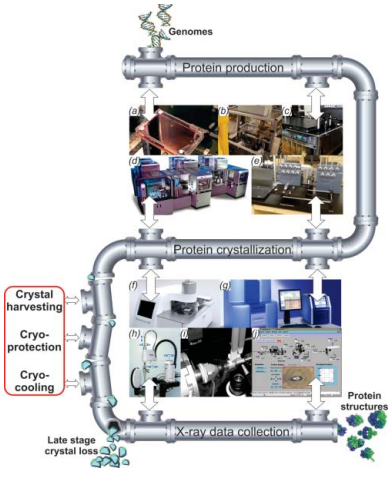
The harvesting of protein crystals is almost always a necessary step in the determination of a protein structure using X-ray crystallographic techniques. However, protein crystals are usually fragile and susceptible to damage during the harvesting process. For this reason, protein crystal harvesting is the single step that remains entirely dependent on skilled human intervention. Automation has been implemented in the majority of other stages of the structure-determination pipeline, including cloning, expression, purification, crystallization and data collection. The gap in automation between crystallization and data collection results in a bottleneck in throughput and presents unfortunate opportunities for crystal damage. Several automated protein crystal harvesting systems have been developed, including systems utilizing microcapillaries, microtools, microgrippers, acoustic droplet ejection and optical traps. However, these systems have yet to be commonly deployed in the majority of crystallography laboratories owing to a variety of technical and cost-related issues. Automation of protein crystal harvesting remains essential for harnessing the full benefits of fourth-generation synchrotrons, free-electron lasers and microfocus beamlines. Furthermore, automation of protein crystal harvesting offers several benefits when compared with traditional manual approaches, including the ability to harvest microcrystals, improved flash-cooling procedures and increased throughput.

1. Introduction

Detailed knowledge of macromolecular three-dimensional structures is essential for understanding how cells work and how diseases progress at the molecular level. A common method used for determining three-dimensional structures is X-ray crystallography, which currently accounts for 88% of all macromolecular structures deposited in the Protein Data Bank (PDB; http://www.pdb.org; as of January 2014, 85 486 of 96 692 structures). Vital to X-ray crystallographic techniques is the growth of protein and other macromolecular crystals. Once grown, the protein crystals are exposed to an X-ray beam and diffraction data are collected. Key to this process is the harvesting of protein crystals, which in the broadest sense includes a series of manipulations that bring the protein crystal from its growth medium into the X-ray beam in a condition suitable for X-ray diffraction. Traditionally, much of this work is performed manually by skilled individuals. However, technological advances in robotics and automation, to a large extent spurred by structural genomics (SG), has fostered the development of high-throughput protein crystallography (HTPX; Terwilliger *et al.*, 2009; Service, 2005). Automation is essential for an efficient HTPX/SG laboratory, playing a key role in all stages from cloning, protein expression and purification (Kim *et al.*, 2004) to crystallization (Mueller-Dieckmann, 2006), data collection and processing (Adams *et al.*, 2011) [reviews of automation include Cymborowski *et al.* (2010), Manjasetty *et al.* (2008) and Blow (2008); a simplified HTPX/SG pipeline is shown in Fig. 1]. Given the plethora of automation systems developed for HTPX/SG, it is somewhat surprising that only limited automation exists for the crystal-harvesting stage (highlighted in Fig. 1). Indeed, protein crystal manipulation represents a unique challenge for automated systems owing to the extremely fragile constitution of



protein crystals and their susceptibility to subtle changes in osmolarity, pH, chemical environment and, to a lesser extent, temperature. Because such fragile micromanipulation tasks are difficult to automate, crystal harvesting is almost always omitted in discussions of HTPX/SG automation. Unfortunately, crystal harvesting also presents several opportunities for late-stage crystal losses and failure (highlighted by the red box and leaking pipeline in Fig. 1). Reasons for these late-stage crystal losses include physical damage to the crystal arising from inappropriate handling by the operator, physical and chemical damage to the crystal owing to poor cryoprotection procedures and loss of diffraction properties during unsuccessful flash-cooling (see §4.2). Almost all of these issues can be addressed by applying suitable automation techniques to control, precisely and reproducibly, the entire crystal-harvesting process.

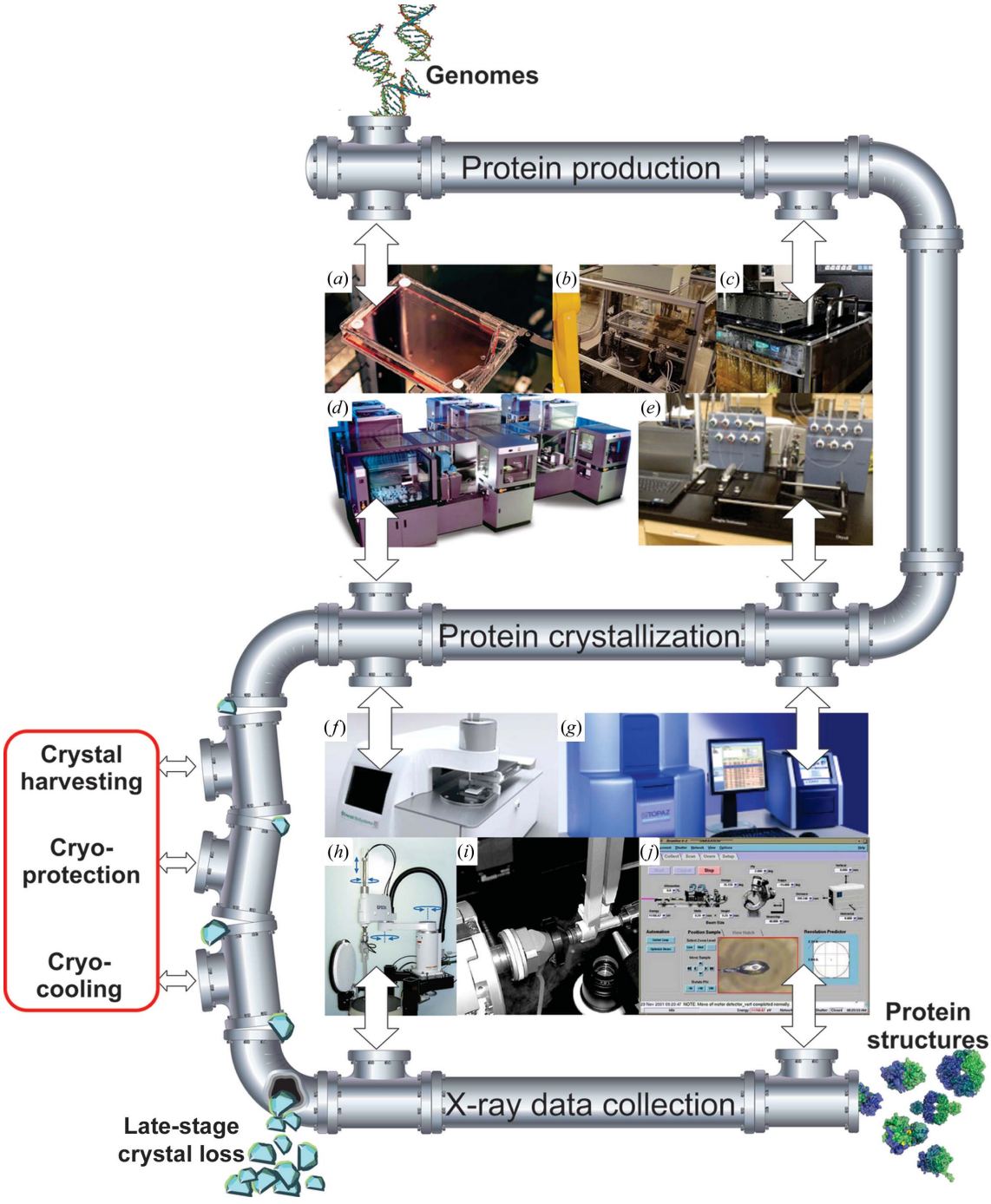


Figure 1
Schematic representation of a typical structural genomics pipeline showing examples of automation currently in use at the JCSG. The three main stages of the pipeline are highlighted as 'Protein production', 'Protein crystallization' and 'X-ray data collection', with examples of automation at each stage: (*a*, *b*) Protein Expression and Purification robot (PEP; GNF Systems, California, USA); (*c*) GNF fermenter (GNF Systems, California, USA); (*d*) CrystalMation crystallization platform (Rigaku, The Woodlands, Texas, USA); (*e*) Oryx8 crystallization system (Douglas Instruments, Hungerford, England); (*f*) Plug Maker (Emerald BioSystems Inc., Bainbridge Island, Washington, USA); (*g*) Topaz crystallizer (Fluidigm, San Francisco, California, USA); (*h*, *i*) Stanford Auto Mounter system (SAM; SSRL, Stanford, California, USA); and (*j*) *Blu-Ice* control system (SSRL, Stanford, California, USA). The wobbly pipeline construction on the left-hand side represents a break in process automation for the 'Crystal harvesting', 'Cryoprotection' and 'Cryocooling' stages (red box).

Current manual crystal-harvesting procedures are far from optimal for typical HTPX/SG use and room for significant technological development exists. It is likely that new technologies will emerge that will revolutionize the way in which data are collected from protein crystals and therefore the manner in which protein crystals are harvested. For example, recent developments in detector technologies enable novel data-collection methods that may in fact obviate the need for classical crystal-harvesting and crystal-mounting techniques (see §1.3). Additionally, *in situ* data-collection strategies may eliminate the need for troublesome cryoprotection procedures (see §3.5), and new data-collection strategies may offer the potential to 'outrun' radiation damage even at room temperature (Rajendran *et al.*, 2011; see §1.3.1). Furthermore, new micro-electromechanical system (MEMS) devices are emerging that may fundamentally change the way in which we approach the manipulation and harvesting of protein crystals (see §1.3.2 and §2.2.12).

1.1. Impact of HTPX/SG

Large-scale HTPX/SG centers are equipped to determine hundreds of novel protein structures every year, and technological advances are constantly being sought to improve the efficiency and throughput at each stage of the HTPX/SG production pipeline. One of the main advantages of organizing structure-determination efforts into large-scale HTPX/SG centers is the centralization of resources and equipment that can be shared by multiple principal investigators. This enables large-scale HTPX/SG centers to leverage robotics and automation on a scale that is not feasible in traditional laboratories supported by a single investigator. In the US, large-scale HTPX/SG centers currently include the JCSG (Joint Center for Structural Genomics; Elsliger *et al.*, 2010), the Midwest Center for Structural Genomics (MCSG; Joachimiak, 2009), the New York Structural Genomics Consortium (NYSGC; Sauder *et al.*, 2008) and the Northeast Structural Genomics Consortium (NSGC; Montelione & Szyperski, 2010). These large-scale centers have contributed significantly to the total number of protein structures deposited in the PDB. Currently, worldwide HTPX/SG efforts account for over 10% of all protein structures deposited in the PDB (Fig. 2*a*). Moreover, this figure is significantly higher for proteins from families with no previous structural coverage, with about 50% of all novel structures coming from HTPX/SG centers (Chandonia & Brenner, 2006). In addition, many synchrotron facilities have (or are in the process of establishing) HTPX/SG capabilities (for a complete list of worldwide macromolecular-capable beamlines and their capabilities, see http://biosync.sbkb.org). Several of these synchrotron facilities are equipped with crystallization facilities and high-flux microfocus beamlines capable of *in situ* data collection (Pohl *et al.*, 2006; Evans *et al.*, 2007; Flot *et al.*, 2010; Heidari Khajepour *et al.*, 2013; Hirata *et al.*, 2010; Mueller *et al.*, 2012; Mueller-Dieckmann, 2006; Rosenbaum *et al.*, 2006; Bingel-Erlenmeyer *et al.*, 2011; see §3.5 for a discussion of *in situ* methods). Such facilities enable industrial and academic users (who are not part of a traditional SG center) to access HTPX/SG automation. Collectively, these HTPX/SG efforts, together with those of the traditional SG centers, make significant contributions to the overall protein structure output of the structural biology community. Advances in areas such as automated protein crystal harvesting will empower such facilities to increase not only throughput but also protein structure output.

1.2. The need for automated protein crystal harvesting

1.2.1. The protein crystal-harvesting process. Crystal harvesting in the broadest sense includes any process whereby a protein crystal is relocated from its growth medium (generally a mother-liquor

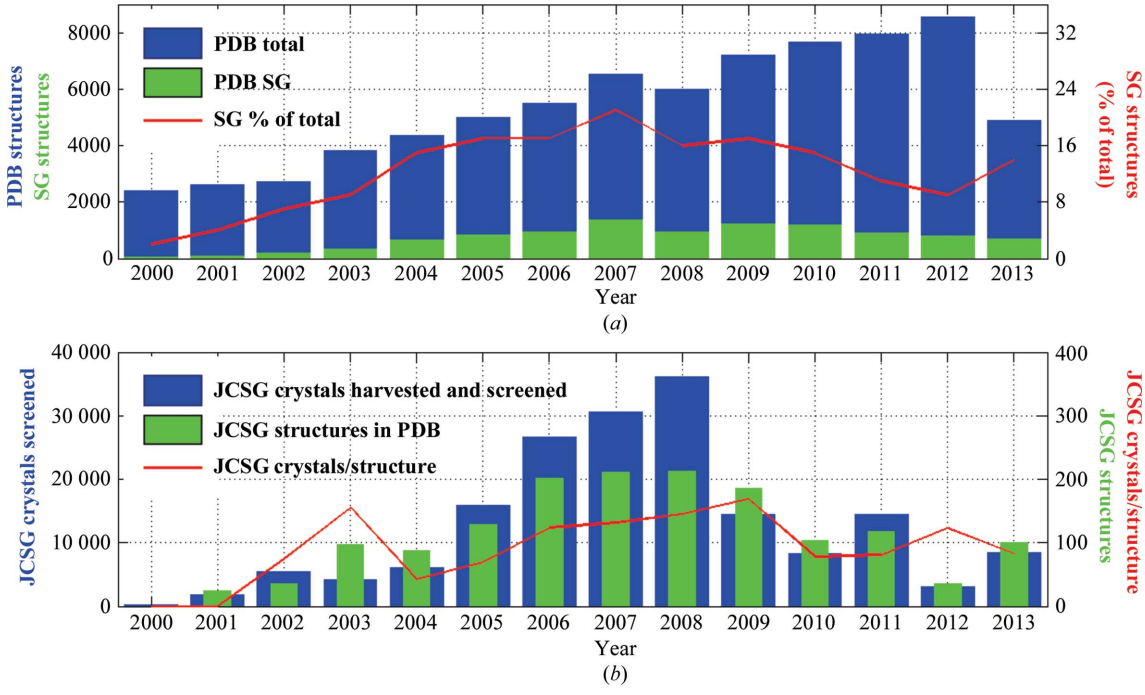


Figure 2
Impact of structural genomics and PDB growth. (*a*) Impact of structural genomics on the total number of protein structures deposited in the PDB; (*b*) number of crystals harvested and screened at the JCSG per protein structure deposited in the PDB. In (*a*) the lower portion of each bar (green) represents the number of structures from structural genomics centers and the upper portion (blue) represents the total number of structures deposited in that year. The red line represents the contribution of structural genomics to the total effort as a percentage (PDB SG/PDB total × 100%). The blue portion for 2013 is artificially low as the data do not include unreleased structures (5129 unreleased structures as of January 2014). In (*b*) the thick blue bars represent the number of crystals harvested and screened for diffraction at the JCSG and the thinner green bars represent the total number of JCSG depositions in the PDB. The red line represents the number of JCSG crystals harvested and screened for every structure deposited in the PDB (JCSG crystals/structure).

