Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

CrystalDirect: a new method for automated crystal harvesting based on laser-induced photoablation of thin films

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Received 23 April 2012
Accepted 10 July 2012

The use of automated systems for crystallization and X-ray data collection is now widespread. However, these two steps are separated by the need to transfer crystals from crystallization supports to X-ray data-collection supports, which is a difficult manual operation. Here, a new approach is proposed called CrystalDirect (CD) which enables full automation of the crystal-harvesting process. In this approach, crystals are grown on ultrathin films in a newly designed vapour-diffusion crystallization plate and are recovered by excision of the film through laser-induced photoablation. The film pieces containing crystals are then directly attached to a pin for X-ray data collection. This new method eliminates the delicate step of 'crystal fishing', thereby enabling full automation of the crystal-mounting process. Additional advantages of this approach include the absence of mechanical stress and that it facilitates handling of microcrystals. The CD crystallization plates are also suitable for *in situ* crystal screening with minimal X-ray background. This method could enable the operational integration of highly automated crystallization and data-collection facilities, minimizing the delay between crystal identification and diffraction measurements. It can also contribute significantly to the advancement of challenging projects that require the systematic testing of large numbers of crystals.

1. Introduction

In the last decade, the field of macromolecular crystallography has been revolutionized by the introduction of high-throughput techniques for protein production, crystallization and synchrotron data collection (Banci *et al.*, 2006; Abola *et al.*, 2000; Rupp *et al.*, 2002; Gräslund *et al.*, 2008; Chayen, 2009; Arzt *et al.*, 2005). Many of these techniques were initially developed within the context of structural genomics projects (Lesley *et al.*, 2002; Terwilliger *et al.*, 2003) and have subsequently been adapted to the study of very challenging systems such as membrane proteins or large multi-protein complexes (Banci *et al.*, 2006; Stuart *et al.*, 2006). The success of these projects often depends on the systematic analysis of large numbers of crystals, sometimes thousands, by X-ray diffraction. This has resulted in a dramatic increase in the number of crystals that are produced and tested on synchrotron beamlines.

Currently, crystallization and data collection often occur at highly automated platforms. However, once crystals appear they have to be transferred from crystallization plates to supports that are compatible with cryogenic X-ray data collection (typically cryoloops), a manual operation that requires an experienced person and can consume a significant amount of time. Moreover, some crystals are damaged or

