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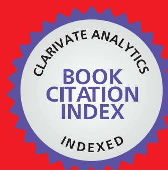

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Chapter 4

X-CHIP: An Integrated Platform for High-Throughput Protein Crystallography

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Additional information is available at the end of the chapter

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1. Introduction

High-throughput protein crystallography can be a time consuming and resource intensive endeavor. Although recent years have seen many advances in the field, screening for suitable crystallization conditions using common commercially available platforms still requires considerable amounts of protein and reagents. Furthermore, diffraction quality testing and data collection typically involve physical extraction and cryogenic freezing of the crystal samples, which may have a significant impact on the integrity of the crystal (Garman, 1999). To acquire high-quality diffraction data, both the crystallization conditions and the cryoprotectants must be further optimized. These steps can be time consuming and are often restricted to experienced users (Alcorn & Juers, 2010). In response to these concerns, the last decade has seen a significant surge of developments in crystallography-aimed microtechnology, specifically the use of crystallization chips. So far, the field is dominated by a range of microfluidic devices (Li & Ismagilov, 2010), with one of the most significant differences between them being the type of crystallization technique they employ. Several devices have been developed, and even commercialized (Topaz® crystallizer, Fluidigm Corp., CA, USA; The Crystal Former, Microlytic Inc., MA, USA) that utilize free interface diffusion (FID) (Hansen *et al.*, 2002). Other chips employ nanochannels to create counter-diffusion crystallization (Hasegawa *et al.*, 2007, Ng *et al.*, 2008, Dhouib *et al.*, 2009) or nanodroplets that simulate batch crystallization (Zheng *et al.*, 2003). There are two clear, parallel implications in all these devices. They are all striving to increase efficiency of the hit identification process, and are offering the possibility of *in situ* X-ray analysis and, in favorable cases, diffraction data collection for structure determination (Zheng *et al.*, 2004, Hansen *et al.*, 2006, Ng *et al.*, 2008, May *et al*, 2008, Dhouib *et al.*, 2009).

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The X-CHIP (Chirgadze, 2009) addresses the same challenges of high-throughput crystallography with an alternative approach, and has a number of unique additional advantages. In contrast to microfluidic chips, the crystallization process takes place on the chip surface, in droplet arrays of aqueous protein and crystallization reagents mixtures under a layer of oil. These microbatch arrays are made possible by altering the chip surface with a unique coating, creating defined areas of varying hydrophobicity. This paper presents the design of the device and accompanying tools for setting up crystallization trials and mounting the chip for data collection, as well as the important benefits, limitations and implications that are inherent to this platform. It also describes proof-of-concept experiments in which this technology was utilized for crystal growth, visual inspection, X-ray diffraction data collection and structure determination of two native and one selenomethionine-labeled protein targets. The presented results show that large, well-diffracting crystals can be grown and high-quality data sets sufficient for structure determination can be collected on a home as well as a synchrotron X-ray source.

2. X-CHIP design and application

The principal idea behind the X-CHIP was to create a platform that presents an alternative to the conventional crystallographic pipeline by consolidating the processes of crystallization condition screening, crystal inspection and data collection onto one device, streamlining the entire process and eliminating crystal handling and arduous cryogenic techniques (Fig. 1).

Figure 1. The X-CHIP was designed and developed as a miniaturized and integrated alternative to conventional methods of crystallization and data collection.

The chip is made from a material chosen for its visual light transparency and relatively low absorption of X-ray radiation. An X-CHIP with a thickness of 0.375 mm absorbed approximately thirty percent of the X-ray intensity of the primary synchrotron beam that was attenuated by 1,800 - 2,000 times to avoid excessive radiation damage to crystals during data acquisition. Designed to be compatible with most standard goniometers, the device

inserts into a chip-base (possessing a machined slit and locking screw) for support and simple mounting (Fig. 1). A plastic receptacle holds multiple chips mounted on bases, providing rigidity for set up, storage and visual inspection of the crystallization drops and can be covered with a special lid to prevent dust contamination. The chip, along with supporting tools, is shown in Fig. 2.