solution) to an environment in which diffraction data can be collected by exposure to an X-ray beam. The process can be separated into several sub-tasks, including looping, cryoprotection, flash-cooling and mounting onto an X-ray source. The looping step involves placing the crystal into a microloop mounted on a standardized mounting pin (for specifications of mounting pins, refer to http://stanford.io/1a7SQgO). The looping process has changed little since the inception of such mounting techniques some 20 years ago (Hope, 1988, 1990; Teng, 1990), although the microloops (or micromounts) are now often made of polyimide materials such as Kapton (Thorne *et al.*, 2003; Georgiev *et al.*, 2006; Kitago *et al.*, 2010). During the crystal-harvesting process, the crystal also typically undergoes a scheme of cryoprotection and flash-cooling to help reduce radiation damage during exposure to the ultrabrilliant and intense synchrotron X-ray beams (Holton, 2009; Garman & Owen, 2006; Garman & Schneider, 1997). The cryoprotection step typically involves supplementing the aqueous mother liquor with a higher percentage of an organic solution such as 2-methyl-2,4-petanediol (MPD), ethanol, glycerol, ethylene glycol, polyethylene glycol or 2-propanol (Petsko, 1975). Once the crystal has been suitably cryoprotected and isolated in the microloop or micromount, the crystal is then flash-cooled to cryogenic temperature. This is typically achieved using liquid nitrogen (LN_2) or a stream of dry gaseous nitrogen, although other cryogens such as liquid propane or helium may be used. The flash-cooled crystal is typically stored in LN_2 until it is mounted on a goniostat for data collection under a cryogenic stream of cold dry gaseous nitrogen. It is important to distinguish the crystal-harvesting stage (*i.e.* looping through to flash-cooling) from the crystal-mounting and screening stage. For the purposes of this review, we will primarily focus on automation of the crystal-harvesting stage (automation of crystal-mounting and screening is briefly discussed in §1.2.2).

1.2.2. Current levels of automation. Reliable automation is available for most of the stages of an HTPX/SG gene-to-structure pipeline, including cloning, protein production and crystallization trials, and X-ray data collection (Fig. 1). A significant break in the automation workflow remains between the automated production of protein crystals and automated X-ray data collection: namely, the automated looping, cryoprotection and flash-cooling of protein crystals (the steps highlighted by the red box in Fig. 1). Automated protein crystal harvesting technologies are still in their infancy (albeit rapidly progressing), and only a handful of mostly semi-automated systems exist (see §2.2). We discuss some of the general system-design features for each of these systems and provide an overview of the most promising systems. Some of these systems present novel technologies that will evolve further and will ultimately allow large-scale HTPX/SG efforts to reach their full potential.

Automated crystal harvesting becomes important when a large number of crystals need to be screened for diffraction, as is the case in any HTPX/SG effort. Although the production of data-quality crystals remains a rate-limiting step in protein structure determination by X-ray diffraction, many automation solutions have been implemented to enable crystal growth on a massive scale: for example, fully integrated platforms offering screen design, plate setup and crystal imaging, such as the CrystalMation system in use at the JCSG (Rigaku Automation, Carlsbad, California, USA; Mallett *et al.*, 2007) and similar systems at The Photon Factory (Hiraki *et al.*, 2005), EMBL Hamburg (Mueller-Dieckmann, 2006) and The Oxford Protein Production Facility (Walter *et al.*, 2005). Once the protein crystals have been grown, they are screened for suitable diffraction properties and data are collected using an X-ray diffractometer. The diffraction-screening and data-collection steps are also highly automated, including the use of fully integrated robotic sample-loading and beamline automation (Beteva *et al.*, 2006; Karain *et al.*, 2002; Rosenbaum *et al.*, 2006; Snell *et al.*, 2004). The use of automated sample-loading systems is now commonplace on many beamlines around the world (for details, see the Robotic Mounting Forum; http://stanford.io/1b5aqkt). For example, the Stanford Auto Mounter (SAM) as used by the JCSG at the SSRL is capable of fully automated sample loading and retrieval and data collection (Cohen *et al.*, 2002). The use of such systems is further simplified by initiatives such as the Universal Puck Project, which aims to standardize the sample and shipping containers used on these beamlines (http://stanford.io/15Kbie4).

1.2.3. The protein crystal-harvesting bottleneck. In contrast to the plethora of automation systems available at all other stages of the crystallization workflow, crystal harvesting remains one of the exclusively manual steps in the traditional X-ray structure-determination pipeline. Protein crystal harvesting is almost always performed by skilled operatives using a stereo microscope to manually identify and harvest crystals. Overall, this process is time-consuming, low-throughput and subject to poor reproducibility. Automated crystal harvesting offers solutions to many of the problems associated with manual crystal-harvesting procedures. The principal benefits of automation are summarized in Table 1, and some of the benefits for a typical HTPX/SG pipeline (*e.g.* that of JCSG) are highlighted below (adapted from Viola, Carman, Walsh, Frankel *et al.*, 2007).

(i) *High throughput*. Automation of crystal harvesting allows many more crystals to be screened when compared with manual procedures and results in a significant saving in person hours. For example, current procedures at the JCSG have resulted in the harvesting and diffraction screening of just over 165 000 crystals (resulting in the determination of 1470 structures as of January 2014). Therefore, each structure requires, on average, around 100 crystals to be manually harvested for every structure solved and deposited in the PDB (Fig. 2*b*). Manual harvesting of 100 protein crystals by a proficient expert takes of the order of 4 h. Clearly, manual harvesting of hundreds of crystals is feasible for single-protein studies in traditional non-HTPX laboratories, but for large-scale HTPX/SG laboratories working on several thousand protein targets in a parallel approach manual looping is suboptimal. Given the high-throughput requirements and high capital investment costs, automation of crystal-harvesting procedures is at present only practical for large-scale HTPX centers or pharmaceutical/biotech research centers. Indeed, several pharmaceutical companies have assembled large-scale automated crystallization platforms that are capable of screening hundreds of drug targets per day. Additionally, automation of crystal harvesting will be beneficial for both fragment-based screening and traditional small-molecule co-crystallization approaches, which rely heavily on high throughput (Rees *et al.*, 2004; Congreve *et al.*, 2005).

(ii) *Fully automated pipeline*. Manual harvesting procedures represent the last bastion of full human intervention in a typical HTPX/SG pipeline, thereby generating a process bottleneck. Automated crystal-harvesting procedures offer an opportunity to close this gap in the automation pipeline. For example, at the JCSG automation is implemented at all other stages of the process, forming a true gene-to-structure pipeline (Elsliger *et al.*, 2010).

(iii) *Smaller crystals*. Automated crystal harvesting will enable the routine harvesting and screening of microcrystals. As structural biology progresses towards ever more challenging targets, such as membrane-protein assemblies and large macromolecular complexes, it will be essential that crystal-harvesting technologies adapt to microcrystals. For example, the JCSG (in collaboration with SQR-1 System Design, Wyoming, USA) is developing a Universal Micromanipulation Robot (UMR) capable of harvesting microcrystals (see

§2.2.6). The JCSG UMR system is capable of extremely high-resolution movement (∼1 µm), allowing the routine harvesting of crystals in the <10 µm range. Microcrystals of this size are hard to manipulate using manual approaches owing to involuntary hand motions (tremors) and excessive dehydration of the crystallization drop over time. High-throughput harvesting of microcrystals will be necessary to complement the microfocus beamlines now available at most synchrotron sources around the world (Axford *et al.*, 2012; Riekel, 2004; Flot *et al.*, 2010; see §4.1 for more on the harvesting of microcrystals).

(iv) *Adaptable to new crystallization methods*. The field of HTPX/SG is constantly in search of new and improved methods for protein crystal growth. This has led to the development of innovative devices based on microfluidics (Hansen & Quake, 2003), including chips (Kisselman *et al.*, 2011), plugs (Gerdts *et al.*, 2008, 2010) and lipidic cubic phase (LCP; Weidong *et al.*, 2004). Harvesting of crystals from such devices and media can often be challenging when performed manually with conventional microloops and tools. Automation of harvesting from such media can be controlled more precisely, as both the motions and tools can be optimized specifically for each device. For example, the UMR system at the JCSG is currently undergoing field testing of its ability to harvest microcrystals and populate SSRL sample-mounting grids (http://stanford.io/15BDJdl). These sample grids were developed at the SSRL specifically for the automated collection of single frames of data (or small rotation angles) from each crystal. Additionally, these grids allow extremely high-density transportation, with up to 1200 crystals contained in a single UniPuck. Harvesting of protein crystals on such a large scale is extremely time-consuming when performed manually and is therefore a prime activity to be targeted for automation.

(v) *Precision liquid handling*. Small-scale liquid dispensing is often required during the crystal-harvesting stage for the addition of cryoprotectants, the soaking of heavy-atom compounds for phasing and the addition of small molecules for co-crystallization studies. Automation facilitates these additions in a precise and controllable fashion. For example, the UMR system at the JCSG has a novel 'cryo-drip' cryoprotection system in which individual drops of low-viscosity perfluoropolyether oil are 'dripped' onto the harvested protein crystal (Viola *et al.*, 2011). Low-volume manipulations such as this are difficult to achieve manually in a reproducible manner. Low-volume liquid dispensing also opens up tremendous possibilities for the automation of high-throughput drug discovery. Such techniques could facilitate the production of crystals soaked in entire libraries of compounds, as routinely used in fragment-based screening approaches (Blundell & Patel, 2004; Congreve *et al.*, 2005).

(vi) *High-speed harvesting*. High speed is essential to limit both dehydration of the crystal once it is removed from the mother liquor (Douangamath *et al.*, 2013) and also to facilitate improved flash-cooling protocols (Warkentin *et al.*, 2006; Warkentin & Thorne, 2007). Additionally, for some crystals it may be advantageous for the crystal to spend as little time as possible in the cryprotectant solution before flash-cooling occurs. For such crystals, speed is of the essence as chemical damage to the crystal may occur. Once the crystal has been harvested and cryoprotected it is essential that it is swiftly moved to liquid nitrogen for flash-cooling without further delay.

(vii) *Reproducibility*. Manual crystal-harvesting procedures are highly variable in nature and often damage fragile protein crystals owing to mechanical stress. Manual procedures are subject to significant shake and jitter, as a result of hand tremor, which may further damage the fragile protein crystal. Such motions are eliminated with the use of automated crystal-harvesting procedures as all movements are steady, precise and repeatable. All robot motions are consistent and software-controlled, therefore ensuring that each crystal is subjected to a similar range of speeds and motions. Controlled motion is essential for reduced inter-operator variability, as one individual may harvest crystals using a different range of speeds and motions when compared with another.

(viii) *Improved flash-cooling*. Automation of the crystal-harvesting and flash-cooling steps further enables the study of the crystal-cooling and cryoprotection process. The production of hyperquenched protein crystals is currently under investigation at the JCSG using the UMR platform with a view to eliminating the need for additional cryoprotection steps (Warkentin *et al.*, 2006; Warkentin & Thorne, 2007). This process simplification will save time and resources and will reduce the need for extra manipulations that can damage the protein crystals (see §4.2 for a further discussion of hyperquenching).

In summary, the high speed, reproducibility and precision liquid handling afforded by robotics systems all lend themselves to closure of the crystal-harvesting process bottleneck. Additionally, automation of crystal harvesting offers the potential for improved handling of microcrystals and reduced late-stage crystal failure (for an outlook, see §4).

1.3. New and emerging technologies likely to have an impact on protein crystal harvesting

1.3.1. New detector instrumentation. Pixel-array detectors (PADs) are effecting a paradigm shift in the way that diffraction data are collected from protein crystals (Broennimann *et al.*, 2006; Flot *et al.*, 2010; Rajendran *et al.*, 2011; Brönnimann *et al.*, 2002, 2003; Eikenberry *et al.*, 2003; Boutet *et al.*, 2012). In turn, such instrumentation is having a profound effect on the way that crystals are harvested and prepared for diffraction studies. A number of data-collection techniques are evolving which in part may obviate many of the steps required to obtain diffraction data from a protein crystal. For example, the fast readout times of PADs (milliseconds) enable diffraction data to be collected in a shutterless mode (continuous crystal rotation and data collection). This minimizes many of the problems associated with acceleration and backlash motion (and other corrections) that may be observed with the stop–start methods employed in the classical rotation technique using traditional goniostats and slow-readout detector configurations. Continuous data-collection methods enable the use of robotics arms as goniostats, thus removing the need for additional crystal-harvesting and pin-mounting steps (Heidari Khajepour *et al.*, 2013). Furthermore, continuous motion during data collection also eliminates any inertia-related issues, which can be problematic for large objects such as entire crystallization plates and associated holders mounted on a robot actuator or goniometer (see §3.5 for a discussion of *in situ* protein crystal growth and mounting). A continuous data-collection mode also allows data collection from streams of slurries of microcrystals, again eliminating the need for traditional microloop-based crystal harvesting (Boutet *et al.*, 2012; Kern *et al.*, 2013). Furthermore, novel techniques such as acoustic droplet ejection (ADE) can be applied to produce such streams of microcrystals (Soares *et al.*, 2011; Roessler *et al.*, 2013; see §2.2.8 for a discussion of ADE techniques).

The increased sensitivity and reduced background of PADs also allows data collection using less intense X-ray exposures, therefore reducing the radiation dose that the crystal is subjected to and thus reducing radiation damage to the crystal. In combination with helical scans (*i.e.* continuous exposure of progressive areas of the crystal during rotation), better cryotemperature data have been obtained (Flot *et al.*, 2010). Furthermore, it is almost possible to 'outrun' radiation damage during room-temperature data collection

Table 1
Benefits of automated crystal harvesting.

Adapted from Viola, Carman, Walsh, Frankel *et al.* (2007).

Specific benefit	Manual crystal harvesting	Automated crystal harvesting	Benefits of automation
High throughput	No	Yes	Allows hundreds of crystals to be harvested. Saves several hundred person hours per year.
Fully automated pipeline	No	Yes	Manual procedures represent a process bottleneck. Closes gap in automation pipeline. True gene-to-structure automation.
Smaller crystals	No	Yes	Allows harvesting of <10 µm crystals. Enables HT use of microfocus/microbeams.
Adaptable to new crystallization and screening methods	No	Yes	Flexible automation adapts to new plate and screen designs (*e.g.* microfluidics).
Precision liquid handling	No	Yes	Automation of soaking of ligands and small-molecule inhibitors for co-crystallization trials.
High-speed harvesting	No	Yes	Reduces time between harvesting and flash-cooling. Reduces damage to crystal from cryoprotectant (osmotic shock *etc.*). Reduces drying out of mother liquor.
Reproducibility	No	Yes	Automation lends itself to reproducible crystal harvesting. Constant robot speeds and controlled motion paths ensure fewer variables for crystal loss and damage.
Improved flash-cooling	No	Yes	High speed and precision of robotics allows hyperquenching of protein crystals. Hyperquenching can result in significant reductions in cryoprotectant requirements. Automated cryoprotection screening and novel methods (*e.g.* cryo-drip).