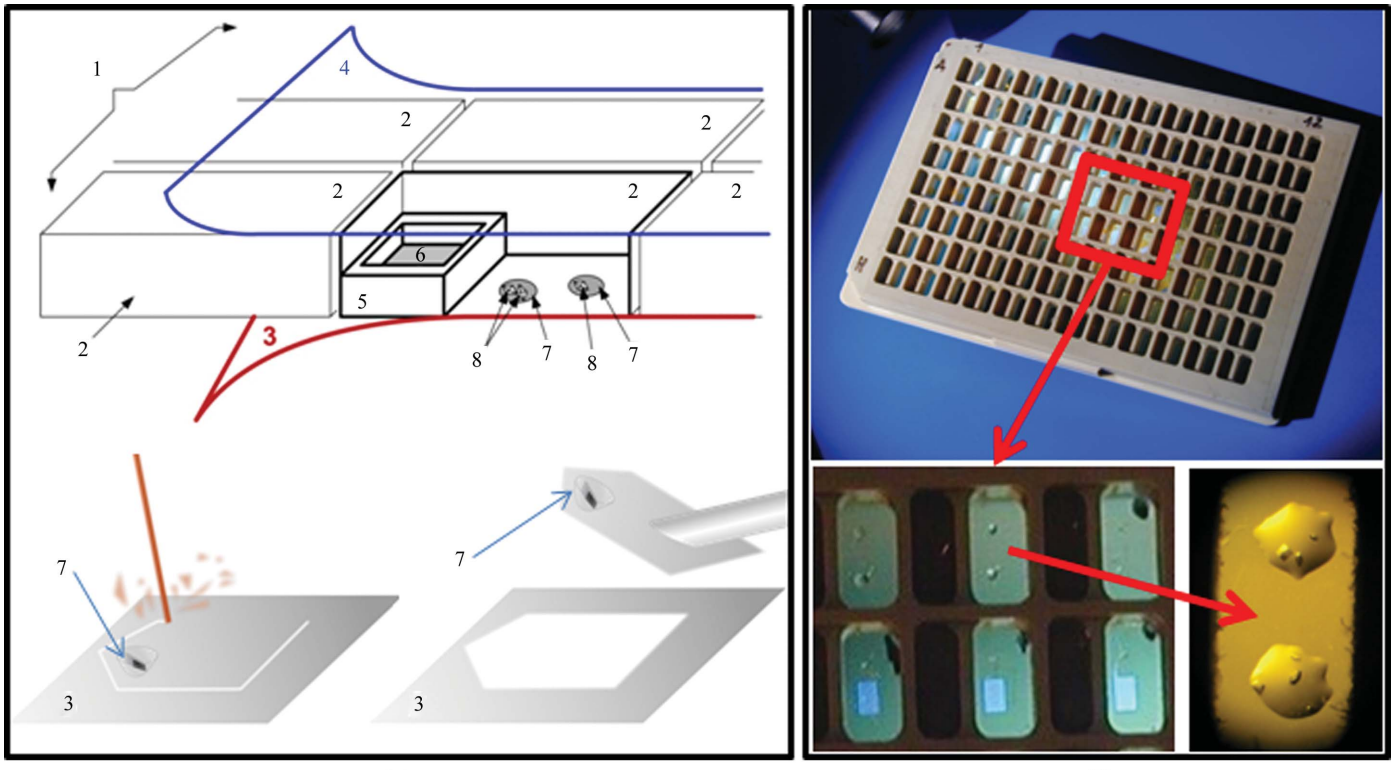
lost during the mounting process. This can be particularly problematic for samples that crystallize forming very thin plates, which tend to be fragile, or for those producing microcrystals, which are difficult to mount in standard loops. In order to circumvent these problems, *in situ* X-ray diffraction experiments were first proposed by McPherson (2000). This approach was later adapted into a number of crystallization formats including 96-well crystallization plates, microcapillaries and chips (Axford *et al.*, 2012; Jacquamet *et al.*, 2004; Ng *et al.*, 2008; Yadav *et al.*, 2005). This approach has been used both for rapid discrimination between protein and salt crystals after initial crystallization screening as well as for full data collection. However, the fact that *in situ* diffraction experiments are carried out at room temperature limits their use to well diffracting crystals. A different approach by Rupp and coworkers is based on the use of a six-axis industrial robot to harvest crystals from crystallization plates into standard crystallization cryoloops in a semi-automated manner (Viola *et al.*, 2007, 2011). Once harvested, the crystals are subjected to an automated cryoprotection treatment and used for cryogenic X-ray data collection (Viola *et al.*, 2011).

Here, we present a novel approach called CrystalDirect (CD) that is designed to enable full automation of the crystal-harvesting process. In this approach, crystals are grown on a support that (i) is directly compatible with X-ray data collection and (ii) can be excised automatically by photoablation and directly attached to a pin for X-ray data collection. Our results demonstrate that the CD method can be used to produce crystals and harvest them, preserving their diffraction quality.

2. Materials and methods

2.1. The CrystalDirect crystallization plates

A new 96-well vapour-diffusion crystallization microplate which allows the growth of crystals on very thin films has been designed (see Fig. 1). The film material and its thickness, 12.5 µm polyimide (Kapton type HN, Dupont), were selected to be compatible with both X-ray data collection (producing extremely low background scattering) and with laser-induced photoablation. The CD plates conform to the standards of the Society for Biological Screening (SBS; meeting standards ANSI/SBS 1-2004 through ANSI/SBS 4-2004 of the American National Standards Institute). The body of the plates was designed to be compatible with injection moulding. However, the prototypes presented in this work were made of polyether ether ketone (PEEK), which is better adapted to prototyping. A simple way to describe the new plates is by comparison with standard 96-well crystallization plates in which crystallization drops are set on shelves (or in crystallization wells). In the CD plates the shelves have been removed and replaced by openings. These openings are closed by the Kapton film, which is glued to the bottom of the plate. Hence, as shown in Fig. 1(*b*),