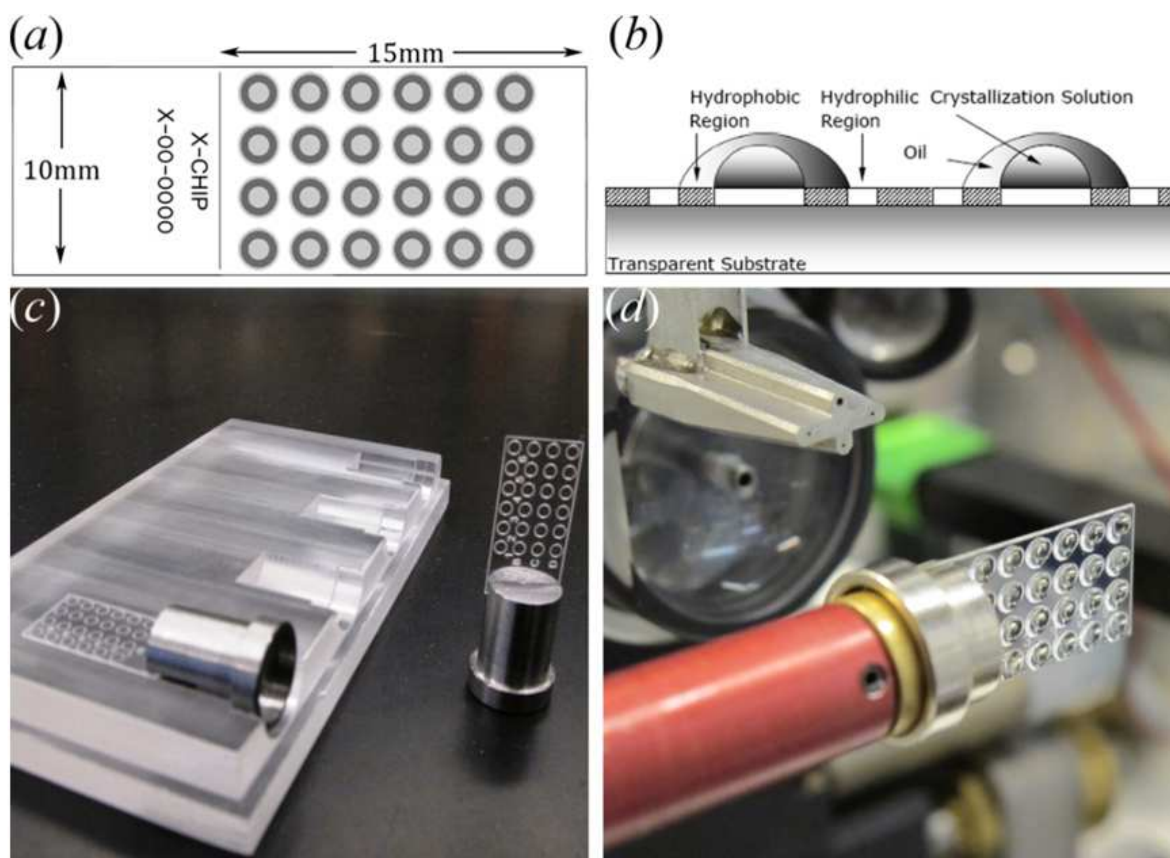

Figure 2. Schematics and images of the X-CHIP (*a*) Top-view schematic of the 24-drop format chip, hydrophilic and hydrophobic areas are shown in light and dark grey, respectively (other formats include 6-drops; not shown) (*b*) Cross-section of the chip (*c*) X-CHIP on a base and a 4-chip receptacle device (*d*) X-CHIP with 24 crystallization drops, mounted on a goniometer.

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The described system applies principles of the microbatch crystallization method, the high effectiveness and unique benefits of which have been described elsewhere (D'Arcy *et al.*, 1996, Chayen, 1998, D'Arcy *et al.*, 2003). On the surface of the chip, circular hydrophilic areas are inscribed in hydrophobic annuli in ordered arrays (Fig. 2*a*, 2*b*). Nanoliter volumes of aqueous protein and precipitant solutions are mixed onto the hydrophilic circle by sequential addition and then covered by an mineral oil layer, which is dispensed on top of the drop and is stabilized on the surrounding hydrophobic ring. The interactions between the aqueous phase, oil layer and coated surface create highly defined droplets of predictable volume and thickness and prevent drops not only from drying but from fusing with each other with each other during crystallization set up and data acquisition. The design of the chip currently uses 1 x 6 and 4 x 6 formats and its size permits visual inspection of the entire chip in one image (Fig. 3*a*).