(Rajendran *et al.*, 2011). The use of such detectors for 'outrunning' radiation damage during room-temperature data collection has enabled the development of *in situ* data-collection methods on several beamlines (see §3.5).

1.3.2. MEMS-based technologies. Micro-electromechanical system (MEMS) devices are now emerging that have the potential to fundamentally change how we approach the harvesting and manipulation of protein crystals and microcrystals (for a review of MEMS technologies, see Madou, 2002). MEMS fabrication technologies are likely to be central to any major advances in the field of automated crystal harvesting. For example, the RodBot system utilizes micrometre-sized magnetized MEMS agents capable of gentle crystal manipulations in solution (Tung *et al.*, 2013, 2014; see §2.2.12). MEMS technology is employed in many of the systems in this review, including the microshovel of the CARESS system and the 'loopless' mounts of the CMM system (see §§2.2.5 and 2.2.7, respectively). Biocompatible MEMS materials such as SU-8 are also used for the construction of the REACH system microgrippers (see §2.2.9; Heidari Khajepour *et al.*, 2013; Ling *et al.*, 2009). SU-8 is a photosensitive epoxy material that is used as a negative photoresist and is commonly used for the construction of biomedical MEMS devices (Nguyen, 2007; Guerin *et al.*, 1997). This material has several attractive properties for this application, including simple MEMS fabrication, high flexibility and very low X-ray scattering. Furthermore, SU-8 is sufficiently flexible to avoid damage to the fragile protein crystal. Additionally, the X-ray background scattering is low enough to allow X-ray data collection while the protein crystal is held in the microgripper.

MEMS devices are ideally suited for the manipulation of biological samples, and other examples include the use of nanometre-precision microgrippers (Beyeler *et al.*, 2007; Agnus *et al.*, 2009), thermally actuated microgrippers (Chan & Li, 2003; Wang *et al.*, 2003) and microgrippers utilizing adhesive forces (Haliyo *et al.*, 2003). Additionally, MEMS fabrication techniques have been used to produce microscale crystallization plates manufactured from silicon substrates using the photolithography techniques commonly used in electronic semiconductor manufacturing (Juárez-Martínez *et al.*, 2002). Full automation of protein crystal harvesting is likely to be dependent on such advances in MEMS and materials technology, and traditional loop-based methods for harvesting protein crystals will be rendered redundant. MEMS tools customized specifically for automated systems will be essential for moving the field forward.

2. Automation of protein crystal harvesting

Automation of protein crystal harvesting is extremely challenging from an engineering perspective and significantly differs from conventional laboratory automation, which generally follows a defined sequence with limited requirement for operational flexibility. Typical laboratory automation processes in an HTPX/SG pipeline are generally simple linear processes that require little sensory feedback to control the process. However, an autonomous system capable of harvesting a protein crystal needs to be able to react in real time to the motion of the crystal and to make intelligent choices on how to proceed given its sensory feedback. In the case of protein crystal harvesting, this requires real-time machine-vision systems that provide closed-loop feedback control of the robot's actuators. The realisation of such a fully autonomous system requires cutting-edge mechatronics development and as such is costly. For this reason, none of the automated crystal-harvesting systems presented in this review have reached full autonomy, and only a few have approached an operator-assisted or semi-automated level of autonomy (see §§2.1 and 2.2 for further discussion of fully automated and semi-automated systems, respectively). The relative advantages and disadvantages of fully automated and semi-automated approaches to protein crystal harvesting are summarized in Table 2.

2.1. Fully automated protein crystal harvesting

Fully automated protein crystal harvesting requires several key steps, including the identification of the target crystal, harvesting of the crystal from the mother liquor and subsequent cryoprotection and flash-cooling. Full automation is the ultimate goal and presents the most promising potential for increased throughput, labor savings and reproducibility. However, as discussed above (see §2), the development of such fully autonomous robotic systems is hindered by certain technological barriers and associated costs, particularly the need for accurate and reliable crystal-imaging algorithms and the need for precise and high-resolution robotic control systems.

Table 2
Advantages and disadvantages of various levels of protein crystal-harvesting automation: fully automated, semi-automated and plate-based *in situ* screening methods.

More + symbols indicate a higher score or cost.

Level of automation	High throughput	Labor saving	Reproducibility	Crystal detection	Cost	Ease of engineering	Damage to crystal	Limited to synchrotron use	Limited data-collection ability	Advantages	Disadvantages
Fully automated	+++	+++	+++	+++	+++	+	+++	+	+	High-throughput. Labor saving. Reproducible.	Relies on crystal image detection. Expensive hardware and software. Difficult engineering.
Semi-automated	+	+	++	+	+	+	++	+	+	Reproducible. Relatively inexpensive.	Medium-throughput. Labor intensive.
Plate-based *in situ*	+++	+++	+++	+++	+	+++	+	+++	+++	Crystal harvesting not required. Limited damage to crystal. Ease of engineering. Very high throughput. Labor saving. Reproducible.	Excess X-ray scattering and adsorption from plate. Reduced rotation angle for data collection. Limited to room-temperature data collection. Relies on crystal image detection or X-ray rastering. Expensive hardware and software. Limited to synchrotron use.

Central to any fully automated protein crystal harvesting system is the detection and identification of protein crystals. Protein crystal identification is difficult to achieve accurately using computers for a number of reasons. Firstly, although protein crystals are often well ordered on a molecular level they are not necessarily well ordered on a macroscopic level, and protein crystals often display disordered edges and surfaces. Automated detection of non-uniform edges and textures, which are often characteristic of diffracting protein crystals, is a nontrivial computational task (Viola *et al.*, 2011). The problem is compounded by movement of the crystals, for example during the harvesting step, as the textures and facets used to identify crystals will be constantly changing as a result of changes in the crystal orientation. This makes real-time feedback of the position of the crystals to the robotic control systems extremely difficult, thus rendering fully automated manipulation of protein crystals an engineering challenge. Secondly, stringent rejection of false positives (*i.e.* non-protein crystal objects) and false negatives (*i.e.* missed protein crystals) in the crystallization drop is essential. Computationally, finding a single protein crystal amongst all of the visual artifacts within a crystallization drop is challenging. Finally, automated crystal-detection algorithms need to be extremely fast and capable of operating in real time as part of the robotics control loop.

Several approaches towards automated crystal detection have been proposed and all generally involve some form of edge-detection algorithm and texture analysis. Example algorithms and approaches include self-organizing neural nets (Kohonen, 1982; Spraggon *et al.*, 2002), machine learning (Liu *et al.*, 2008), Fisher linear discriminant analysis (Cumbaa & Jurisica, 2005; Fisher, 1936), Bayes object classification (Wilson, 2002), decision trees (Bern *et al.*, 2004) and support vector machines (Zhu *et al.*, 2004). Although much work has been carried out in this field, the success rates remain relatively low, with ∼15% error rates being common (Wilson, 2002; Cumbaa & Jurisica, 2005; Bern *et al.*, 2004). Clearly, the current best crystal-detection practices are not reliable enough for routine HTPX/SG pipeline application and are insufficient to enable fully autonomous protein crystal harvesting. It is likely that full automation of the crystal-harvesting process will require improved methods of crystal detection that do not solely rely on image-based computer-vision methods. For example, alternate crystal-detection methods such as spectroscopic imaging (Nagarajan & Marquardt, 2005), birefringence (Bodenstaff *et al.*, 2002), second-harmonic generation microscopy (Kissick *et al.*, 2013), UV fluorescence (Dierks *et al.*, 2010; Desbois *et al.*, 2013), two-photon fluorescence (Madden *et al.*, 2011) and X-ray diffraction rastering (Stepanov *et al.*, 2011; Hilgart *et al.*, 2011) are potential methods capable of enabling fully automated protein crystal harvesting.

2.2. Semi-automated protein crystal harvesting

Many of the technical difficulties associated with a fully autonomous crystal-harvesting system can be mitigated by the simpler design features of a semi-automated crystal-harvesting system (Table 2). Semi-automated (or 'operator-assisted') crystal-harvesting systems are dependent on human input. Such systems eliminate the need for complex computer-vision and robotic guidance systems and instead rely on human user input to identify protein crystals and to partially guide and supervise the robot control systems. Semi-automated crystal-harvesting systems therefore benefit from simpler designs and reduced construction costs, while maintaining many of the benefits of automation. However, the major downsides of semi-automated approaches are the lower throughput and the labor-intensive nature of skilled human-assisted operation (compared with a fully autonomous system requiring no human input).

Searches of the internet, patent and literature databases reveal 14 semi-automated crystal-harvesting systems, all with varying levels of deployment and automation (see Table 3 for a summary of published systems in order of publication date). In the following section, we will discuss the key features of each system with a focus on the suitability of their technologies for use in an HTPX/SG environment. Some of these systems were specifically designed for HTPX/SG use (*e.g.* 'chopsticks', ACH, CARESS, REACH and UMR; see Table 3 for abbreviations) and two were designed for remote telerobotic operation in space (PCGDS and CPPI). Each of the systems offers a diverse set of solutions to the various technical challenges faced in protein crystal harvesting in an HTPX/SG environment. Readers who are not interested in the technical details of each system may proceed to §3 for an assessment of the techniques employed.

2.2.1. Protein Crystal Growth Demonstration System (PCGDS). The PCGDS system was developed by a team from The University of Washington in collaboration with the Boeing Missile and Space Division (Huntsville, Alabama, USA) as a prototype system for handling protein crystals onboard the International Space Station (ISS; Hannaford *et al.*, 1997; Fig. 3*a*). Protein crystals grown in microgravity are subject to reduced convection and sedimentation rates, and it has been claimed that space-grown crystals can be of better quality than their earth-bound counterparts (Lorber *et al.*,

Table 3
Examples of semi-automated protein crystal-harvesting systems.

System	Developer	URL	Key features	Harvesting mechanism	Reference
Protein Crystal Growth Demonstration System (PCGDS)	University of Washington and Boeing Missiles and Space Division, USA	N/A	Remote telescience/telerobotics harvesting onboard ISS. Microcapillary-based harvesting.	Microcapillary	Hannaford *et al.* (1997)
Crystal Preparation Prime Item (CPPI)	Oceaneering Space Systems, Houston, TX, USA and University of Alabama at Birmingham, USA	http://bit.ly/17psIji	Remote telescience/telerobotics harvesting onboard ISS XCF. 'Liquid–liquid bridge'-based system to isolate crystals for harvesting.	Microcapillary, microtool	Gittleman *et al.* (1999)
Two-fingered micro-hand ('chopsticks')	KEK and AIST, Japan	http://bit.ly/11i6xmV	Microgrippers operated in 'chopstick' fashion. Crystal manipulated with chopsticks and placed into loop or microtool.	Microgripper, microtool	Hiraki *et al.* (2005), Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki, Mizukawa *et al.* (2004)
Automated Crystal Harvester (ACH)	The Ohio State University, USA and University of Limerick, Ireland	N/A	'Capillary-loop coordinating mechanism'. Harvesting of membrane proteins from LCP.	Microcapillary, microtool	Weidong *et al.* (2004)
Columbia Automated Robotic Environment for Streak Seeding (CARESS)	NESG, Columbia University, USA	http://bit.ly/10Z7uS4	Silicon microshovel. Automated streak-seeding. Micropipette-based pickup.	Microcapillary, microtool	Georgiev & Allen (2008), Georgiev *et al.* (2004, 2005, 2006)
Universal Micromanipulation Robot (UMR)	Square One System Design, Wyoming, USA and JCSG, The Scripps Research Institute, California, USA	http://bit.ly/11lf1dX	Semi-autonomous microtool-based harvesting. Flexible plate and cassettes/puck configurations. Automated data tracking, plate opening, 'cryo-drip' cryoprotection and flash-cooling. High-resolution robotics (1 µm).	Microtool	Viola, Carman, Walsh, Frankel *et al.* (2007), Viola, Carman, Walsh, Miller *et al.* (2007), Viola *et al.* (2011)
Capillary-top Mounting Micromanipulator (CMM)	Nagoya University and Hokkaido University, Japan	http://bit.ly/1a20g6v	'Loopless' mounting. Reduced X-ray absorption at longer wavelengths. Improved S-SAD phasing.	Microcapillary, microtool	Kitago *et al.* (2005, 2010), Price (2013), Watanabe (2006)
Acoustic Droplet Ejection (ADE)	National Synchrotron Light Source (beamline X25) and Labcyte Inc., Sunnyvale, California, USA	http://1.usa.gov/1bOALE1	'Acoustic' mounting of micro-crystal slurries. Enabling technology for FEL beam-lines. 'Conveyor-belt' loading of crystals.	ADE	Soares *et al.* (2011), Roessler *et al.* (2013)
Robotic Equipment for Automated Crystal Harvesting (REACH)	European Synchrotron Radiation Facility (beamline FIP-BM30A) and NatX-ray, Saint Martin d'Hères, France	http://bit.ly/12nZZb1	Semi-autonomous micro-gripper-based harvesting. Automated data tracking, plate opening, cryoprotection and flash-cooling. Direct X-ray data collection from plates and samples held in microgrippers.	Microgripper	Heidari Khajepour *et al.* (2013)
Conventional optical tweezers (COT)	Diamond Light Source, Oxfordshire, England	N/A	Lens-based 'optical trap' mounting of microcrystals in 10 µm range.	COT, micromesh	Wagner *et al.* (2013)
Fiber optical tweezers (FOT)	RIKEN SPring-8 Center, Hyogo, Japan	N/A	Fiber-based 'optical trap' mounting of microcrystals in the 5–30 µm range. 'Touchless' mounting.	FOT, microtool	Hikima *et al.* (2013)
RodBot	Multi-Scale Robotics Laboratory, Institute for Robotics Design and Intelligent Systems, ETH, Zurich, Switzerland	N/A	MEMS device. Mobile magnetic device. Fluid-flow manipulation and mounting of crystals of any size.	MEMS, microtool	Tung *et al.* (2013, 2014)
Crystal Harvester (CH)	Bruker, Billerica, Massachusetts, USA	N/A	Commercial product. Micromanipulator-based.	Microtool	N/A
Station for Automated Microscopy and Imaging (SAMI)	FMP Products Inc., Greenwich, Connecticut, USA	http://bit.ly/12uWFO9	Commercial product. Twin-loop micromanipulator primarily for small-molecule crystallography.	Microtool	N/A

2002; Ng *et al.*, 2002; Normile, 1995). The main objective of the PCGDS system was to demonstrate the feasibility of telerobotics, specifically robotics controlled by means of standard internet protocols (IPs). Using such a system, it would be possible to control microgravity protein crystallization experiments onboard the ISS from a remote control station based on the earth. The benefits of the system would include freeing up crew time for other duties onboard the ISS and also freeing up valuable communication bandwidth to the ISS (alternate sources of standard IP connectivity would be utilized instead of standard ISS communication channels). This system utilizes microcapillaries to harvest protein crystals from standard 24-well CrysChem plates (Hampton Research, Aliso Viejo, California, USA) using a Mini-DD Robot (University of Washington) connected to a linear motion base with 25 µm positioning resolution. The end-effector of the robot is fitted with a microcapillary system controlled by a Model 210 syringe pump (KD Scientific, Holliston, Massachusetts, USA). A series of cameras provide visual feedback for operation of the system, including one to monitor the position of

the target crystal and the pipette tip, a second to provide an overview of the robotics work area and a third for close inspection of the microcapillary tip to ensure successful crystal capture.