(*a*) (*b*)

Figure 1
The CD crystallization microplate. (*a*) Schema of the CD plate (top) and CD harvesting principle (bottom). Components are indicated by numbers: 1, crystallization plate; 2, crystallization cells; 3, crystallization film; 4, top sealing film; 5, crystallization solution reservoir; 6, crystallization solution; 7, crystallization drop; 8, crystal. (*b*) View of the CD plate prototype (top), detail of some of the wells (bottom left) and the crystal-growth area (bottom right). As can be noted, some film areas have been excised to recover crystals.

each of the 96 crystallization cells of the CD plate contains a reservoir that holds the crystallization solution and a section of film where crystallization drops are deposited. Once the crystallization experiments have been set up, the plate is sealed with a transparent film (Crystal Clear sealing film; Hampton Research), as for standard crystallization microplates. The CD plates are compatible with commercial crystallization and microplate imaging robots.

2.2. Crystallization experiments

Lysozyme (Sigma–Aldrich catalogue No. L6876) was dissolved in 0.1 *M* sodium acetate pH 4.8 to a concentration of 40 mg ml^{-1}. Thaumatin was dissolved in 0.1 *M* MES pH 6.5 to a concentration of 20 mg ml^{-1}. To avoid interference of the cryocooling treatment in evaluation of the harvesting technique, both lysozyme and thaumatin crystals were grown under cryoconditions. The crystallization solution for lysozyme was 30%(*w*/*v*) MPEG 5000, 1.0 *M* sodium chloride, 50 m*M* sodium acetate pH 4.5 and that for thaumatin was 0.1 *M* HEPES pH 7.5, 0.7 *M* potassium/sodium tartrate, 20% glycerol. Sitting-drop vapour-diffusion crystallization experiments were carried out on CD plates using a Cartesian PixSys 4200 (Genomic Solutions) robot at the High Throughput Crystallization Laboratory of the EMBL Grenoble Outstation (https://embl.fr/htxlab; Dimasi *et al.*, 2007). Nanovolume crystallization experiments were performed by depositing 100 nl sample and 100 nl crystallization solution on the inner surface of the films. The reservoirs were filled with 30 µl crystallization solution either manually or with the help of a TECAN Genesis pipetting robot. Once the experiments had been set up, the plate was sealed on its upper side with CrystalClear sealing film (Hampton Research catalogue No. HR4-506). The experiments were incubated at 293 K. Regular inspections were carried out either manually of with the help of automatic imaging systems (Rigaku Minstrel III). Control experiments were performed on CrystalQuick microplates (Greiner Bio-One) using the same protocol. CD plates were also used for primary crystallization screening of proteins that had never been crystallized previously. A total of 768 different crystallization conditions from commercially available screens, including Crystal Screen, Crystal Screen 2, Crystal Screen Lite, PEG/Ion, MembFac, Natrix, Quick Screen, Grid Screens Ammonium Sulfate, Malonate, PEG 6000, PEG/LiCl and MPD (from Hampton Research), JCSG+ and PACT screens (from Qiagen), and a 24-condition custom-made grid screen that assays sodium formate (from 0.8 to 3.2 *M* in 0.8 *M* unit increments) against pH (from 4.0 to 9.0 in one-unit increments) were tested. In these experiments, two crystallization drops were typically set up in each cell equilibrating against the same reservoir solution.

2.3. The CrystalDirect crystal-harvesting bench

The CD crystal-harvesting bench was built with five major components (Fig. 2).

(i) A femtosecond laser (Amplitude S-pulse).

(ii) A laser scanner (Sunny TSH8615D1064) and control software to focus and move the laser beam along the cutting area.