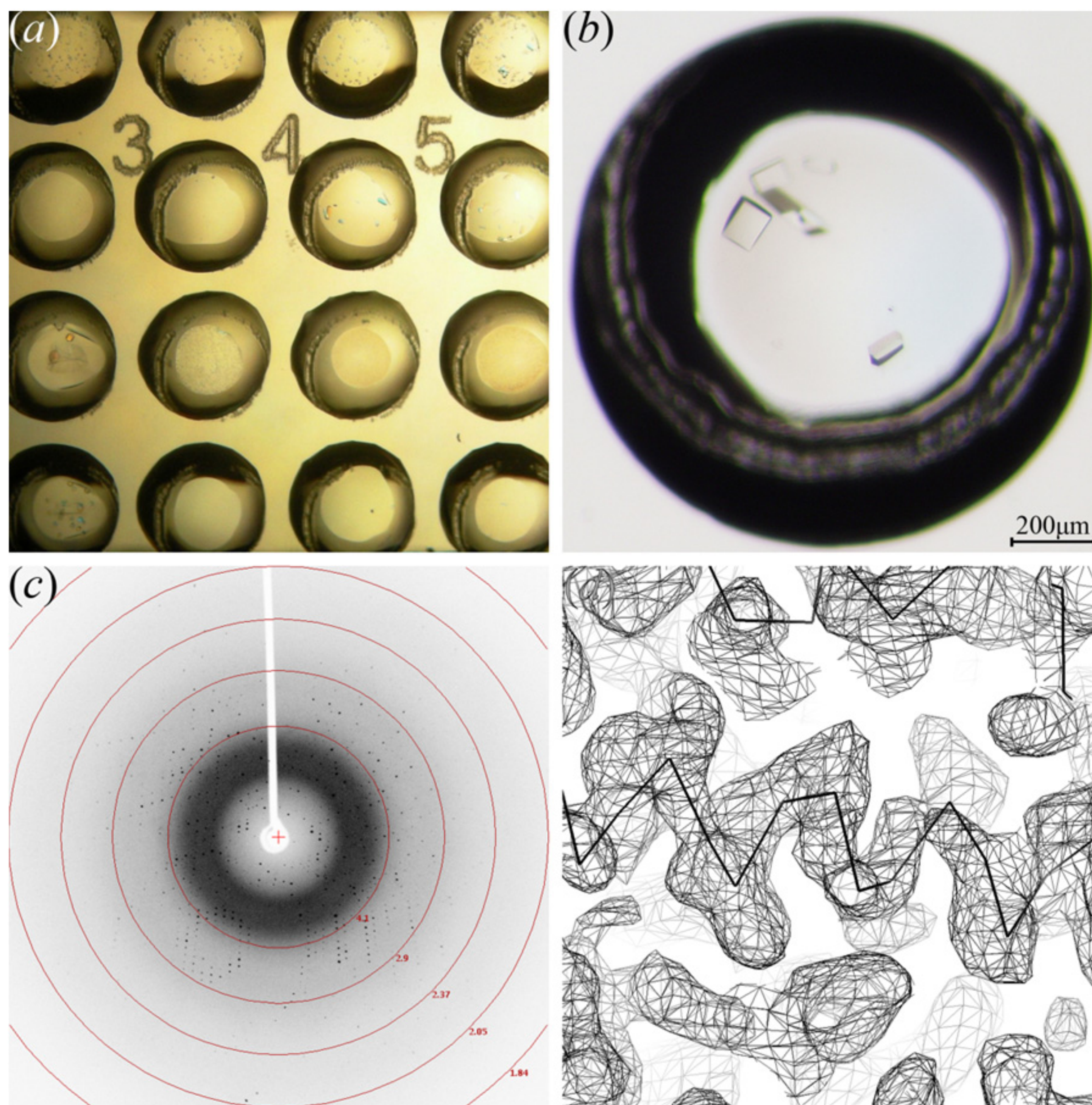

Figure 3. Experimental results: (*a*) section of a 4x6 X-CHIP with a two-dimensional optimization of two crystallization conditions for native PA0269, taken two weeks after initial set up (*b*) crystals of EphA3 grown overnight, crystal size approximately 250μm in length (*c*) On-the-chip diffraction image for an EphA3 crystal, collected on a Rigaku FR-E rotating anode with R-AXIS HTC detector (*d*) Part of an experimental electron density map generated using the SAD PA0269SM data set collected directly from the crystal grown on the X-CHIP, superimposed with the protein C_{α}-trace, shown as a black solid line.