The PCGDS was reported as a testbed system used for harvesting 0.5 mm plastic cubes, and its performance with protein crystals is unknown (Hannaford *et al.*, 1997). Therefore, we will critically assess the microcapillary approach to protein crystal harvesting further in our review of the ACH and CARESS systems (see §§2.2.4 and 2.2.5, respectively).

2.2.2. Crystal Preparation Prime Item (CPPI). The CPPI system was built by Oceaneering Space Systems (Oceaneering International Inc., Houston, Texas, USA) under contract to the University of Alabama (http://bit.ly/17psIji; Gittleman *et al.*, 1999; Fig. 3*b*). The system was also designed to operate as a telescience/telerobotic facility in a similar fashion to the PCGDS system (see §2.2.1). The system was built to fly onboard the ISS as part of the X-ray Crystallography Facility (XCF; McDonald *et al.*, 1997). The XCF consists of four main subsystems ('Prime Items') housed in modular ISS rackmount cases: (i) the Crystal Growth Prime Item (CGPI) for the growth of protein crystals in microgravity environments, (ii) the CPPI for crystal harvesting, (iii) the X-ray Diffraction Prime Item (XDPI) for data collection in microgravity environments and (iv) the Command Control and Data Prime Item (CCDPI) (McDonald *et al.*, 1997). As a telescience facility, the system was designed to enable the remote preparation and mounting of protein crystals in a microgravity environment and as such was not intended to be fully autonomous. The core components of the system include a robotic arm, a tool cartridge system containing the crystals, a fluid-management system and a flash-cooling system. The system is encased in a sealed and temperature-controlled environment ensuring contamination-free operation in the harsh environs of space. The protein crystals are loaded (at high density) into a series of 12 cartridge tools that also contain the protein crystal-harvesting loops. The fluid-management system consists of 12 pipettes arranged opposite to each other in two banks of six in a tip-to-tip arrangement. The pipettes tips are brought together such that a liquid–liquid bridge is formed between the two pipette tips. This produces a liquid–liquid gap ranging in size from 0.127 to 1.27 cm. It is this liquid–liquid bridge region between the two pipettes that forms the working area for protein crystal harvesting and preparation. Liquid is slowly pumped between the two pipette tips until a protein crystal suitable for harvesting is isolated in the liquid–liquid bridge. This bank of pipettes is also used for the addition of cryoprotectant to the mother-liquor solutions. A camera and a robotic arm (OM3198 micromanipulator; Oceaneering International Inc., Houston, Texas, USA) are trained on this liquid–liquid bridge area containing the protein crystals, allowing

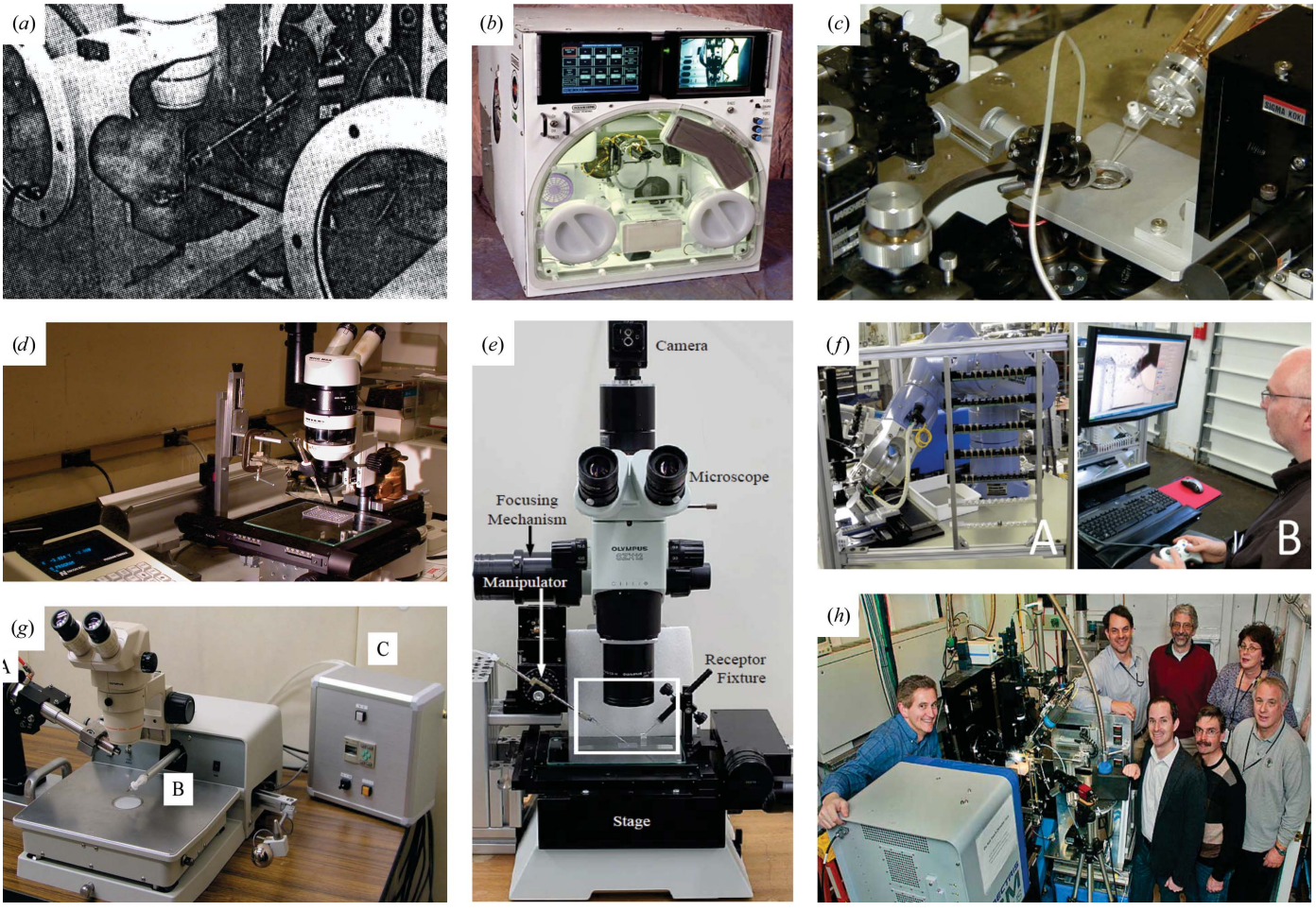


Figure 3
Examples of semi-automated protein crystal-harvesting systems. (*a*) Protein Crystal Growth Demonstration System (PCGDS), (*b*) Crystal Preparation Prime Item (CPPI), (*c*) two-fingered micro-hand ('chopsticks'), (*d*) Automated Crystal Harvester (ACH), (*e*) Columbia Automated Robotic Environment for Streak Seeding (CARESS), (*f*) Universal Micromanipulation Robot (UMR), (*g*) Capillary-top Mounting Micromanipulator (CMM), (*h*) Acoustic Droplet Ejection (ADE). See Table 3 for references, URLs and further details.

the operated-assisted harvesting of the protein crystals by an earth-based scientist. Once harvested in the microloop, the protein crystal is snap-cooled using a custom-designed conduction cooling unit and then stored in a carousel capable of holding 24 crystals maintained at −180°C. Alternatively, crystals can be placed in a microcapillary storage container, without flash-cooling, for further study at room temperature.

The CPPI system has the advantage of being a rugged and reliable system that is capable of harvesting, flash-cooling and storing crystals in extreme environments such as onboard the ISS. The system is dependent on a liquid–liquid bridge for harvesting of protein crystals and as such is not a generally applicable technology for harvesting directly from crystal-growth plates without further modification. The CPPI system led to the design of the ACTOR (Rigaku, The Woodlands, Texas, USA) robotics system, which is capable of automatic sample mounting, X-ray screening and data collection (http://bit.ly/ZtH4u3).

2.2.3. Two-fingered micro-hand ('chopsticks'). The 'chopsticks' micromanipulator was designed by a collaboration between scientists from The High Energy Accelerator Research Organization (KEK) and The National Institute of Advanced Industrial Science and Technology (AIST) in an attempt to fully automate protein structure solution (Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki & Mizukawa, 2004; Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki, Mizukawa *et al.*, 2004; Tanikawa & Arai, 1999; Tanikawa *et al.*, 1997, 1999; Price, 2013; Fig. 3*c*). The 'chopsticks' system is centered on the use of a two-fingered micro-hand system in which each micro-finger ('main-finger' and 'sub-finger') can operate independently in a fashion analogous to chopsticks (Tanikawa & Arai, 1999; Tanikawa *et al.*, 1999; Fig. 4*a*). The crystal-harvesting procedure relies on the operator-assisted control of the chopsticks using a joystick controller. The crystal is carefully held between the main-finger and the sub-finger and is then placed in a traditional microloop for flash-cooling and X-ray data collection. A series of algorithms are used to control the camera focus (Ohba *et al.*, 2000), detect the crystal position and determine the best orientation for handling the crystal (Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki, Mizukawa *et al.*, 2004). Many of these operations are performed automatically, including opening of the fingers to a size appropriate for the target crystal, closing of the fingers to carefully hold the crystal and transfer of the crystal to the target microloop. The system displayed a 90% success rate when harvesting 8 µm glass beads (Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki, Mizukawa *et al.*, 2004). This is a respectable success rate given that the beads are significantly smaller than typical protein crystals (10–300 µm range).

The 'chopstick' approach to crystal harvesting is an attractive concept that overcomes several of the problems associated with traditional microloop-based approaches. Firstly, the system precisely controls the contact pressure exerted on the target crystal, therefore

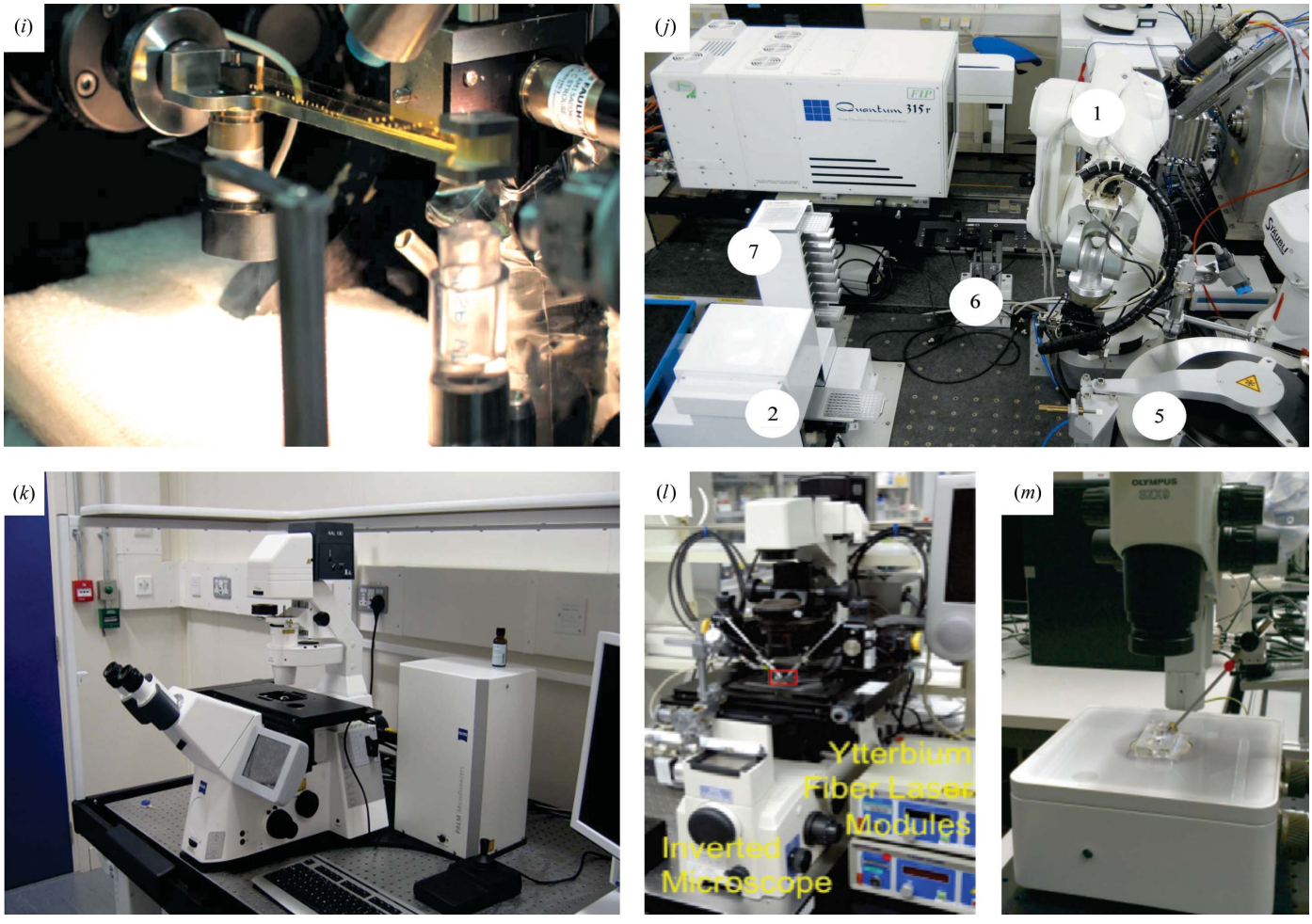


Figure 3 (continued)
Examples of semi-automated protein crystal-harvesting systems. (*i*) ADE conveyor belt, (*j*) Robotic Equipment for Automated Crystal Harvesting (REACH), (*k*) conventional optical tweezers (COT), (*l*) Fiber optical tweezers (FOT) and (*m*) RodBot. See Table 3 for references, URLs and further details.

reducing the potential for physical and structural damage to the fragile crystal. Secondly, the micro-fingers are in an 'open' configuration when approaching the crystal, therefore reducing the likelihood of the operator or system having to 'chase' the crystal around the mother liquor of the growth drop. Such a 'chase' is commonly observed when using traditional microloop-based