(iii) A microplate support with a manual micrometric stand allowing precise adjustment in three dimensions. This stand was used to align the film area containing the crystal with the working area of the laser scanner.

(iv) A pin holder mounted on a second micrometric stand adjustable in three dimensions that was used to bring the tip of the pin into contact with the film. The pins used in these experiments were compatible with the SPINE sample-holder standard (Cipriani *et al.*, 2006), but with a hollow shaft and with the tip cut on the bias (see Fig. 2). The pin holder allowed the application of a vacuum to the tip of the pin (through its hollow shaft) in order to facilitate initial attachment of the film to the pin. The cut on the bias provided a larger area of contact, which was required for reliable attachment of the film, and an optimal angle relative to the axis of the pin for exposure to X-rays.

(v) A digital camera was installed under the microplate support to facilitate alignment of the crystals into the working area of the laser scanner and to monitor the whole process.

The pin was maintained on the holder using a permanent magnet and was recovered manually after

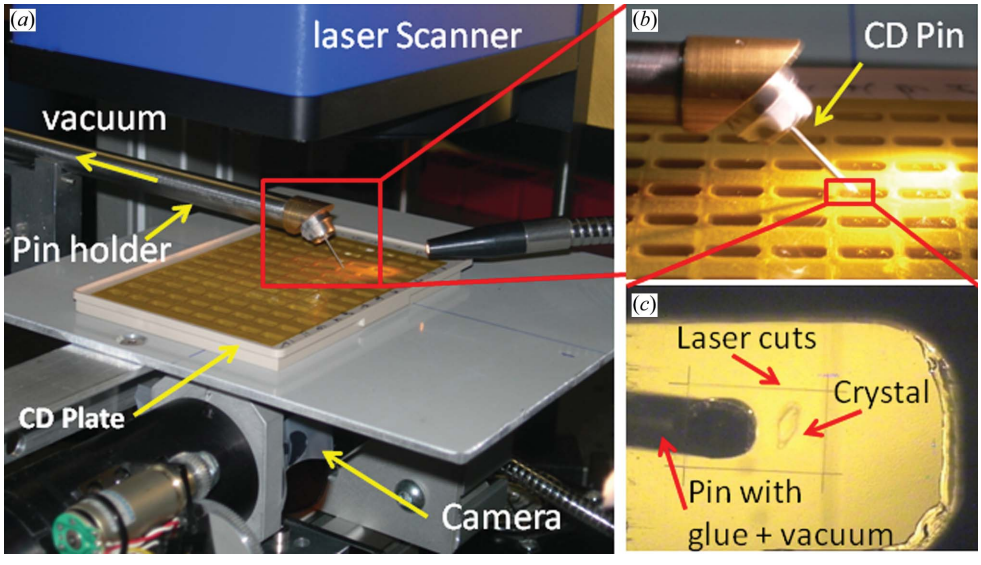


Figure 2
The CD harvesting bench. (*a*) General view of the CD harvesting bench with major components indicated (the laser source is outside the frame). (*b*) Detail of the CD pin in contact with the crystallization film on the back of a CD plate. (*c*) Part of the film from which a crystal has been excised and attached to a CD pin (viewed from the camera shown in *a*). At this point the pin with the mounted crystal can be recovered.

the harvesting process. The laser and scanner were empirically tuned to efficiently ablate the Kapton film with minimal energy. This was performed by adjusting the energy of individual laser pulses above the photoablation threshold of the Kapton film and by optimizing the laser pulse frequency, the linear speed of the laser beam on the film and the number of photoablation passes.

2.4. Diffraction and data collection

X-ray data-collection experiments were carried out on the ID14-14, ID29, ID23-1 and BM14 beamlines of the European Synchrotron Radiation Facility. Data were processed using the *XDS* software package (Kabsch, 2010). Successive rounds of automated and manual model refinement were applied using the programs *REFMAC*5 (Murshudov *et al.*, 2011) and *Coot* (Emsley *et al.*, 2010).