3. Materials and methods

Previously investigated targets, the protein kinase domain of human Ephrin Receptor Tyrosine Kinase A3 (EphA3) (Davis *et al.*, 2008) and the *Pseudomonas aeruginosa* alkylhydroperoxidase D protein (PA0269) (McGrath *et al.*, 2007) were selected as model proteins to demonstrate the feasibility of crystal growth and *in situ* data collection using the X-CHIP. Following several rounds of on-chip optimization, both projects were

crystallized at conditions similar to those in the cited literature. EphA3, at 15 mg ml^{-1}, and native PA0268, at 10 mg ml^{-1}, were crystallized using a 1:1 (vol/vol) with 0.2M $(NH_4)_2SO_4$, 0.1M Hepes pH 7.5, 25% PEG3350 and 0.8M $(NH_4)_2SO_4$, 0.1M sodium citrate pH 4.0 respectively. For crystallization drop set up, a protein sample volume of 200-250nl was mixed with an equal volume of precipitant solution and then covered by an mineral oil volume of 0.75-1.25µl; all solutions were dispensed with a Gilson P2 pipette. Prior to set up, the chip was inserted into the holder, which was afterward covered with a lid to prevent contamination. Chips were stored for 1-4 weeks at room temperature without significant evaporation and transported by road to the synchrotron beamline. Crystals both from stored chips as well as from chips set up at the synchrotron were used to collect diffraction data sets.

In-house data sets were collected on a *Rigaku FR-E* Superbright rotating anode X-ray source equipped with a *Rigaku R-AXIS HTC* image plate as a detector (Rigaku, The Woodlands, TX, USA). The synchrotron data sets were collected on the *IMCA-CAT ID-17* beamline at the Advanced Photon Source (APS) facility with appropriate beam attenuation, using the *Pilatus 6M* detector (Dectris Ltd., Baden, Switzerland). The X-CHIP was manually mounted onto the goniometers as shown in Fig. 2*d*. Individual samples were initially optically centered, and then centered using diffraction to refine crystal position. In both cases the cryo-stream was blocked and data collection was performed at room temperature (~298K).

4. Results

Two important aspects of the described system were investigated throughout this study; the capacity of the chip to produce diffraction quality crystals and the feasibility of diffraction data acquisition (*in situ*) of sufficient quality for *de novo* structure determination. To assess the first task, the reproducibility of previous crystal hits obtained by sitting drop vapor diffusion technique was tested. For both EphA3 and PA0269 projects, vapor diffusion crystallization conditions resulted in high-quality crystals on the X-CHIP (Fig. 3*a*, 3*b*). For PA0269, on-chip optimizations further improved the crystal size and quality and decreased the number of crystals per drop (Fig. 3*a*). These results demonstrated that the X-CHIP can be successfully used to obtain and optimize crystallization hits and grow single crystals that are large enough for straightforward data collection.

Proof-of-concept experiments for on-the-chip data collection were carried out on the rotating anode source and the synchrotron beamline. The initial data collection trials on the in-house X-ray source led to the acquisition of a complete EphA3 data set. While the experiment was conducted at room temperature, diffraction data could still be obtained with sufficient completeness, even for crystals of such low symmetry space group as $P2_1$ (Table 1). At the synchrotron beamline, data sets for EphA3, PA0269 and a PA0269 selenomethionine derivative (SAD) were collected. The high sensitivity and ultrafast readout time of the *Pilatus 6M* detector allowed complete data sets to be collected quickly at room temperature without significant degradation of the sample and with excellent processing statistics. Owing to the finely focused beam (50 x 50µm), it was possible to collect

data from multiple small crystals grown within the same drop, without any obvious impact on the diffraction quality of neighboring crystals. A particularly interesting result can be observed by comparing the mosaic spread between the X-CHIP and the benchmark data (*i.e.* cryo loop) in Table 1. It is evident that the mosaic spread was consistently lower for data set experiments collected using the chip, and in the case of PA0268 was as low as 0.046°. Furthermore, based on resolution range alone, EphA3 crystals only started showing radiation damage after as much as ten minutes of continuous X-ray exposure, more than twice the time needed for obtaining a full data set (data not shown).