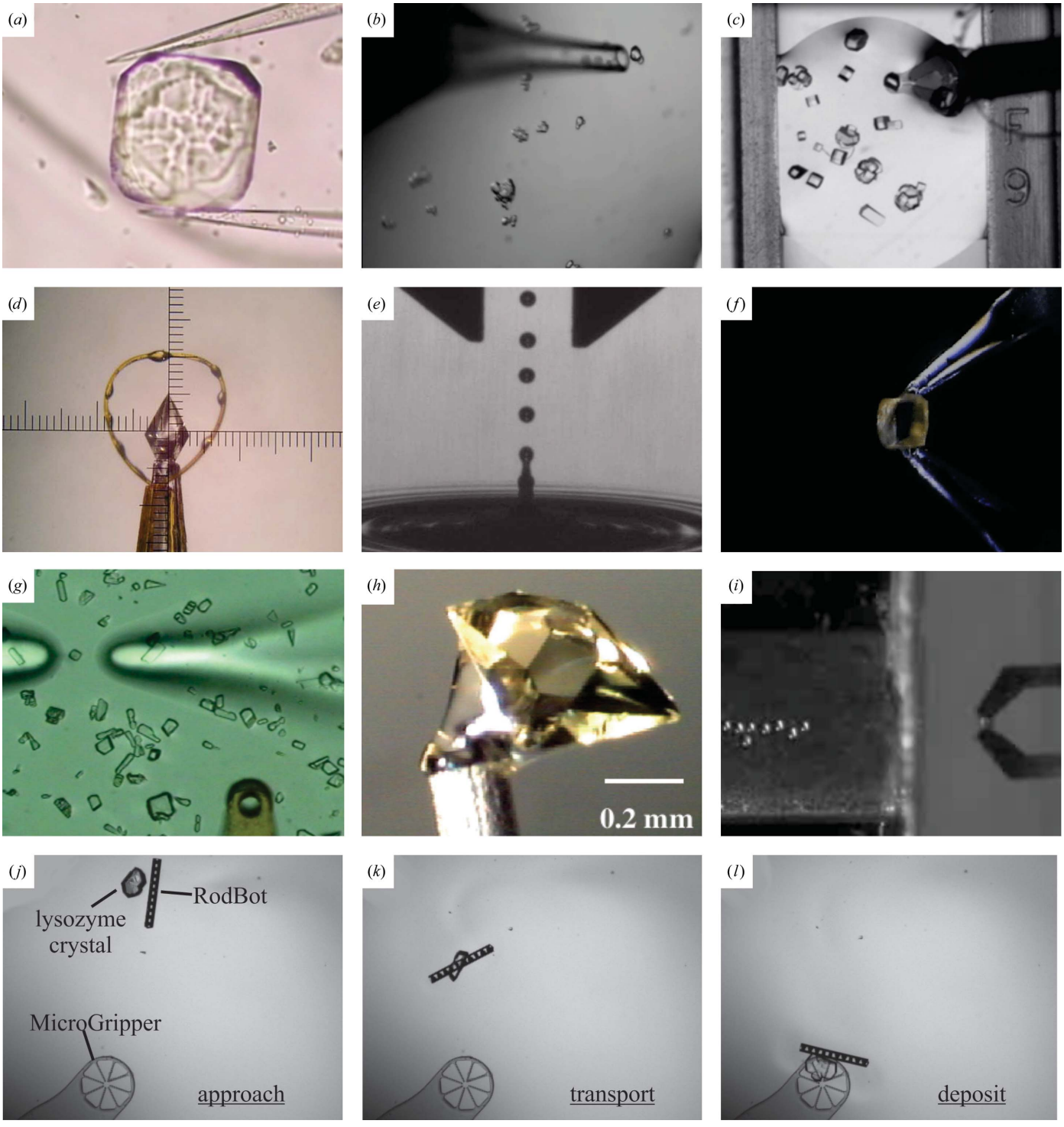


Figure 4
Examples of protein crystal-harvesting mechanisms. (*a*) Microgripper ('chopsticks'; http://bit.ly/13lEQh7; Ohara, Ohba, Tanikawa, Hiraki, Wakatsuki, Mizukawa *et al.*, 2004); (*b*) microcapillary (ACH; http://bit.ly/1b572WM; Weidong *et al.*, 2004); (*c*) microtool (UMR; http://bit.ly/17pws4n; Viola *et al.*, 2011); (*d*) 'loopless' (CMM; http://bit.ly/1a20g6v; Kitago *et al.*, 2005); (*e*) Acoustic Droplet Ejection (ADE; Soares *et al.*, 2011); (*f*) microgripper (REACH; Heidari Khajepour *et al.*, 2013); (*g*) optical tweezers (FOT; Hikima *et al.*, 2013); (*h*) 'crystal catcher' (Kitatani *et al.*, 2008); (*i*) non-crystallographic application of force-sensing microgripper (Femtotools; http://bit.ly/17yY9YK; Beyeler *et al.*, 2007); and (*j*–*l*) manipulation of a crystal using the RodBot system (Tung *et al.* (2013, 2014). In (*j*), the RodBot begins to rotate, generating a vortex that is used to capture the crystal, in (*k*) the RodBot transports the captured captured crystal towards the loop and in (*l*) the RodBot stops rotating and the crystal is released into the loop for harvesting.

approaches and is largely attributed to fluid-flow and bow-wave effects caused by movement of the microloop. Similar microgrippers have also been investigated for use in the REACH system, which employs recent advances in MEMS technology to fabricate nanometre-precision microgrippers (Beyeler *et al.*, 2007; Agnus *et al.*, 2009; see §2.2.9). A movie of the system in action is available at http://bit.ly/13lEQh7.

2.2.4. Automated Crystal Harvester (ACH). The ACH is a microcapillary-based protein crystal-harvesting system designed at The Ohio State University as part of a platform for automating membrane-protein crystallization (Muthusubramaniam *et al.*, 2004; Weidong *et al.*, 2004; Fig. 3*d*). The ACH system was amongst the first automation solutions to address the difficulties associated with the manipulation of nanolitre volumes of highly viscous lipidic cubic phase (LCP) growth media, as commonly used in membrane-protein crystallization. The ACH system was also the first to move away from standard microloop-based harvesting procedures in favor of a 'capillary-loop coordinating mechanism'. Using this system, the crystal is first harvested using a microcapillary and then transferred to a traditional microloop for flash-cooling and X-ray data collection (Weidong *et al.*, 2004; Fig. 4*b*). The ACH system uses a series of vision- and focus-based object-tracking functions to monitor the position of the loop, microcapillary and crystal during the mounting stage. Crystallization drops (<10 µl) are visualized in 72-well microbatch plates using an Eclipse E400 microscope (Nikon, Tokyo, Japan) fitted with an *X*–*Y* stage controlled by a MAC 2000 micropositioner (Ludl Electronic Products Ltd, Hawthorne, New York, USA). The microcapillary is attached to a micropump to provide negative and positive pressure for operation of the aspirate and dispense stages, respectively. The *X*–*Y* stage is used for high-resolution positioning (100 nm) of the crystals so that they can be manipulated using the microcapillary and transferred to the microloop for X-ray data collection.

The microcapillary approach to crystal harvesting offers several advantages over traditional microloop-based methods. Firstly, the microcapillary approach provides a more robust and reliable 'pickup' phase as the crystal follows the liquid flow of the micropump and the operator does not have to chase 'stubborn' crystals around the drop (as discussed above for the 'chopsticks' system in §2.2.3). Secondly, the overall process is mechanically less damaging to the protein crystal because physical contact with the harvesting device is minimized as the protein crystal remains in solution. This mechanism also ensures that the crystal remains in the protective environs of the mother liquor at all times, without the risk of dehydration. Additionally, microcapillary approaches, such as the ACH and the CARESS system (see §2.2.5), are not unduly affected by high-viscosity media such as LCP and therefore offer additional advantages for harvesting from such media.

2.2.5. Columbia Automated Robotic Environment for Streak Seeding (CARESS). The CARESS system was amongst the first semi-autonomous protein crystal-harvesting systems specifically designed for HTPX/SG use at the Northeast Structural Genomics Consortium (NESG; http://bit.ly/10Z7uS4; Georgiev & Allen, 2008; Georgiev *et al.*, 2004, 2005, 2006; Mezouar & Allen, 2002; Fig. 3*e*). The CARESS system was initially designed for automated streak-seeding of crystallization drops in 96-well format plates using custom-designed silicon microshovels (Georgiev *et al.*, 2006). Streak-seeding is a powerful method used for generating diffraction-quality crystals (Stura & Wilson, 1991); however, the process is highly manual in nature and time-consuming. In order to automate streak-seeding protocols for use in an HTPX/SG environment, the CARESS system uses custom-made microshovels fabricated from a single-crystal silicon wafer. Silicon microtools offer several advantages for this application, including simple fabrication in a variety of shapes and sizes and the ability to form a rigid and precise tool for calibration of the end-effector position (Georgiev *et al.*, 2006). The CARESS system utilizes an MP-285 micropositioner (Sutter Instrument Company, Novato, USA) with three degrees of freedom (DoFs) for high-resolution positioning (40 nm) of the silicon microshovel end-effector. The crystallization plates are mounted on a motorized ProScan stage (Prior Scientific, Rockland, Massachusetts, USA) and visualization is performed using an SZX12 microscope (Olympus, Tokyo, Japan). The source seed crystals are first placed on a 22 mm coverslip and the system then takes an image of the drop and automatically locates the crystals using edge-detection algorithms (Georgiev *et al.*, 2005). Two crystals are selected, and the microshovel automatically touches them and then automatically proceeds to streak the target drops arranged on a 96-well coversheet (Neuro-Probe Inc., Gaithersburg, Maryland, USA or Molecular Dimensions Ltd, Newmarket, England). The system is capable of streak-seeding ~6.5 wells per minute and produced results comparable to manual streak-seeding protocols (Georgiev *et al.*, 2006). The system was further developed to enable semi-automated protein crystal harvesting by mounting a glass micropipette on the micromanipulator end-effector (Georgiev & Allen, 2008) in a fashion similar to the ACH device (Weidong *et al.*, 2004; see §2.2.4). The glass micropipette is actuated using a CellTram Vario micro-injector (Eppendorf, Hamburg, Germany) controlled by a stepper motor. The micro-injector is capable of very small volume aspirate and dispense steps (2 nl). Using a series of computer-controlled and visual feedback loops, the user guides the micro-injector until the crystal is drawn inside the micropipette (Georgiev & Allen, 2008). The crystal is then dispensed from the micropipette into a microloop resting in a microbridge filled with an appropriate cryoprotectant.

This micropipette approach to crystal harvesting offers similar advantages over the traditional microloop-based methods to those discussed in §2.2.4 for the ACH system. Primarily, these advantages include easier automation, less damage to the crystal and adaptability to various crystal-growth media such as LCP. Movies of the system in action are available at http://bit.ly/1b572WM (harvesting) and http://bit.ly/19KPLGi (streak-seeding).

2.2.6. Universal Micromanipulation Robot (UMR). The UMR workcell was engineered by Square One System Design (SQR-1 System Design, Wyoming, USA) with current design and development input from the authors of this article (http://bit.ly/11lf1dX; Viola, Carman, Walsh, Frankel *et al.*, 2007; Viola, Carman, Walsh, Miller *et al.*, 2007; Viola *et al.*, 2011; Fig. 3*f*). The UMR workcell is a flexible semi-autonomous protein crystal-harvesting system that is currently undergoing field testing and further development at the Joint Center for Structural Genomics (JCSG) at The Scripps Research Institute (California, USA). The workcell is built around a TX60 six-axis serial robotic arm (Stäubli, Pfäffikon, Switzerland) that is used in a semi-automated three-step harvesting and flash-cooling process: (i) selection of an appropriately sized end-effector microtool, (ii) presentation of the microtool in close proximity to the drop for user-guided harvesting and (iii) automated cryoprotection and flash-cooling of the harvested crystal and loading into the storage dewar. The system is highly flexible: it is capable of loading crystals into both SSRL cassettes or UniPucks and can accept any standard SBS footprint crystallization plate. The system utilizes novel machine-vision algorithms (Labview, National Instruments, Austin, Texas, USA), which are used to locate the crystal within the crystallization plate and to aid in user-guided control of the harvesting process. The workcell has several additional features that aid the crystal-harvesting workflow, including data tracking of all harvested crystals

and their pin locations to a database (plate, well, pin and cassette locations), automated plate sealing-tape opening and a novel automated 'cryo-drip' procedure using low-viscosity oil (Viola *et al.*, 2011; a video of the cryo-drip procedure is available at http://bit.ly/15TSskw). The automated plate sealing-tape opening system reduces dehydration of the mother liquor in the drop owing to the small aperture of the opening in the plate sealing-tape (∼3 mm). This small opening is possible owing to the restrained working envelope of the robot motions, which is capable of operating in a much more confined space than is possible by hand. The sealing-tape puncture can therefore be significantly smaller than the opening necessary for manual looping, which generally involves removal of the entire tape covering a crystallization well. The smaller opening in the sealing tape means that the useful life of the crystallization drop is extended approximately tenfold (Viola *et al.*, 2011).

The primary objectives of the UMR workcell include (i) optimization of the flash-cooling of protein crystals and (ii) enabling routine high-throughput harvesting of microcrystals. Central to the first goal is the automated 'cryo-drip' system, in which single drops of perfluoropolyether oil are automatically 'dripped' from a solenoid valve onto the harvested crystal located in the microtool. Combined with the high-speed robotics motions, this technique offers several advantages over traditional manual flash-cooling approaches, including the potential for hyperquenching (see §4.2 for more on hyperquenching). The second goal of enabling HTPX/SG use of microcrystals is facilitated by the small step size of the robot-arm motions (1 µm), which enables the harvesting of protein crystals of <10 µm in size (see §4.1 for more on the harvesting of microcrystals). Furthermore, in a similar fashion to the REACH system (see §2.2.9), the UMR platform also provides an opportunity for data collection directly from harvested crystals or *via in situ* data-collection methods (see §3.5 for more on *in situ* methods). A movie of the system in action is available at http://bit.ly/17pws4n.

2.2.7. Capillary-top Mounting Micromanipulator (CMM). The CMM system was developed by a team from Hokkaido University and Nagoya University as a system for facilitating 'loopless' mounting of protein crystals for optimal long-wavelength experiments (http://bit.ly/1a20g6v; Kitago *et al.*, 2005, 2010; Price, 2013; Watanabe, 2006; Fig. 3*g*). The sulfur anomalous signal (S *K* absorption edge, 5.02 Å) used for sulfur single-wavelength anomalous diffraction (S-SAD) phasing increases at longer wavelength X-rays (Dauter *et al.*, 2002; Dodson, 2003). However, the scattering and absorption of X-rays by the aqueous solvent and the microloop also increases at longer wavelength X-rays. The resulting reduction in the accuracy of the recorded diffraction intensities can readily obscure the already weak sulfur anomalous signal. Aiming to reduce the background scatter for sulfur anomalous data collection, a 'loopless' crystal-mounting method was devised in which the protein crystal is harvested using a nylon microloop mounted on top of a glass micropipette. The microloop is arranged so that the micropipette opening is located in the center of the microloop (Kitago *et al.*, 2005; Fig. 4*d*). Once the crystal has been harvested in the microloop, the mother liquor is aspirated away from the crystal by applying negative pressure to the micropipette. This results in a mother liquor-free crystal resting on the tip of the micropipette. The crystal is then flash-cooled in the cryostream and the nylon microloop is removed. Modified CrystalCap bases (Hampton Research, Aliso Viejo, Calfornia, USA) that enable this mounting method have been produced (Watanabe, 2006), but the process still remains complex and time-consuming. A system to automate this procedure was built around a model 7200CR Dual Head Epoxy Die Bonder (West Bond, Anaheim, USA; Kitago *et al.*, 2010; Price, 2013). The die bonder provides an optical microscope, an *X*–*Y* stage and a micromanipulator *Z* arm on which the modified CrystalCap base is mounted. The micromanipulator arm remains stationary and the *X*–*Y* stage (holding the plate containing the crystal to be harvested) is moved manually by the operator to harvest the crystal into the microloop. Once harvesting is complete, a series of automatic events are triggered 100 ms apart. Firstly, the vacuum line connected to the micropipette opens, removing the mother liquor from the crystal. Secondly, a high-speed air-driven shutter is actuated on the cryostream (Giraud *et al.*, 2009). The precision and high speed of these events ensures that the crystal is efficiently flash-cooled.