3. Results

The CrystalDirect (CD) approach relies on the use of new supports, CD plates, for crystallization experiments that allow the growth of crystals on ultrathin films for minimal background X-ray scattering and hence are directly compatible with X-ray data collection. Crystals are recovered by excising the film area that contains them through laser-induced photoablation and are attached to a pin for X-ray data collection.

The CD plates can be used in the same way as other currently available vapour-diffusion crystallization plates, except that the crystallization drops are deposited on the surface of a thin film rather than on a plastic shelf or cup (see Fig. 1 and §2). In order to validate their capacity to support crystal growth, CD plates were used to carry out crystallization experiments with two model proteins, thaumatin and lysozyme, using a Cartesian crystallization robot. In both cases optimal crystallization solutions were used (see §2). Both proteins produced large single crystals that appeared after a few hours of incubation. These crystals were similar in morphology and size to those obtained under the same conditions using standard crystallization plates. CD plates have also been used in primary screening experiments with hundreds of crystallization solutions from commercial screening kits (see §2), leading to the identification of crystallization conditions for two proteins that had never previously been crystallized (data not shown). This demonstrates that CD plates are appropriate for crystal growth and that they are compatible with the wide variety of chemicals used in crystallization experiments.

Once crystals have grown, the film areas containing them have to be excised by laser-induced photoablation and attached to pin supports that are compatible with X-ray diffractometers. The process of crystal recovery involves the following steps. Firstly, a CD plate is positioned on the harvesting bench