	EphA3	***EphA3***	**EphA3**	***PA0269 SM****	**PA0269**	**PA0269SM ***
Crystal mount method	X-CHIP	*Cryo Loop*	X-CHIP	*Cryo Loop*	X-CHIP	X-CHIP
Sample Temperature (K)	295	*100*	295	*100*	295	295
X-ray Source	Rigaku FR-E	*BM-17 APS*	ID-17 APS	*ID-17 APS*	ID-17 APS	ID-17 APS
λ (Å)	1.54	*1.00*	1.00	*0.97934*	1.00	0.97938
Detector	R-AXIS HTC	*MarCCD M300*	Pilatus 6M	*ADSC Q210*	Pilatus 6M	Pilatus 6M
Space group	$P2_1$	$P2_1$	$P2_1$	$P6_322$	$P6_322$	$P6_322$
Resolution (Å)	2.00	*1.93*	1.95	*1.75*	1.95	1.95
High resolution shell (Å)	(2.10-2.00)	*(2.03-1.93)*	(2.05-1.95)	*(1.84-1.75)*	(2.05-1.95)	(2.05-1.95)
Data Collection Time (min)	100	*29*	5.3	*74*	3.0	3.3
$\Delta\phi_{total}$ (°)	100†	*190*	160	*185*	90	100
Mosaic spread (°)	0.100	*1.048*	0.360	*0.364*	0.046	0.160
Completeness (%)	85.3†	*98.4*	96.2	*99.8*	100	100
Multiplicity	2.4	*3.6*	2.7	*10.6*	9.5	10.3
<I/σ(I)>	4.5 (2.2)	*12.9 (5.0)*	7.5 (2.7)	*19.4 (3.5)*	12.1 (2.7)	20.8 (3.9)
R_{merge} (%)	8.8 (34.6)	*4.9 (9.7)*	10.9 (34.6)	*7.5 (49.9)*	8.2 (47.5)	6.9 (50.2)

Benchmark data is in *italic font*.
Values in parentheses refer to the highest resolution shell.
*Single-wavelength anomalous dispersion (SAD) data collection, using anomalous signal from seleno-methionine.
† Completeness of 99% was achievable from the same set of crystals with a total oscillation angle of 140 degrees.

Table 1. Summary of selected data sets.

For crystallization of EphA3 and PA0269, paraffin oil was used to coat the crystallization drops after protein and precipitant solution had been dispensed. Other oils have been explored, such as Hampton's Al's Oil (50/50 paraffin/silicon oil mixture), silicon oil and a 50/50 mix of paratone/paraffin oils. Higher viscosity oils (paraffin, paratone/paraffin) performed better on the X-CHIP by being highly restricted to the hydrophobic ring boundaries. The thinner silicon oil was found to flow outside of these boundaries causing drop merging. Al's oil required more careful application compared to higher viscosity oils, but proved to stay within the hydrophobic boundaries. Crystallization conditions containing ethanol, 2-methyl-2,4-pentanediol (MPD) and detergents were also tested on the X-CHIP. Ethanol tolerance was tested with a 5-30% gradient using paraffin oil as a cover. The phase separation within the crystallization drops remained intact for the entire gradient range. Crystallization drops containing MPD in combination with different oils tolerated up to 8% before they began to disperse beyond the hydrophobic area the hydrophobic area. While this can exclude some MPD based conditions from being used on the X-CHIP, the impact on the overall versatility is low since most commercially available initial screens from Hampton Research and Emerald Biosystems (e.g. Wizard I&II, Index, Crystal) only have an average of 5-6% total conditions containing MPD. Detergent tolerance was tested with n-dodecyl-N,N-dimethylamine-N-oxide (DDAO) and n-octyl-β-D-glucoside in combination with a paraffin oil covering. Separation between the phases remained intact with the 0.05% n-octyl-b-D-glucoside condition but, DDAO was not tolerated even at very low concentrations.