This CMM or 'loopless' crystal-mounting method has several advantages over traditional techniques. These include easier centering of the crystal in the X-ray beam owing to a lack of lensing effects (Lavault *et al.*, 2006) and improved flash-cooling of harvested crystals owing to the removal of excess aqueous solution around the protein crystal (Warkentin & Thorne, 2009; Pellegrini *et al.*, 2011). Movies of the system in action are available from the IUCr electronic archive and at http://bit.ly/19KXFiK.

2.2.8. Acoustic Droplet Ejection (ADE). The ADE system was developed by Labcyte Inc. and adapted for protein crystal harvesting on beamline X25 of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, USA (http://1.usa.gov/1bOALE1; Soares *et al.*, 2011; Fig. 3*h*). The system is used to acoustically transfer ∼2.5 nl drops of microcrystal slurry onto micromesh mounts. The micromeshes are then flash-cooled and X-ray data are collected using a microfocus beam with raster-scanning techniques (Hilgart *et al.*, 2011). The ADE system consists of a modified Echo liquid-handling system (Labcyte Inc., Sunnyvale, California, USA) coupled to a robotics system for loading and unloading of plates. An *X*–*Y* stage is also used to precisely position the target plate containing the protein crystals within the ADE system.

The ADE method utilizes the energy of focused sound waves to eject droplets from the surface of a liquid (Wood & Loomis, 1927; Fig. 4*e*). The volume of the ejected droplet is proportional to the wavelength of the sound energy supplied, and the velocity of the ejected drop is proportional to the amplitude of the sound energy supplied (Soares *et al.*, 2011). Therefore, the size, speed and ejection distance of the ejected drops can be precisely controlled, allowing regulation of the density of microcrystals on the micromesh. ADE methods have previously been used for the manipulation of protein crystals, for example using matrix microseeding techniques (Villaseñor *et al.*, 2010). The ADE system was used to mount slurries of microcrystals used to determine the structures of insulin and lysozyme to resolutions of 1.9 and 1.8 Å, respectively. It was reported that the quality of the microcrystals was not affected by the energy of the ADE technique, as shown by the overall high quality of the diffraction data (Soares *et al.*, 2011).

Fourth-generation X-ray microbeam and free-electron laser (FEL) sources present new challenges, particularly in the small size and the large number of crystals required. Novel protein crystal-mounting technologies such as the ADE system will be essential for extracting the full benefits from these new high-brilliance X-ray sources (see §4.1 for more on the harvesting of microcrystals). A step forward in this direction has recently been achieved by the commissioning of a conveyor-belt system that utilizes the ADE technology (Roessler *et al.*, 2013; Fig. 3*i*). Using this system, droplets of microcrystal slurry are dispensed at a high rate onto a Kapton (polyimide) conveyor-belt assembly that is attached to a standard goniometer base. It has been reported that crystals of between 20 and 200 µm in size were successfully dispensed onto the conveyor belt. Systems such as the ADE conveyor belt offer the potential for an unprecedented speed of data collection from multiple crystals. In combination with the high-

speed readout capabilities of PADs, it will be possible to combine partial data sets collected in extremely fast succession from multiple crystals. High-speed sample change enabled by ADE technology will further enable radiation damage to be 'outrun', even during data collection at room temperature (see §1.3.1 for a discussion of PADs and their implications for radiation damage). A movie of an ADE system in action is available at http://bit.ly/11H7zcq.

2.2.9. Robotic Equipment for Automated Crystal Harvesting (REACH). The REACH system was developed by a collaboration between NatX-ray (Saint Martin d'Hères, France) and scientists from beamline FIP-BM30A (Roth *et al.*, 2002) at the European Synchrotron Radiation Facility (ESRF), France (http://bit.ly/12nZZb1; Heidari Khajepour *et al.*, 2013; Fig. 3*j*). The REACH system is capable of semi-autonomous crystal harvesting, cryoprotection and flash-cooling of individual crystals, and is additionally capable of *in situ* data collection directly from the plate. The REACH system is based on the commercially available G-Rob system that utilizes a six-axis robotics arm (Stäubli, Pfäffikon, Switzerland) for orientation of plates during *in situ* data collection (see §3.5 for a further discussion of *in situ* methods). The robot arm also acts as a goniometer for data collection from flash-cooled and capillary-mounted samples. The use of a serial robotic arm (defined as a serial manipulator consisting of a 'shoulder', an 'arm' and a 'wrist') as a goniometer for data-collection purposes is an attractive solution for streamlining the workflow. This tightly coupled setup is capable of speeding up the steps between crystal harvesting, flash-cooling and data collection, as no tool change is required. However, such setups can present an engineering challenge given the larger spheres of confusion (SoCs) associated with such serial devices when compared with traditional high-resolution goniometers (Davis *et al.*, 1968; He, 2009; Sanishvili *et al.*, 2008). Recent developments employing an air bearing as the last axis of the robot arm have reduced the SoC values down to a few micrometres (D. Nurizzo, unpublished work). Additionally, NatX-ray has recently developed a new prototype capable of an SoC of 6 µm (J.-L. Ferrer, personal communication). These concerns necessitate a careful matching of X-ray beam and crystal sizes (see §3.5 for more on the SoC).

Central to the REACH system is the two-finger piezoelectric microgripper developed by Femto-ST (Besançon, France; Agnus *et al.*, 2009; Fig. 4*f*). The microgripper has a resolution of 1 µm and an opening range of 0–500 µm, allowing it to harvest all but the largest protein crystals. In contrast to the microgripper developed for the 'chopsticks' system (see §2.2.3), which operates with six degrees of freedom (DoFs; technically three DoFs + three DoFs), the REACH microgrippers operate with two DoFs (in the 'open' and 'closed' planes). All other motions of the REACH system are controlled by the six-axis robot arm. In order to facilitate a good grip on the crystal, the very end of the microgrippers was modified by the addition of concave pads (60 µm wide × 30 µm thick) composed of SU-8 (see §1.3.2). The system is also equipped with a fully motorized inverted-microscope system capable of automated plate screening and crystal tracking from any SBS-format crystallization plate (Visualization Bench). After the plates have been imaged, the user directs the robot arm and microgripper end-effectors towards the target crystal using a game-pad controller in a similar fashion to the UMR system (see §2.2.6). The crystals can be cryoprotected before or after harvesting, and once the crystal has been captured it is rapidly transferred to the liquid-nitrogen stream. The crystal position is pre-centered in the X-ray beam, ensuring that the sample is ready for direct X-ray data collection while held in the microgrippers. Alternatively, crystals can be harvested using the microgrippers and transferred into traditional microloops for storage or data collection elsewhere. The system was reported as being capable of harvesting crystals in the 40–400 µm range in a similar time frame to manual methods. However, significant time savings were reported when harvesting was coupled to direct data collection from samples held within the microgripper.

Fully integrated systems such as REACH and UMR pave the way towards the realisation of a fully automated HTPX/SG pipeline. Both of these systems are under active development and share common features, including automated tape opening, semi-autonomous user-assisted robotics harvesting, automated cryoprotection and automated sample cooling. However, a key difference between the UMR and the REACH system is the use of a microgripper system, which offers the potential for easier and more flexible crystal harvesting. Additionally, the REACH system is coupled directly to an X-ray source, thus allowing more rigorous *in situ* study of the effects of harvesting and flash-cooling procedures. In fact, there has been a recent explosion in the development of *in situ* data-collection devices at beamlines around the world, and components of the REACH/G-Rob system are now in use at The École Polytechnique Fédérale de Lausanne (EPFL; Switzerland), Laboratorio Nacional de Luz Sincrotron (LNLS; Brazil), ESRF (France), Centre National de la Recherche Scientifique (CNRS/CBS; France) and Brookhaven National Laboratory (BNL; USA). A movie of the system in action is available from the IUCr electronic archive or at http://bit.ly/130SUip.

2.2.10. Conventional optic tweezers (COT). An optical trap (or tweezers) (OT) is produced when a laser is tightly focused using high numerical aperture (high-NA) lenses. In the early 1970s, it was shown that focused lasers could generate optical forces in the pico-newton range. These forces were capable of levitating and displacing micrometre-sized dielectric objects from water and air (Ashkin, 1970). Dielectric particles subject to this focal point experience two forces owing to the scattering of incident photons: (i) a scattering force in the direction of the incoming beam and (ii) a gradient force generated by fluctuating dipoles in the target dielectric object. The net effect of these two forces is a positive buoyancy force that balances gravity such that the object is held within the trap at the position of equilibrium. Laser-based systems have previously been used for the manipulation of protein crystals in seeding experiments (Bancel *et al.*, 1998, 1999) and also in numerous other biological applications (for reviews, see Neuman & Block, 2004; Lang & Block, 2003). The main advantage of OT techniques for protein crystal harvesting is that they can be used to manipulate fragile microcrystals without mechanical contact (Fig. 4*g*). A conventional OT (COT) can be defined as an OT formed by focusing using conventional optical lenses. This definition is useful for making a distinction from a second class of OT which uses fiber optics for focusing (FOT; see §2.2.11).

A COT system for microcrystal harvesting and mounting was developed at the Diamond Light Source, England (DLS; Wagner *et al.*, 2013; Fig. 3*k*). As discussed previously, mounting of microcrystals is exceptionally difficult by hand, and microcrystallography beamlines, such as beamline I24 at DLS, necessitate new crystal-mounting technologies (Evans *et al.*, 2007). The COT system uses a modified PALM MicroTweezers microscope (Zeiss, Oberkochen, Germany) fitted with 63× water-immersion objective lenses and a 1.5 W 1064 nm Nd:YAG laser (Wagner *et al.*, 2013). The system was modified by the addition of a SmarAct micromanipulator to allow precise mounting of the microloop.

As with any technique utilizing radiation, it is essential to monitor the experimental samples for radiation damage. Indeed, the laser radiation produced by the COT system was sufficient to cause damage to the polyimide film mounts at relatively low powers (<50 mW). It was reported that 50 mW of laser power was required to levitate a 10 µm CPV polyhedron crystal (Wagner *et al.*, 2013). It is

Table 4
Harvesting mechanisms used in current semi-autonomous protein crystal-harvesting systems and their relative advantages and disadvantages.

Examples of each harvesting mechanism are shown in Fig. 4. More + symbols indicate a higher score or cost.

	Design features						Outlook		
Harvesting mechanism	Semi-automated systems	Physical damage to crystal	Ease of harvesting	Ease of automation	Cost	Compatibility with data collection	High throughput	Ability to harvest microcrystals	Reduced late-stage failures
Microcapillary	PCGDS, CPPI, ACH, CARESS, CMM	+	+++	+++	+	+	++	+++	+++
Microtool	UMR, CH, SAMI	+++	+	++	+	+++	+	+	+
Microgripper	Chopsticks, REACH	++	+++	++	+++	+	++	+++	+++
Acoustic Droplet Ejection	ADE	+	+++	+	+++	+	+++	+++	+++
Optical trap	COT and FOT	++	+++	+	+++	+	++	+++	+++
MEMS	RodBot	+	+++	+++	+	+++	+++	+++	+++
In situ	REACH	+	+++	+++	+	+++	+++	+++	+++

interesting to note that these laser power figures are fivefold higher than the figures reported for the FOT system (§2.2.11). Operation of the laser at powers below 120 mW was reported as a good compromise between minimized laser damage and ability to levitate the crystal. Furthermore, laser harvested and mounted crystals of CPV polyhedrin were used to obtain a structure with better resolution (1.5 Å) than the original structure deposited in the PDB (2.1 Å; Coulibaly *et al.*, 2007). These results suggest that laser damage can largely be mitigated by the use of carefully controlled OT techniques.

COTs suffer from several major disadvantages, including the requirement for bulky and expensive high-NA lenses, which limits the flexibility and placement of the device. Additionally, the limited focal range of high-NA objectives means that only samples mounted in thin or shallow media can be manipulated. Furthermore, the power constraints imposed by such systems limits them to the harvesting of only the smallest crystals without the risk of radiation damage. In fact, the COT system is limited to the harvesting of protein microcrystals directly from glass cover slips mounted onto the microscope. Therefore, COT systems are likely to be unable to harvest larger crystals directly from drops in standard crystallization plates. This limits the potential of COT-based systems as a universal method for full automation of protein crystal harvesting. It is likely that the flexibility of placement and increased focal range afforded by FOT-based systems may better serve a role in full automation of protein crystal harvesting (see §2.2.11).

2.2.11. Fiber optic tweezers (FOTs). The FOT system was developed by a team of scientists at SPring-8, Japan as a system for manipulating and mounting protein microcrystals on BL32XU (Hikima *et al.*, 2013; Hirata *et al.*, 2010; Fig. 3*l*). FOTs eliminate many of the disadvantages of COTs and allow a more flexible placement of OT technology (Xin *et al.*, 2012; see §2.2.10 for a discussion of OT technology). The FOT system developed at SPring-8 uses a twin-lensed fiber approach (Taguchi *et al.*, 2000) consisting of two near-infrared (1064 nm) ytterbium fiber laser modules YLM-1-1064 (IPG Lasers GmbH, Burbach, Germany) each connected to a lensed fiber NSOM probe (Nanonics Imaging Ltd, Jerusalem, Israel). The FOT is manipulated using an M200 *XYZ* micromanipulator (Suruga Seiki Co. Ltd, Tokyo, Japan) and the process is visualized using an Eclipse TE-2000-U microscope (Nikon, Tokyo, Japan). A third micromanipulator is used to position a microloop in the target crystallization drop for receiving the harvested crystal from the FOT.

Although no physical damage to the crystals was observed during manipulation, cooling of the target crystallization plate was required to avoid vaporization of the drop by the laser. Although the power required to manipulate similarly sized protein crystals using the FOT system (10 mW) is significantly less than required by a COT (50 mW), the energy emission of the FOT was still large enough to occasionally melt the polyimide film of the micromount (Bancel *et al.*, 1998, 1999; Wagner *et al.*, 2013; Hikima *et al.*, 2013). In order to assess possible photodamage, thermal damage and oxidative damage caused by the near-IR laser, X-ray data sets were collected from lysozyme and thermolysin crystals mounted using the FOT. It was concluded that no significant changes to the molecular structures were observed and therefore that the damage incurred by the FOT manipulations was minimal (Hikima *et al.*, 2013).