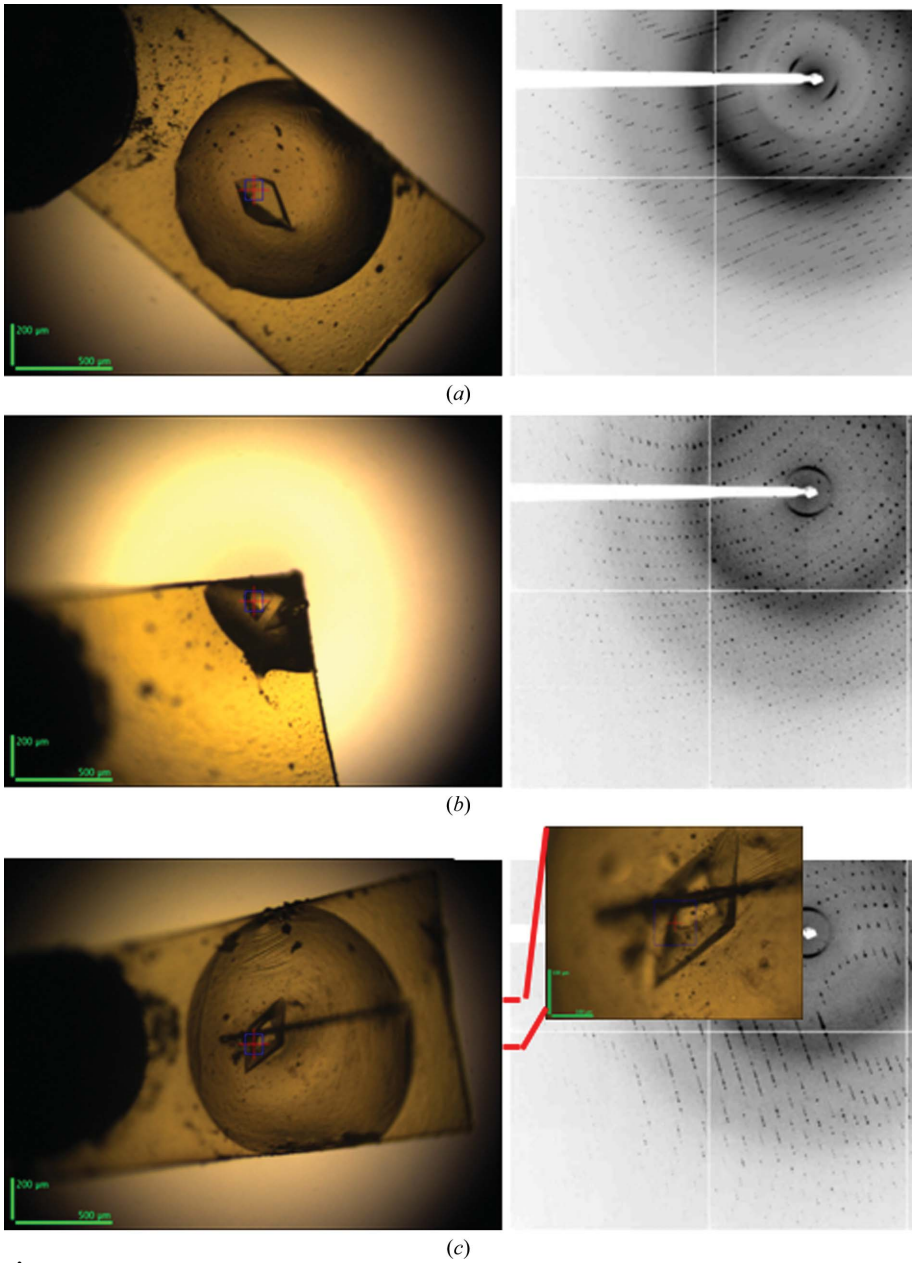


Figure 3
Crystals mounted using the CD method and their diffraction patterns. (*a*) Film area containing a full 100 + 100 nl crystallization drop with a crystal inside. (*b*) Part of a drop containing two crystals. (*c*) A crystal with a linear ablation pattern applied to the central part. The diffraction pattern corresponds to the area proximal to the ablated zone. The red cross indicates the beam position.

Table 1
Crystallographic data-collection and refinement statistics.

	Lysozyme		Thaumatin	
	CD	Control	CD	Control
PDB code	4b0d	4axt	4axr	4axu
Beamline	ESRF ID29	ESRF ID14-4	ESRF ID23-1	ESRF ID23-1
Detector	PILATUS 6M	ADSC Quantum 315r	ADSC Quantum 315r	ADSC Quantum 315r
Wavelength (Å)	0.900	0.979	0.976	0.976
Temperature (K)	100	100	100	100
Space group	$P4_32_12$	$P4_32_12$	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)	a = 78.25, c = 37.92	a = 78.24, c = 37.47	a = 57.98, c = 150.38	a = 58, c = 150.52
Mosaicity (°)	0.071	0.153	0.123	0.092
Resolution range (Å)	39–1.1 (1.16–1.10)	37.48–1.1 (1.16–1.10)	45.91–1.38 (1.45–1.38)	46.94–1.38 (1.45–1.38)
Total reflections	649397	600121	451302	473132
Unique reflections	48335	47356	46257	46432
Completeness (%)	100 (99.9)	99.3 (96.7)	86 (31.9)	86 (33.8)
Average multiplicity	13.4 (8.8)	12.7 (7.2)	9.8 (1.9)	10.2 (2.1)
$\langle I/\sigma(I)\rangle$	14.8 (1.5)	18.5 (3.1)	26.6 (2.7)	24.2 (3.8)
$R_{\rm p.i.m.}$†	0.02 (0.46)	0.02 (0.23)	0.01 (0.20)	0.02 (0.14)
Wilson B factor (Å^2)	10.2	8.7	12.2	8.0
R factor on F between data sets‡	0.28		0.28	
Refinement statistics				
Resolution range (Å)	34.1–1.1	25.6–1.1	45.9–1.38	45.9–1.38
Working/test reflections	45810/2441	44908/2388	43835/2333	43988/2345
$R_{\rm work}/R_{\rm free}$	0.19/0.21	0.16/0.18	0.13/0.17	0.15/0.19
No. of non-H atoms	1179	1300	1888	1919
Protein/water atoms	1012/167	1012/288	1613/275	1613/306
R.m.s.d. from ideal values§				
Bond lengths (Å)	0.026	0.023	0.026	0.023
Bond angles (°)	2.342	2.049	2.013	1.945
Average B factor, all atoms (Å^2)	17.21	16.44	15.84	13.245
Ramachandran statistics¶ (%)				
Favoured	99.2	99.2	98.5	99.0
Allowed	0.8	0.8	1.5	1.0
Forbidden	0	0	0	0
Rotamer outliers¶ (%)	3.8	6.7	0.6	0.6
Clashscore¶	7.14	7.65	1.33	2.36
R.m.s.d., all atoms (Å)	0.1		0.1	
Real-space fit				
Average residue-based real-space correlation coefficient, nonwater residues††	0.99	0.99	0.99	0.99
Residue-based real-space R value††	0.12	0.09	0.11	0.10