5. Discussion

The series of initial experiments on the X-CHIP crystallization platform described above demonstrated the chip’s applicability for high-throughput protein crystallography and provided insight into the benefits and limitations of this system. Crystallization using the microbatch method on the chip was shown to be suitable for crystal growth and also offered additional benefits. Oil covered drops evaporate very slowly (days to weeks), simplifying both manual and automated set up. Furthermore, changing the composition of the top oil layer with various oil mixtures makes it possible to vary the rate of water evaporation over a wide range, adding another favorable dimension to crystallization screening (D'Arcy *et al.*, 2004). Inherently, the system is economical since crystallization hit determination and optimization trials require up to five times less volume of protein sample and five hundred times less reagent solution than standard vapor diffusion methods. Theoretically, the volumes can be decreased even further by incorporating robotic liquid handling systems, but are currently limited by the accuracy of manual dispensing. In addition, the simplicity of the device results in low manufacturing costs and the platform design eliminates the time and expenses associated with cryogenic techniques. The small size of the chip offers more convenient and faster visual inspection, as all the crystallization drops can be viewed under a microscope simultaneously. Furthermore, the system design provides a non-invasive means of diffraction testing and screening, as the developed device can be mounted on most in-house and synchrotron beamline data acquisition systems without any modification of

the chip or adjustments to that system. These capabilities of the X-CHIP make it a potentially useful platform for high-throughput initiatives such as fragment-based screening by co-crystallization.

The X-CHIP system has the potential to completely remove the "user factor" between crystal growth and X-ray diffraction data collection, eliminating crystal manipulation. The feasibility of *in situ* data collection has significant implications. Firstly, data collection at room temperature eliminates the need for the tedious and often limiting step of cryo-condition optimization with the added advantage that crystal structures determined at room temperature are more representative of the physiological state. Additionally, experimentally obtained SAD data displayed excellent processing statistics, clearly of sufficient quality for *de novo* structure determination. Interestingly, in at least one of the cases investigated, undisturbed crystals have shown significantly lower mosaic spread than that of cryogenically frozen samples, suggesting the potential application of this system to samples of high sensitive or ones with a large unit cell (Table 1). Once mounted on the goniometer, navigation along the chip and alignment of any crystal in the drops is quite straightforward, presenting the potential for data collection in a high-throughput mode. This approach eliminates the necessity for mounting of individual loops as in conventional robotics systems and may save hours of valuable synchrotron beam time. Finally, the elimination of manual crystal handling opens the opportunity for full automation of the crystallization to data acquisition pipeline allowing streamlining of the entire process.

Current developments on the project are aimed at scaling down the drop volumes of the X-CHIP system. Attempting to do so using manual set up has proven to be challenging, but application of a liquid handling robotics system can address this issue. The *Mosquito* crystallization robotic system (Molecular Dimensions Ltd., Suffolk, UK) has already been used to successfully set up crystallization experiments with total drop volumes as low as 200nl. The X-CHIP is also being applied to the crystallization trials of additional protein targets. As a point of interest, experiments with highly sensitive and/or small crystal samples could greatly benefit from the use of this system, as the non-invasive data collection approach would likely resolve many problems that arise from crystal handling. We are also exploring the application of the chip for projects in which low mosaic spread is essential for successful outcome.

6. Conclusions

From the initial studies of the device it is evident that not only does the X-CHIP have the potential to increase efficiency and offer on-the-chip *in situ* data collection for *de nevo* structure determination, but it also has a range of additional benefits including the opportunity for full automation. Even though the recent growth of microchip crystallization technology has seen the development of several useful devices, the X-CHIP platform offers previously unprecedented simplicity with comparable or in some cases even better performance. As seen in the recent growth of commercially available and published

microchip crystallization devices, there is continuing interest in improving the efficiency and effectiveness of the diffraction pipeline. With its intuitive design, minimalistic support platform and compatibility with most existing beamline setups, the X-CHIP platform offers a new integrated tool for protein crystallization and X-ray diffraction data collection.

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