The FOT system was reported to be successful at transferring crystals in the 5–30 µm range, which is larger than the 10 µm reported for the COT system (Hikima *et al.*, 2013; Wagner *et al.*, 2013). However, crystals larger than 30 µm could not be manipulated by the FOT system (Hikima *et al.*, 2013), again suggesting that OT manipulation techniques may be limited to use on microcrystals. FOT techniques are not hindered by bulky objective lenses and limited focal depths (as is the case with their COT counterparts). Therefore, FOT techniques still represent an interesting avenue towards the full automation of harvesting, particularly for microcrystals.

2.2.12. RodBots. As discussed above (§1.3.2), recent advances in MEMS fabrication technologies provide an opportunity for interesting and potentially revolutionary concepts in automated protein crystal harvesting. One such concept is the RodBot system developed by the Multi-Scale Robotics Laboratory, Institute for Robotics Design and Intelligent Systems, ETH, Zurich (Tung *et al.*, 2014; Fig. 3*m*).

The RodBot system consists of a rod-shaped microrobot that is used to transport the crystal to the microloop, where it can then be harvested and flash-cooled in the traditional fashion (Figs. 4*j*, 4*k* and 4*l*). RodBots are wireless mobile devices available in a variety of sizes (typically 55 × 55 × 500 µm), with the device size being matched to the target crystal size. RodBots are made of SU-8 material embedded with sticks of a soft magnetic material. The RodBots are driven by low-frequency magnetic fields generated by an octopole electromagnetic coil assembly located under the crystallization plate. The magnetic field causes the RodBot to rotate (in a low Reynolds number liquid), thus generating fluid currents. These fluid currents result in a rising flow ahead of the RodBot, forming a stable fluid vortex above it. Crystals are lifted up by these currents, trapped in the vortex and then transported to a desired location in the droplet, for example onto a microloop, where they can then be harvested (Fig. 4*j* shows the 'approach' phase, Fig. 4*k* shows the 'transport' phase and Fig. 4*l* shows the 'deposit' phase).

The RodBot method has been shown to work in solutions of various viscosities ranging from those of pure water to concentrated high-molecular-weight PEGs. The gentle fluidic force acting on the crystal is spread smoothly over the whole surface of the crystal, thus eliminating the potential for localized physical damage to the crystal (as is often observed when applying mechanical contact and force during the harvesting process with a traditional microloop). The

RodBot is capable of transporting crystals ranging from a few micrometres to submillimetre in size. The RodBots can be manoeuvered in any format of crystallization plate, making the system compatible with existing crystallization hardware. The system can be used as a stand-alone platform or can be integrated into larger crystal manipulation and harvesting platforms. The RodBot system is an exciting technology that addresses several of the key issues associated with the manipulation of fragile protein crystals. The system is highly flexible and cost-effective and has the potential to facilitate the development of fully automated harvesting systems.

The reader should also be aware of other commercially available systems, including the Crystal Harvester (CH; Bruker, Billerica, Massachusetts, USA) and the Station for Automated Microscopy and Imaging (SAMI; FMP Products Inc., Greenwich, Connecticut, USA). These systems are not included in the detailed summary above owing to a lack of peer-reviewed material. A summary of all of the crystal-harvesting systems is provided in Table 3.

3. Assessment

The systems discussed in §2.2 demonstrate several of the engineering challenges that need to be overcome in the design of an automated protein crystal-harvesting platform. In their simplest form, these engineering challenges include (i) identification of the target protein crystal and (ii) manipulation of the fragile protein crystal. A variety of visualization and imaging methods have been explored for identifying protein crystals, including the use of machine-vision, edge-detection and vision-focus algorithms. Additionally, a number of different approaches for manipulation of the protein crystals have been demonstrated, including the use of microcapillaries, microtools, microgrippers, ADE, OT and MEMS devices. Each of these mechanisms has their advantages and disadvantages for protein crystal harvesting (for a summary, see Table 4).

3.1. Microcapillary harvesting methods

Microcapillary harvesting methods, as employed in the PCGDS (§2.2.1), CPPI (§2.2.2), ACH (§2.2.4), CARESS (§2.2.5) and CMM (§2.2.7) systems, have the advantage of being the easiest and cheapest to automate (Table 4 and Fig. 4*b*). Microcapillary-based systems require only a simple pump or syringe system, as routinely used in well established micro-injection techniques. Another major advantage of the microcapillary approach is the ease and reliability of crystal harvesting and the lack of crystal damage. As discussed above for the ACH system (§2.2.4), the microcapillary approach provides the easiest mechanism of crystal 'pickup' using only gentle fluid aspiration and minimal collateral damage. The major downside to the microcapillary harvesting approach is the incompatibility of capillaries with current cryoprotection and flash-cooling protocols. Therefore, such systems require an extra manipulation step in which the crystal is transferred to a traditional microloop or microtool for flash-cooling and X-ray data collection.

3.2. Microtool harvesting methods

Microtool harvesting methods, as used by the UMR (§2.2.6), CH and SAMI systems, are an attractive option for use in automation platforms. Microtools are cheap and relatively easy to automate and, probably most importantly, are compatible with current flash-cooling and X-ray data-collection protocols (Table 4 and Fig. 4*c*). However, in contrast to many of the other methods used for crystal 'pickup', a major disadvantage of microtools is the difficulty of crystal capture and manipulation. As discussed above for the 'chopsticks' system (see §2.2.3), crystal-harvesting methods using traditional microtools and microloops can result in a 'chase' of the crystal around the drop. This is a result of fluid flow and bow waves caused by the harvesting tool, and can often lead to damage of the target crystal and collateral damage to other crystals within the drop. Some of these issues can be circumvented by direct growth of crystals within the cryoloop or micromount and this approach offers an exciting avenue towards future automation efforts (Berger *et al.*, 2010). The issue of unwanted fluid flows and associated collateral crystal damage are largely eliminated by the microcapillary, microgripper, ADE, OT and MEMS methods of crystal manipulation.

3.3. Microgripper harvesting methods

Microgripper-based harvesting approaches, such as the 'chopsticks' (§2.2.3) and REACH (§2.2.9) systems, offer a potential for the easiest route towards automation of protein crystal harvesting and manipulation (Table 4 and Figs. 4*a*, 4*f* and 4*i*). Microgrippers offer many advantages over traditional microtool approaches, including ease of crystal 'pickup' and the potential for reduced collateral damage to the crystal and the crystallization drop. The downsides of the microgripper approach include the high cost of microgrippers with force-feedback control capability (which are essential for limiting damage to the fragile crystals; Beyeler *et al.*, 2007) and the low compatibility with current flash-cooling and data-collection protocols. However, as discussed above in §2.2.9, developments with the REACH system suggest that it is possible to collect X-ray data directly from crystals mounted in the microgrippers (Heidari Khajepour *et al.*, 2013).

3.4. Emerging harvesting methods (ADE, OT and MEMS)

The microtool and microgripper approaches to crystal manipulation all involve actual mechanical contact of the harvesting tool with the fragile protein crystal, resulting in some of the issues discussed. However, many of the issues associated with the use of mechanical contact can be mitigated by using other non-mechanical forces to manipulate the protein crystal. Recent technological advances in the COT (§2.2.10), FOT (§2.2.11), ADE (§2.2.8) and MEMS (§§1.3.2 and 2.2.12) techniques have enabled non-mechanical mechanisms of crystal manipulation using sound, light and fluid flow, respectively (Table 4 and Figs. 4*e*, 4*g* and 4*j*–4*l*). Such techniques have the advantage of being less physically hazardous to the crystal and also provide more precision for the harvesting of microcrystals. However, both the ADE and the COT techniques require expensive and bulky control and focusing systems and are limited in both the thickness of the target material and the sizes of crystals that can be manipulated. Additionally, the amount of incident energy (light or sound) must be carefully considered to prevent thermal damage and photo-damage to the fragile protein crystal. Recent advances with FOT techniques remove the need for bulky high-NA focusing systems, allowing more flexibility in the placement of the harvesting tool, and also permit crystal harvesting from a wider range of plates and sample sizes (Xin *et al.*, 2012; Wagner *et al.*, 2013).

Another interesting optical approach to crystal harvesting is the Crystal Direct (CD) method (Cipriani *et al.*, 2012). This method uses laser-induced photoblation of ultrathin films of Kapton (12.5 µm) to extract protein crystals from custom-designed crystallization plates (Cipriani *et al.*, 2012). The Kapton film forms the support surface for protein crystal growth, and once excised by the laser the film can be mounted on a traditional pin and goniometer for X-ray data collection. The CD method is an attractive solution for the automated analysis of large numbers of microcrystals, for example using raster-scanning techniques to analyze the whole of the excised drop (Hilgart

et al., 2011). Additionally, this mounting technique also eliminates the need for manipulation tools to enter the drop, and therefore eliminates possible damage to the crystal caused by mechanical stress. Other laser-based methods have also been developed, primarily for excision of the microloop and mother liquor surrounding the crystal (Kitano, Matsumura *et al.*, 2005; Kitano, Murakami *et al.*, 2005). Such techniques require expensive equipment, and simpler tool-free crystal-mounting methods, such as the Crystal Catcher (CC), have been demonstrated (Kitatani *et al.*, 2008). The CC device is used to inject a mixture of vinyl adhesives onto the end of a mounting pin, which is then used to capture the target crystal from the mother liquor without mechanical damage (Fig. 4*h*). The CC device shares many of the benefits of the 'loopless' harvesting system detailed previously (see §2.2.7) and offers an alternative approach for manipulating protein crystals, particularly for S-SAD data collection, where low background scattering is required.

MEMS techniques, as demonstrated by the RodBot system (§2.2.12), are exciting new techniques that provide an alternate means for protein crystal manipulation using novel and more gentle forces. The novelty of the RodBot system is in the use of hydrodynamic fluid flows to capture and move the crystals without any direct physical contact. Manipulation of the RodBot agent is achieved entirely by magnetic fields, so many of the complications of driving and controlling mechanical components in the vicinity of the fragile protein crystal are largely avoided. Integration of MEMS subsystems, such as the RodBot, into current harvesting platforms appears to be a promising avenue for future development.

The emerging crystal-harvesting technologies described above, along with the new detector and MEMS technologies detailed elsewhere in this article (see §1.3), are likely to revolutionize the way in which protein crystals are harvested and X-ray data are collected. One such technology-driven shift, which is already under way, is *in situ* crystal growth and data collection. Many of the engineering challenges discussed throughout this review are in fact mitigated by the use of *in situ* crystal growth and mounting techniques. While not strictly methods of crystal harvesting, *in situ* crystal growth and data-collection methods eliminate the need for extensive crystal manipulation, and offer a simple pathway towards full automation. We will now briefly discuss some of these *in situ* solutions, with a particular focus on their suitability for automation.

3.5. *In situ* protein crystal growth and mounting

In situ crystal growth and data collection is dependent on a close coupling between the crystal-growth medium and a suitable X-ray source (for a review of *in situ* methods, see Fiedler *et al.*, 2012). Recent advances in both home and synchrotron X-ray sources, together with allied developments in plate design and materials technology, have enabled a surge in interest in *in situ* methods. Many diverse solutions to *in situ* protein crystal growth and data collection have been presented, ranging from simple SBS-format plates (Society for Biological Screening; meeting standards ANSI/SBS 1-2004 through ANSI/SBS 4-2004 of the American National Standards Institute) through to complex microfluidic chips (for reviews of microfluidics, see Li & Ismagilov, 2010; Leng & Salmon, 2009; Sauter *et al.*, 2007). The use of *in situ* methods does away with removal of the crystal from the mother liquor and therefore mitigates many of the engineering challenges associated with protein harvesting (advantages and disadvantages are summarized in Table 2). More importantly, the need for physical contact and further manipulations of the fragile protein crystal are eliminated, thereby limiting late-stage damage to the crystal. Such techniques can be used to expedite the structure-determination process by facilitating the search for well diffracting crystals and aiding in the systematic analysis of parameters affecting diffraction quality, such as dehydration (Douangamath *et al.*, 2013; Lobley *et al.*, 2012). Furthermore, *in situ* approaches have been used to solve several viral and ligand-bound structures where traditional manual mounting methods have previously failed (Axford *et al.*, 2012; le Maire *et al.*, 2011).

The simplest form of *in situ* device consists of a plastic SBS plate, in which crystals are grown, that is capable of being mounted on an X-ray source. Diffraction data are then collected directly from the crystal in the plate. Examples of simple plate designs used for *in situ* diffraction include the X-Chip (Kisselman *et al.*, 2011; Chirgadze *et al.*, 2012), Crystal Former (Microlytic, Burlington, Massachusetts, USA; Stojanoff *et al.*, 2011), In-Situ-1 (MiTeGen, Ithaca, New York, USA), topographic drop-pinning plate (Soliman *et al.*, 2011), *in situ* crystallization plate (Watanabe *et al.*, 2002), CrystalQuick X (Greiner Bio-One, Monroe, North Carolina, USA; le Maire *et al.*, 2011) and CrystalSlide (Greiner Bio-One). Despite the benefit of eliminating crystal manipulation, several downsides exist to *in situ* plate-based approaches, including the scattering of incident X-rays by the plate material and mother liquor. Additionally, the design of traditional SBS-format plates is such that there is a limit to the angle of rotation that the crystal can be subjected to and therefore a reduction in the amount of useful data that can be collected from a single crystal. Furthermore, plate-based *in situ* devices are limited to room-temperature data collection, as the crystal cannot be cryoprotected and flash-cooled within the plate. Issues with X-ray scattering and rotation ranges can be mitigated by the use of custom well designs and plate materials. For example, materials with low birefringence and X-ray scattering properties can be used, such as the cyclic olefin copolymer used for the CrystalQuick X plate (Greiner Bio-One; Bingel-Erlenmeyer *et al.*, 2011; le Maire *et al.*, 2011). Additionally, the profile of the wells in these plates is designed to allow up to 80° of data to be collected (le Maire *et al.*, 2011). However, a viable solution for *in situ* data collection from cryocooled samples has yet to be presented. As discussed previously in §1.3.1, it is likely that the high-speed readout of PAD detector technology will render low-temperature requirements redundant owing to the 'outrunning' of radiation damage.