† $R_{\rm p.i.m.} = \sum_{hkl}\{1/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$ (Weiss, 2001). ‡ Howell & Smith (1992). § Engh & Huber (1991). ¶ Chen *et al.* (2010). †† Kleywegt & Jones (1996).

(see §2) and a cell containing crystals is aligned within the laser scanner working area. A small amount of glue is applied to the tip of the CD pin, which is then mounted on the pin holder. The position of the pin relative to the crystal and the area of the film to be excised are then selected. Excision of the film takes place in two steps. Firstly, an initial cut is made with the laser on the side proximal to the base of the pin. The pin is then placed into contact with the film and a vacuum is applied through the pin to facilitate adhesion of the film. The excision of the film is then completed with three additional cuts (see Fig. 2). At this point, the harvesting process is complete and the pin with the film containing the crystal(s) is recovered and cryocooled in liquid nitrogen. A video of this sequence is available as Supplementary Material.[1]

Fig. 3 shows examples of crystals mounted using the CD method and their diffraction patterns. Table 1 shows data-collection and refinement statistics for representative thaumatin and lysozyme crystals mounted using the CD and the standard manual fishing method. As can be appreciated, the diffraction properties of crystals recovered using the two methods are similar. Laser-induced photoablation has been widely used in material processing applications, as it allows the removal of materials with micrometre precision with negligible thermal effects on or other kinds of degradation of the remaining material (Chichkov *et al.*, 1996; Georgiou & Koubenakis, 2003). Indeed, our results indicate that as expected the CD harvesting process did not influence the diffraction quality of the crystals even when the laser was used to ablate areas close to a crystal (see Fig. 3*b*). To further verify this point, the laser was used to cut through the crystals themselves and X-ray diffraction patterns were collected from areas close to and far away from the ablated region (see Fig. 3*c*). In all of the experiments performed, the two areas showed similar diffraction properties, confirming the absence of detectable damage in areas adjacent to the ablated region. Similar results were obtained in experiments in which pulsed

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: KW5049). Services for accessing this material are described at the back of the journal.

lasers were used to modify the shape of crystals (Kashii *et al.*, 2005; Kitano *et al.*, 2004).

In the experiments presented here, crystallization conditions compatible with cryocooling were used and crystals were directly flash-cooled in liquid nitrogen and used for X-ray analysis, with the aim of minimizing the potential effects of post-harvesting treatments on diffraction quality. However, once recovered using the CD method the samples can be subjected to post-harvesting treatments, including cryoprotection. As illustrated in Fig. 3, we were able to recover either full crystallization drops or parts of them and to collect either one or several crystals on a single film piece. For simplicity, the excision pattern used for these experiments was rectangular and of a constant size (as shown in Fig. 3). However, no limitations apply to the shape of the excision pattern and its size can easily be set to match that of the crystals.

Although the CD plates have been designed to enable automation of the crystal-harvesting process, they may also be used for standard manual mounting (see Supplementary Fig. 1). For this purpose, a section of the film containing the crystallization drop can be excised with a scalpel and placed under a microscope, where crystals can be recovered with standard loops. This eliminates the inconvenient access angle associated with some 96-well crystallization plates. Alternatively, the excised film can be glued directly to a CD pin and used directly for X-ray diffraction experiments (see Supplementary Fig. 1). The CD plates are also ideal for *in situ* X-ray diffraction experiments, as the X-ray background scattering associated with the support is significantly reduced compared with standard crystallization microplates.