In situ screening of SBS-format plates is now possible at many synchrotrons around the world, including the ESRF (beamline FIP-BM30A; REACH system as detailed in §2.2.9; Heidari Khajepour *et al.*, 2013; Jacquamet *et al.*, 2004; le Maire *et al.*, 2011), the Swiss Light Source (SLS; beamline X06DA; Bingel-Erlenmeyer *et al.*, 2011), DLS (beamline I04-1; Axford *et al.*, 2012; Lobley *et al.*, 2012), Helmholt-Zentrum Berlin (HZB; beamline BL14.1; Mueller *et al.*, 2012) and the Australian Synchrotron (AS; beamlines MX1 and MX2; http://bit.ly/GJum4P). Although *in situ* methods are generally limited to use on high-flux synchrotron X-ray sources, several solutions have also been developed for 'in-house' use, including the PX scanner (Agilent Technologies, Santa Clara, California, USA; Skarzynski, 2009), PlateMate (Rigaku, The Woodlands, Texas, USA; Hargreaves, 2012) and an LCP plate holder designed specifically for screening membrane-protein crystals (Agirre *et al.*, 2008). The use of such techniques in-house often requires exposure times of 10–20 min, limiting their use to the pre-screening of crystals for differentiation of salt and protein. Nonetheless, the diffraction obtained from such low-dose experiments can be used as a predictor of diffraction strength and for prioritization of crystals for further analysis at a synchrotron source (Bourenkov & Popov, 2006; Fiedler *et al.*, 2012).

Although simple SBS-format plates offer the greatest flexibility and compatibility with existing crystallization platforms, more

complex microfluidic devices for *in situ* crystal growth and data collection have been developed. These include the Phase Chip (Shim *et al.*, 2007), Microfluidic Chip (Dhouib *et al.*, 2009), Microfluidic Device for Kinetic Optimization (Hansen *et al.*, 2006), Fluidigm Chip (May *et al.*, 2008) and the Microcapillary Protein Crystallization System (MCPS; Emerald BioSystems Inc., Bainbridge Island, Washington, USA; Gerdts *et al.*, 2008, 2010). These systems all offer the advantage of low-volume protein requirements (typically in the low microlitre to nanolitre range). Furthermore, these devices can be customized specifically for optimization of automated *in situ* X-ray data collection; for example, the Diffraction Capable Chip (DCC; Fluidigm, San Francisco, California, USA; May *et al.*, 2008). Alternative non-plate-based approaches include the use of microcapillaries (Yadav *et al.*, 2005; Gavira *et al.*, 2002) and polyester tubing (Kalinin & Thorne, 2005). Commercial examples of the microcapillary approach are available, such as the CrystalHarp (Molecular Dimensions Ltd, Newmarket, England).

In order to accurately assess the effect of crystal handling and flash-cooling protocols during the harvesting process, it is essential to compare diffraction properties of the same crystal both before and after such manipulations. This is not trivial to achieve in a controlled and reproducible fashion without the use of automation. Clearly, *in situ* plate-based methods have many advantages in this area. The REACH system (§2.2.9) has made considerable progress towards this goal, allowing the user to test the diffraction properties of the crystal both in the plate before harvesting (*in situ*) and also directly from a robotically harvested crystal using the six-axis robot arm as a goniometer. However, as discussed above (§2.2.9), the use of a serial robotic arm for both crystal harvesting and direct data collection is an engineering challenge owing to the large spheres of confusion (SoCs) associated with such devices.

The SoC of a goniometer is defined as the minimum spherical volume required to enclose the movement of the crystal when all axes are fully rotated (Davis *et al.*, 1968; He, 2009). Typical values for commercially available goniometers are in the range 1–100 µm and those for modern piezo-based devices are in the submicrometre range (Davis *et al.*, 1968; He, 2009). For microcrystals, the SoC value should be smaller than the beam size or the crystal size (whichever is the smaller), such that the crystal remains within the X-ray beam during the entire rotation range required for data collection (He, 2009). High SoC values may also lead to issues with data collection from large crystals owing to fluctuations in the scattering volume as a result of the crystal moving within the beam. Both of these situations contribute systematic errors that can lead to poor scaling and inaccurate data. Such problems are further exacerbated by the smaller beams now common on modern microfocus beamlines. For example, on the GM/CA minibeam at beamline 23ID-B (Advanced Photon Source; APS) it has been shown that low SoC values are critical for microcrystallography, with a 10 µm crystal and a 10 µm beam requiring SoC values of 2 µm or less (Sanishvili *et al.*, 2008). Therefore, careful matching of sample size and beam size is essential. The robotics arm utilized in the REACH system is reported to have an SoC of better than 13 µm. Robotically harvested and mounted crystals of lysozyme and NikA–FeEDTA resulted in data quality comparable to that obtained using traditional manual approaches (Heidari Khajepour *et al.*, 2013). Obviously, the SoC requirements are dependent on the application, beamline and sample size; therefore, the general applicability of data collection using a robotic arm-mounted sample remains a focus of study. As noted in §1.3.1, backlash-free continuous data collection using PADs allows much greater flexibility in the design of novel robotic goniostats.

4. Outlook and remaining challenges

In conclusion, automated protein crystal harvesting offers three main benefits over traditional manual approaches: (i) the ability to harvest microcrystals that cannot be easily harvested by hand, (ii) reduced late-stage crystal failures owing to poor crystal handling and cryoprotection and (iii) increased throughput and closure of the automation bottleneck (for a summary, see the 'outlook' columns on the right-hand side of Table 4). We will now assess the future of automated crystal harvesting with a particular focus on these main areas of benefit.

4.1. Ability to harvest microcrystals

Depending on the actual unit-cell dimensions, the lower theoretical boundary of protein crystal size suitable for structure determination by X-ray methods is around 1.2 µm (Holton & Frankel, 2010). Microcrystals of this size are commonly observed during routine crystallization screening and are often overlooked as potential data-collection candidates. Instead, these crystals are often flagged as 'crystal hits' for follow-up crystallization optimization (often referred to as 'fine screening'). For high-importance targets such as protein–protein complexes, protein–nucleic acid complexes, virus assemblies and membrane proteins, this crystallization optimization step can often take many years to perfect (if ever!). In contrast, the direct use of these microcrystals for structure determination on microfocus beamlines and FEL sources offers an attractive solution. Manual harvesting of microcrystals is exceptionally difficult and is almost impossible for crystals in the single-digit micrometre range. It is often difficult to isolate such small crystals from each other and it is also very difficult to remove excess mother liquor. The use of micromeshes has largely been adopted to overcome some of these issues, as excess mother liquor can be wicked away (or replaced with oil). Additionally, a large number of crystals spread over the surface of a micromesh can easily be analyzed using raster-scanning techniques to identify individual crystals for data collection (Hilgart *et al.*, 2011; Stepanov *et al.*, 2011).

Recent advances in fourth-generation synchrotron sources, particularly the increased availability of microfocus beamlines and FEL sources, has empowered new ways of solving protein structures from crystals not previously amenable to X-ray methods (Seibert *et al.*, 2011; Chapman *et al.*, 2011; Hunter & Fromme, 2011; Rasmussen *et al.*, 2007; Sawaya *et al.*, 2007; Boutet *et al.*, 2012). Microcrystals are increasingly viable candidates for structure determination and have been used to determine the structures of several proteins from X-ray data collected on FEL sources, including lysozyme (Boutet *et al.*, 2012), mimivirus particles (Seibert *et al.*, 2011), photosystem I (Chapman *et al.*, 2011) and photosystem II (Kern *et al.*, 2013). Owing to the high energies involved, these techniques are generally destructive to the protein crystal and therefore require a large number of crystals for the collection of a complete data set. The harvesting of microcrystals for these new methods represents a unique technical challenge to the structural biology community, especially given their small size and the requirement for large numbers. Platforms such as the ADE system (see §2.2.8) have been developed to address the need for rapid mounting of large numbers of microcrystals. Additionally, we are currently developing the UMR system (see §2.2.6) to harvest into SSRL grids. These grids consist of a 200 µm thick piece of polycarbonate containing 75 laser-etched holes with diameters of 125, 200 and 400 µm (http://stanford.io/15BDJdl). This allows extremely high-density shipping of crystals, with 1200 crystals contained in a single UniPuck and 8400 in a single shipping dewar. Automated crystal-harvesting technologies will be essential to

enable the routine use of microcrystals on this scale of throughput, and high-speed mounting of microcrystals is likely to remain a significant engineering challenge in the years ahead.

4.2. Reduced late-stage failure and improved cryoprocedures

Late-stage crystal failures can be defined as crystals that failed to diffract sufficiently well for successful structure determination owing to physical and/or chemical damage to the crystal. This damage may be the result of poor handling during the harvesting and mounting phase or owing to poor cryoprotection procedures. The true number of crystals lost to late-stage crystal failure is extremely difficult to ascertain, as information on the diffraction state of the crystal is required both *before* and *after* such manipulations take place. Few systematic studies on such failures have been carried out, although several attempts to analyze the quality of crystals before harvesting have been made using *in situ* methods (Douangamath *et al.*, 2013; Heidari Khajepour *et al.*, 2013; see §3.5). Given the hundreds of thousands of crystals that are screened for diffraction every year (see §4.3), the rescue of even a very small percentage of these failures could have a significant impact on structure-determination rates. Key to the analysis of these late-stage failures is a better understanding of current cryoprotection protocols and closing of the gap between crystal harvesting and data collection.

Cryoprotection and crystal-cooling protocols, which have enabled and revolutionized data collection from protein crystals at cryogenic temperatures, have changed little since the inception of standard looped-based harvesting methods decades ago (Garman & Owen, 2006; Petsko, 1975; Teng, 1990). However, the speed, precision and reproducibility afforded by modern robotics and liquid-handling systems have the potential to revolutionize the way that protein crystals are flash-cooled. Currently, ∼80% of all protein crystals harvested at the JCSG require addition of cryoprotectant to prevent damaging ice-crystal formation. Specifically, cryoprotection prevents the formation of hexagonal ice crystals that can lead to physical damage to the protein crystal. Such damage can result in data with reduced resolution and increased mosaicity. The extra cryoprotection step is both time-consuming and potentially harmful to the crystal, so alternate methods of crystal cooling are being investigated. One such technique is the 'cryo-drip' protocol employed by the UMR system (see §2.2.6), which is being investigated as a method for the routine production of hyperquenched protein crystals in an HTPX/SG environment.

Hyperquenching occurs when crystals are cooled at extremely high rates (>10 000°C s^{-1}) such that amorphous ice is the predominant bulk water species (Warkentin *et al.*, 2006; Warkentin & Thorne, 2007). The 'cryo-drip' method used by the UMR system (see §2.2.6) facilitates the production of hyperquenched crystals owing to two principal factors: (i) the removal of excess mother liquor from the microtool and crystal and (ii) the coating of the crystal in a thin layer of oil. Removal of excess aqueous solution from the crystal limits the formation of damaging hexagonal ice, and the thin coating of oil around the crystal acts as an insulating layer. The insulating oil layer limits direct exposure of the protein crystal to the slow rate of cooling observed in the film boiling regime of the liquid nitrogen (and also the gas cushion on the surface of the liquid nitrogen), thus raising the Leidenfrost temperature (Gakhar & Wiencek, 2005). Slow cooling rates in the film boiling regime and the gas cushion phases are largely responsible for the formation of damaging crystalline ice. It has been shown that crystals treated with a layer of insulating oil are cooled directly in the more efficient nucleate boiling regime (Gakhar & Wiencek, 2005). In the nucleate boiling phase, the crystal undergoes a much higher rate of cooling, such that hexagonal ice crystals are not given the chance to assemble and instead unstructured amorphous ice is produced.

Automated harvesting platforms, such as the UMR, offer a unique combination of precision liquid handling and high-speed robotics that can be fine-tuned to optimize hyperquenching protocols in a precise and reproducible manner. For example, the high-speed motion of the robotics arm during the liquid-nitrogen plunge step further assists the insulating effect of the oil layer. The crystal passes through the thin gas cushion above the liquid nitrogen at such a high speed that the cooling effect of the gas phase has a limited effect on the overall rate of cooling of the insulated crystal. Therefore, the majority of the cooling is achieved during the more efficient nucleate boiling phase and hyperquenched crystals are produced. One of the main advantages of hyperquenching protein crystals is the reduction in cryoprotectant that is required to produce ice-free diffraction. For example, it has been demonstrated that a 22% reduction in cryoprotectant is possible using hyperquenching protocols (Warkentin *et al.*, 2006). Given that even higher rates of cooling may be achieved by even higher speed robotics and automation, it is possible that the need for additional cryoprotectant can be eliminated altogether, further streamlining the HTPX/SG pipeline (Warkentin & Thorne, 2007).

4.3. Increased throughput

A conservative estimate suggests that hundreds of thousands of protein crystals are manually harvested and screened for X-ray diffraction every year. For example, the PDB is currently growing at a rate of approximately 7000 structures per year (Fig. 2*a*), and experiences at the JCSG suggest that approximately 100 crystals are screened for diffraction for every structure deposited in the PDB (Fig. 2*b*). Therefore, as a very crude estimate, these data suggest that of the order of 700 000 crystals are screened every year for diffraction. Obviously, this is an estimate based on JCSG HTPX/SG throughput levels, focused on targets tailored for SG. It is likely that challenging targets from traditional laboratories may require the screening of significantly more than 100 crystals. Furthermore, the global market in cryopins is estimated to be around two million per year (http://prn.to/16Kbhqp), suggesting that our estimate is indeed a conservative one. Whatever the true number, it is clear that extremely large numbers of protein crystals are harvested and screened for diffraction both at synchrotrons and home sources around the world. Automation offers the potential to streamline this diffraction-screening process and increase throughput. Furthermore, as discussed in §1.1, HTPX/SG efforts currently contribute approximately 10% of all protein structures deposited in the PDB every year (Fig. 2*a*). Large-scale HTPX/SG centers form a unique opportunity for the consolidation of advanced technology, equipment and resources on a scale that is not feasible in traditional laboratories. Closure of the automation bottleneck at these large-scale centers is likely to produce fewer late-stage crystal failures, resulting in both increased throughput and output. Small increases in HTPX/SG output are likely to have a significant impact on the overall number of structures deposited in the PDB. Furthermore, methods developed by HTPX/SG centers will continue to introduce process improvements that can be adopted by traditional small-scale laboratories.

4.4. Final appraisal

Several novel approaches to crystal harvesting have been presented, ranging from semi-autonomous systems utilizing robotics arms through to simple micromanipulator and micropipette-based

systems. All of these systems are in essence engineered to work around one simple problem: the manipulation of protein or other macromolecular crystals in a way that overcomes their inherently poor structural, physical and chemical stability. No single approach so far appears to have mastered the actual mechanics of the crystal manipulation step. Unless an alternate technology emerges, it is likely that a universally applicable approach will have to combine several of the technologies presented. Such technologies are likely to facilitate a true 'gene-to-structure' automation solution, providing a single platform for the growth of protein crystals and the subsequent collection of X-ray diffraction data.

Approximately 14 documented protein crystal-harvesting systems exist, and most of these are proof-of-concept or demonstration systems that are no longer in routine use and are not commercially available. Components of the FOT (Zeiss, Oberkochen, Germany) and ADE (Labcyte Inc., Sunnyvale, California, USA) systems are commercially available, but to the best of our knowledge REACH/G-Rob (NatX-ray, Saint Martin d'Hères, France) and UMR (SQR-1 System Design, Wyoming, USA) are the only fully integrated semi-automated protein crystal-harvesting systems that are currently commercially available. Given the massive global effort in the production and screening of protein crystals, it is remarkable that the majority of protein crystal harvesting is still carried out by hand. Further developments in automation will be necessary for improved crystal-harvesting and cryoprotection methods.

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