4. Discussion

The use of automation and high-throughput approaches is currently pervasive in macromolecular crystallography. However, crystal mounting is one of the operations which has not yet been fully automated. The CrystalDirect approach that we present here uses a redesigned crystallization microplate that allows the growth of crystals on a very thin film which is compatible with both X-ray measurements and photoablation. Pieces of film are precisely excised to isolate crystals and are directly attached to a pin for X-ray diffraction experiments. This approach eliminates the complicated step of 'crystal fishing', thus simplifying the process of crystal recovery.

The results presented here demonstrate that laser photoablation can be used for the recovery of crystals of biological molecules and represents an initial validation of the CD method. Contrary to other approaches, none of the operations involved in the CD harvesting protocol require perception or complex movements. This makes it possible to conceive a fully automated crystal-recovery process in which the only input required from the operator is to locate the crystals and decide on the optimal area of the film to be excised. These actions could be operated off-line through a remote computer interface, for example at the time the crystallographer evaluates crystallization drops, and the harvesting parameters recorded for further use at the time of harvesting. To fully attain this goal, the automation of post-harvesting treatments, including cryoprotection, will be necessary. Methods for robotized crystal cryocooling, including hyper-quenching and drip-cryoprotection, have already been described (Viola *et al.*, 2011) and could be incorporated into a fully automated crystal-harvesting system. Alternative approaches that preclude the need to add cryoprotectant solutions have also been proposed and could be explored in this context (Kim *et al.*, 2005; Pellegrini *et al.*, 2011).

The CD method could facilitate and speed up the analysis of large numbers of crystals. However, it also has some additional advantages over standard mounting methods. Firstly, this mounting process does not involve mechanical stress on the crystals, since no tool enters the crystallization drop or contacts the crystals. This is important when working with very fragile crystals such as thin plates. Secondly, it is not affected by the size of the crystals, making it possible to easily recover microcrystals. As mentioned above, whole crystallization drops or parts of them can be recovered using this method and there is no limitation on the size or the shape of the excised areas. Hence, it is possible to select optimal excision patterns to match the size and shape of a single crystal and recover it individually, even when it is growing in contact with other crystals. This also minimizes the amount of mother liquor recovered with the crystal, thus contributing to reducing background scattering and facilitating cryocooling. Alternatively, multiple crystals could be mounted in a single film and exposed to X-rays independently through the use of kappa goniometers (Cipriani *et al.*, 2007). Moreover, the CD approach may also allow precise pre-orientation of the crystals relative to the pin, which can contribute to optimal data collection for crystals that have previously been characterized or when multiple crystals are recovered in a single film piece. The CD plates can also be used for manual crystal recovery and for *in situ* X-ray diffraction experiments, providing easier access to crystals in the first case and significantly lowering the background scattering commonly observed with standard crystallization plates in the second case.

We believe that the CrystalDirect approach could significantly contribute to the integration of automated crystallization screening facilities and automated X-ray data-collection units. This integration could considerably reduce the delay between the identification of crystals and their measurement using X-rays. It would also facilitate the advancement of highly challenging projects such as those dedicated to the study of membrane proteins or large macromolecular complexes that are rapidly becoming a major focus of research in structural biology.

We would like to thank Vincent Rouffiange (Amplitude Systemes), Ioan Hacala, Marc Faucon and Charly Loumena (Alphanov) for providing access to their laser-application laboratories and for their advice and assistance. We would like to thank the European Synchrotron Radiation Facility and the Grenoble Outstation of the European Molecular Biology Laboratory for access to the Macromolecular Crystallography

beamlines. We would like to thank Dr Andrew McCarthy (EMBL) and Dr Hassan Belrhali (EMBL) for support during data collection. We would like to thank Dr Stephen Cusack (EMBL) for his continued support of this project. This work was partially supported by the P-CUBE project of the European Commission (FP7/2007-2013; Grant No. 227764).

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